Quasi Steady-State Approximations in Intracellular Signal Transduction – a Word of Caution

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Abbreviations:
QSSA, Quasi steady-state approximation; sQSSA, standard QSSA; tQSSA, total QSSA; MM, Michaelis-Menten; MAPK, mitogen activated protein kinase; MAPKK, MAPK kinase; MKKK, MAPK kinase kinase; MKP, MAPK phosphatase; ERK, extracellular signal-regulated kinase; MEK, MAPK and ERK kinase; NGF, nerve growth factor; EGF, epidermal growth factor.

Keywords: Michaelis-Menten kinetics, quasi steady-state assumption, enzyme signaling networks, double phosphorylation, MAPK cascade.

Summary

The main goal of computational biology, and in particular of Systems Biology, is to define a comprehensive model that can accurately represent the experimental data and serve as a tool to generate and test hypotheses. Consequently, a high accuracy of the model is necessary in order to give a satisfactory prediction, both by a qualitative and quantitative point of view. Enzyme reactions play a pivotal role in intracellular signal transduction. Many enzymes are known to possess Michaelis-Menten (MM) kinetics and the MM approximation is often used when modeling enzyme reactions. However, it is known that the MM approximation is only valid at low enzyme concentrations, a condition not fulfilled in many *in vivo* situations. Thus, using the MM approximation with its parameter values obtained from *in vitro* experiments will often lead to false conclusions when simulating *in vivo* systems. Recently several other mathematical approaches, such as the total quasi steady-state approximation (tQSSA), have been developed for enzymes with MM kinetics. These new approximations are valid not only whenever the MM approximation is, but moreover in a greatly extended parameter range. Starting from a single reaction and arriving at the mitogen activated protein kinase (MAPK) cascade, we give several examples of biologically realistic scenarios where the MM approximation leads to quantitatively as well as qualitatively wrong conclusions, and show that the tQSSA improves the accuracy of the simulations greatly. Moreover, we discuss the use of approximations in reverse engineering and the biological importance of our findings.

Introduction

Every living cell responds to external stimuli, like hormones, ions, heat shock, etc., which are transduced by a complex intracellular molecular network. When an external ligand binds a plasma membrane receptor, intracellular second messengers interacting with membrane receptors are activated, and by means of biochemical reactions

transduce the signal.

In the last decade many mathematical models have been formulated to investigate the behavior of complex intracellular biochemical networks. Many of those are based on the well-studied MAPK cascade (see for example [1–5]), and although not crucial for the results presented here, this ubiquitous signalling pathway will be given special attention in the following.

The aim of such modeling (which is an integral part of the 'Systems Biology' large scale project) is roughly twofold: to reproduce and study some particular phenomena observed experimentally (like bistability, oscillations, ultrasensitivity, hysteresis, etc.) and to investigate the properties of these networks as information processing and transducing devices. As a hope for the future, this modeling could be used for pharmaceutical scopes (first of all drug discovery) as a reliable tool to make predictions about the effects of drugs on the biochemical networks, thus shortening the preclinical phase. This goal is related to the ambitious project of a "Virtual Cell" ([6], http://www.vcell.org/) or "Silicon Cell" ([7], http://www.siliconcell.net/), which aims at simulating the behavior of whatever cell as closely as possible to the physiological reality: "A silicon cell is a precise replica of (part of) a living cell" (cited from http://www.siliconcell.net/).

Surprisingly, the mathematical formulation of these highly interconnected enzyme reactions is usually based on *in vitro* studies of isolated reactions, without a serious criticism of the delicate passage from the kinetics of simple reactions to the kinetics of a network of reactions shared by several cascades in a crowded molecular environment [8]. This can be justified when analyzing underlying mechanisms (e.g., the importance of feedback or the creation of oscillations), where the exact kinetic expressions and parameters are less important since one is usually only interested in the qualitative behavior that the system can perform. However, in the light of the Silicon Cell project, which aims at being a both qualitative as well as quantitative precise representation of the living cell, the use of correct parameters, kinetic expressions and initial conditions (i.e., steady-state concentrations of molecular species) becomes cru-

cial. This is the subject of the present work.

One of the principal components of the mathematical approach to Systems Biology is the model of biochemical reactions set forth by Henri in 1901 [9–11] and Michaelis and Menten in 1913 [12], and further developed by Briggs and Haldane in 1925 [13]. This 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.

This scheme is mathematically represented by a system of two nonlinear ordinary differential equations (ODEs), corresponding initial conditions and two conservation laws as shown in the Methods section. 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.

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 assumption* or *approximation* (standard QSSA, sQSSA)), which is valid when the enzyme concentration is much lower than either the substrate concentration or the Michaelis constant K_M [14, 15]. This condition is usually fulfilled for *in vitro* experiments, but often breaks down *in vivo* [16, 17]. We refer to the Methods section for the mathematical formulation of scheme (1), and to [18] for a nice, general review of the kinetics and approximations of (1).

The advantage of a 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 numerical simulations greatly, especially for large networks as found *in vivo*. Moreover, the kinetic constants in (1) are usually not known,

whereas finding the kinetic parameters for the MM approximation is a standard *in vitro* procedure in biochemistry [19]. 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) [20, 21], which is valid for a broader range of parameters covering both high and low enzyme concentrations, have been introduced recently. Tzafriri [21] showed that the tQSSA is at least roughly valid for any set of parameters in the case of the reaction in (1). Importantly, the tQSSA uses the same parameters (V_{max}, K_M) as the sQSSA. Hence, the parameters found *in vitro* from the MM approach can be used by the tQSSA for modeling *in vivo* scenarios.

The roles of V_{max}, the maximal reaction velocity, and K_M, the Michaelis constant describing the concentration of the substrate at which the reaction rate is half maximal, become essential when characterizing biochemical reactions *in vitro* as well as *in vivo*. Moreover, descriptions of cooperative reactions, inhibition and many other biochemical processes have exploited the fundamental ideas of the MM scheme, i.e., the sQSSA and the parameters V_{max} and K_M (see, e.g., [19]). However, since these approximations cannot be expected to be valid *in vivo*, employing the tQSSA to these more complex situations would be preferable. Tzafriri & Edelman [22] studied the completely reversible enzyme reaction in terms of the tQSSA. We have recently derived the tQSSA for fully competitive reactions [23].

In this paper we show that the use of the sQSSA can lead to gross quantitative as well as qualitative wrong conclusions even in the case of simple networks. The tQSSA is shown to estimate the behavior significantly better, and therefore we propose to use this approximation when modeling intracellular signalling networks.

Results

Our investigation applies to every biochemical network which includes enzyme reaction cascades. Many mathematical models have been applied to the phosphorylation and dephosphorylation reactions with special attention to the MAPK cascade, for which there exist many experimental [24–26] and theoretical results [1–5]. We discuss the validity limits of the sQSSA and the advantage of the tQSSA, starting from subcomponents of this cascade and finally arriving at the full cascade including feedback. To fully appreciate the differences between the sQSSA and the tQSSA we recall the simpler and very general cases of, firstly, a single reaction, and then two reactions with substrates competing for the same enzyme. All mathematical expressions are given in the Methods section or the Appendix.

For the single reaction represented in (1) it has been known for many years that the sQSSA (MM approximation) holds when the initial substrate concentration is much higher than the initial enzyme concentration ($S_T \gg E_T$), but it was later realized that this is not a necessary condition; if the enzyme concentration is much less than the Michaelis constant, then the sQSSA also holds [14, 15]. This is summarized in the validity criterion $E_T \ll S_T + K_M$, which loosely says that the sQSSA holds at low enzyme concentrations (with respect to either the substrate concentration or the K_M value).

This can not be expected to hold *in vivo*, and the tQSSA has been introduced in order to have an approximation applicable to *in vivo* reactions as well [20, 21]. This approximation holds for a much larger region of parameter space, and is in fact always roughly valid [21]. Importantly, the tQSSA coincides with the sQSSA when the latter is expected to hold, i.e., at low enzyme concentrations. Fig. 1 shows that the tQSSA approximates the full system very well also for high enzyme concentrations where the sQSSA fails.

[Figure 1 about here.]

Competing substrates and the double phosphorylation mechanism

A theoretically well-studied example of a slightly more complicated network is the case of fully competitive reactions [14, 23, 27], i.e., reactions with competing substrates, S_1 and S_2, also known as substrate-inhibitor systems,

$$\begin{aligned} S_1 + E &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E + P_1, \\ S_2 + E &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E + P_2, \end{aligned} \tag{2}$$

where S_i, C_i and P_i represent substrate, enzyme-substrate complex and product ($i = 1, 2$) for the two competing reactions. Note that this reaction scheme also covers competitive inhibition (for $k_2 = 0$ with S_2 being the inhibitor).

The validity criterion of the sQSSA for (2) is known [14,27], and says basically that the sQSSA holds at low enzyme concentrations as in the case of a single, noncompetitive reaction, which can be seen as a special case of (2) which negligible inhibitor concentration. We [23] extended the region of validity by employing the tQSSA to (2), and as shown in Fig. 2 it approximates the full system very well. We have not found any values of the parameters for which numerical simulations show that our tQSSA breaks down dramatically. Of importance, our approximation captures the competition as does the competitive sQSSA (18) and in contrast with the single reaction tQSSA (10), but also at intermediate or high enzyme concentrations where the sQSSA does not hold anymore (Fig. 2). However, when the competition can be neglected due to, e.g., low substrate concentrations, the single reaction tQSSA does indeed estimate the full system well (not shown).

Our results are immediately applicable to, e.g., successive reactions catalyzed by the same enzyme, such as nonprocessive or distributive double phosphorylation or dephosphorylation processes, as seen for example in the MAPK cascade [28–32]. The reaction scheme can be seen as a special case of (2) with $P_1 = S_2$ and is summarized as

$$S_1 \xrightarrow{E} S_2 \xrightarrow{E} P, \tag{3}$$

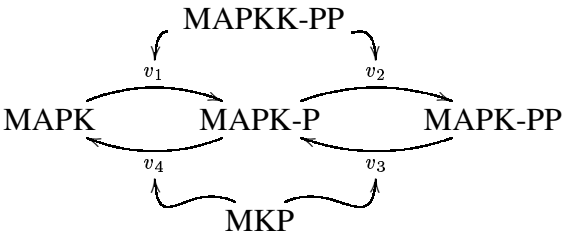
where it is usually assumed that at the beginning only S_1 is present. Here S_1 and S_2 compete for the same enzyme, E. For the case of the MAPK cascade one can think of, e.g., diphosphorylated and thus activated MAPKK (MAPKK-PP, here E) phosphorylating MAPK (here S_1) twice, producing first mono-phosphorylated MAPK (MAPK-P, here S_2) and then double-phosphorylated MAPK (MAPK-PP, here P). See also scheme (4) below.

In the MAPK cascade literature every single reaction is often treated by a MM approximation for an isolated reaction of the form (1), not only without any *a priori* examination of its applicability, but also neglecting the other terms involved in the double reaction and, in particular, the important fact that, for example, MAPK and MAPK-P are competing substrates for MAPKK-P (however, see [31,32]). This means that even when the sQSSA for (3) holds, the neglect of the competition leads to wrong estimations of the behavior, and can only be expected to be an even greater problem when the sQSSA breaks down, in which case the tQSSA should be used. This situation is illustrated in Fig. 2B, which shows that even when both the non-competitive sQSSA and tQSSA as well as the competitive sQSSA fail, the competitive tQSSA is an excellent approximation.

[Figure 2 about here.]

The double phosphorylation as well as double dephosphorylation of MAPK was recently modelled taking into consideration the competition between the pools of MAPK with different phosphorylation states [31,32]. We model this process by assuming that (2) holds for both the phosphorylation as well as the dephosphorylation processes as in [31]. In [32] both (2) as well as a more complicated process of phosphorylation were considered, but this further step is not of our interest here although applying the tQSSA to this more complicated scheme would be interesting. Similarly, we follow [31] and model the dephosphorylation by (2) instead of the slightly more complicated scheme from [32] for the sake of simplicity.

Thus, we are studying the scheme



(4)

where the reaction rates $v_1 - v_4$ are assumed to follow Michaelis-Menten kinetics with competition between MAPK and MAPK-P for activated MAPKK (MAPKK-PP), and between MAPK-PP and MAPK-P for the generic phosphatase MKP [33]. Using parameters from [32, Fig.1], we see in Fig. 3A that in this case the competitive sQSSA underestimates the duration of the transient phase before reaching the steady state. Furthermore, it underestimates the steady state level of MAPK-PP. However, this underestimation is not a feature of the sQSSA, since lowering the total MAPK concentration to $[\text{MAPK}]_T = 50$ results in an (even more pronounced) overestimation of the steady state level (Fig. 3B), which of course can be of equal importance as an underestimation. Notably, the tQSSA fits both the dynamic behavior as well as steady state levels very well in both cases. Remark the counter-intuitive result that a ten times *lower* total MAPK concentration in Fig. 3B yields a more than two times *higher* level of activated MAPK, showing the strength and utility of mathematical modeling. To illustrate the importance of a reliable estimation of the MAPK levels, we remark that is has been shown experimentally that the dynamics of MAPK activity is crucial for the fate of the cell [24, 34, 35]. For example, PC12 cells proliferate in response to transient MAPK activation, while they differentiate when the activated MAPK levels are sustained [36]. We follow this up in the next section and in the discussion.

[Figure 3 about here.]

The MAPK cascade

[Figure 4 about here.]

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 [33,37], 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 as mentioned in the previous section.

Looking at the complete MAPK cascade, shown in Fig. 4, it is clear that all the problems arising in the simpler cases described in the previous sections may occur.

[Figure 5 about here.]

One of the most interesting phenomena in biochemical networks is the appearance of (sustained) oscillations experimentally observed, for example, in glycolysis, in intracellular calcium or in circadian networks. Recently, these oscillatory phenomena have been investigated theoretically for signal transduction networks like MAPK cascade [3].

Several authors suppose that MAPK-PP acts, by means of a feedback mechanism, on the first layer of the MAPK cascade, and in some cases this feedback has been shown experimentally, for example in NIH3T3 cells, where Raf-1, a MKKK, was found to be inactivated by ERK, a MAPK [38]. See also [35, 39] for reviews. Kholodenko [3] introduced a noncompetitive inhibition of this kind. The mathematical model of this complex network (with or without feedback) was built using the non-competitive MM approximation, and it was shown that oscillations could occur for several parameter values.

However, the appearance of oscillations could depend on the way in which the model has been formulated. We compare the network with the full system of reactions to both the competitive sQSSA [31, 32] and the competitive tQSSA [23]. Note that the tQSSA used here is an *ad hoc* approach (see Appendix C), since a truly valid tQSSA has not been found yet for large networks such as the MAPK cascade. In contrast to Kholodenko [3], we model the negative feedback as a competitive inhibition with inhibition constant K_I to allow the use of the competitive tQSSA (see Fig. 4 and Appendix C). Our simulations confirm that the cascade can reach a steady state as well as oscillate also in this case. However, with parameters very similar to [3] the (competitive) sQSSA approximation can lead to qualitatively wrong conclusions such as oscillations when the full system is steady (Figs. 5B and C), or quantitative wrong estimations of, e.g., the amplitude of the oscillations (Fig. 5A), or the steady-state levels of MAPK-PP (Fig. 5D), while the full system is in general much better approximated by the (competitive) tQSSA. However, for some parameters the tQSSA approach also fails qualitatively (Fig. 5B), or with respect to the period of the oscillations (Fig. 5A).

In Table 1 we summarize the ranges of the inhibition constant K_I for which MAPK-PP oscillates in the three cases. It is seen that the solution of the full system undergoes oscillations for a very narrow range of this parameter, while the use of the sQSSA yields oscillations for a much larger range, also for values for which the solution of the full system does not perform rhytmic behavior (Figs. 5B and D). However, the competitive tQSSA also fails to predict the behavior for some parameters, but the range for which this occurs is markedly reduced compared to the sQSSA (Table 1). New improved tQSSAs should be developed in order to get a better representation of the full system.

[Table 1 about here.]

The great majority of authors using the sQSSA usually neglects the concentration of the complexes, as expressed, e.g., in the conservation law $[\text{MAPK}]_T = [\text{MAPK}] + [\text{MAPK-P}] + [\text{MAPK-PP}]$ [3, 32], but this is only valid at low enzyme concentrations.

We suppose that this is the major reason for the poor prediction of the sQSSA. The complex concentrations are indeed significant in our simulations of the full system as shown in Fig. 6A. This figure shows the complex (MAPK-P)-(MAPKK-PP) of reaction 8 in Fig. 4, the substrate MAPK-P and the free enzyme MAPKK-PP. In contrast with the sQSSA, the tQSSA considers the complex concentrations, and it is seen from Fig. 6A that this is necessary, since the total substrate concentration is comparable with the complex concentration.

Taking the complex concentrations into account is not only important for the generation of oscillations, but, as it could be expected, also for the steady-state concentrations obtained for the full system, the sQSSA and the tQSSA at high values of the inhibition constant K_I (Table 1). With the parameters used here the competitive sQSSA overestimates the steady-state level of activated MAPK (Fig. 5D) as in the simpler case considering only the last level of the cascade (Fig. 3B). On the other hand, the competitive tQSSA estimates this level well (Figs. 5C and D). As mentioned in the previous section, the correct estimation of activated MAPK-PP has important implications for predicting further downstream effects. We follow this question up in the discussion.

[Figure 6 about here.]

A second problem of both the QSSAs is the fact that the complex never enters a steady state during the oscillations. In Fig. 6B we show the time derivatives of the concentrations from panel A, which measure the rate of change. The assumption that the complex concentration changes much more slowly than the substrate lies at the heart of the QSSAs, but this does not hold in general; as seen in Fig. 6B the rate of change of the complex is comparable to that of the substrate, the total substrate and the kinase. We believe that this is why the tQSSA also fails for some parameters and, moreover, sometimes estimates the period of the oscillations badly (Fig. 5B). Consequently, it would in some cases be preferable to model the network by means of the full system. The implications of this approach and its flaws will be faced in the discussion.

Discussion

Far from being a collection of serial chemical reactions, the higher eukaryotic intracellular signal transduction networks are very intricate and highly complex. The increased amount of data and knowledge about these networks has made mathematical modeling and computational methods increasingly important in Systems Biology, and has led to projects such as the Silicon Cell, which aims at being a precise replica of the living cell. This means using experimentally found data and reproducing both qualitative and quantitative behavior of the cell.

So far most of the models describing enzyme reactions, e.g., in the MAPK cascade, have been based on the classical Michaelis-Menten approximation (sQSSA) and many of these did not consider competition between substrates. These approaches were taken, although parameters and initial conditions were chosen so that the validity criterion for the sQSSA no longer held and the competition could not be neglected. As exceptions, we mention Hatakeyama et al. [31] and Markevich et al. [32], who treated the problem of substrate competition in terms of the sQSSA for two substrates competing for the same kinase, but they did not consider the region of validity of the sQSSA and neglected the enzyme-substrate complexes.

Although it was known that the sQSSA will often be invalid *in vivo*, the sQSSA approach was necessary for many years, since no better approximations were known, but this has changed recently with the introduction of the tQSSA. This approach was first applied to the simplest reactions [20, 21], and later to increasingly more complex schemes such as reversible reactions [22] and fully competing systems [23].

We have here presented the application of the tQSSA to biologically realistic networks, and shown that it is superior to the sQSSA in all the presented cases. We did not formally investigate the validity of the tQSSA for all the reaction networks examined, and found in fact that the tQSSA has its limitations as well (Table 1), probably related to the fact that the complexes do not always enter a quasi-steady state (Fig. 6B). However, based on our simulations we feel confident in saying that compared to the

sQSSA it provides a more accurate estimate of the behavior of enzyme networks. For example, it was found that the tQSSA estimates the steady state levels of activated MAPK very well (Figs. 3 and 5A), while the sQSSA often fails dramatically. We believe that the main reason for this is the fact that the tQSSA incorporates the complex concentrations while the sQSSA does not, as stated for example in the conservation law $[\text{MAPK}]_T = [\text{MAPK}] + [\text{MAPK-P}] + [\text{MAPK-PP}]$ [3, 32].

To illustrate the importance of a reliable estimation of the MAPK dynamics and steady-state levels, we remark that is has been suggested that both the duration and intensity of the activated MAPK is crucial for the fate of the cell ([40] and references therein). For example, rat PC12 cells differentiate if stimulated by NGF and proliferate if stimulated by EGF [41, 42], although the cognate receptors use the same signaling cytoplasmic network to transduce the signal to the nucleus. In the two cases, the most evident difference is that NGF induces a sustained MAPK (ERK) activity, while EGF induces a transient MAPK (ERK) activity (see [36] for a review). Recently, it was also shown that PC12 [42] and Kaposi Sarcoma [43] cells are sensitive to the strength of the MAPK signal indicating a threshold phenomenon, which means that even minor changes in the levels of activated MAPK can have dramatic consequences.

We showed that the choice of the approximation scheme could dramatically change the size of the parameter range in which oscillations occur in the MAPK cascade with a competitive, negative feedback (Fig. 5A and Table 1).

If any Silicon Cell should help to discover pharmaceutically sensitive targets and reproduce the effects of drugs on these targets, the quantitative aspects of the model would have to be carefully studied and resolved, for example in estimating the size of the above parameter windows. For instance, continuing the example of the MAPK cascade with inhibitory feedback, assume that we wish to apply a drug in order to create oscillations. Lowering the K_I value would appear promising on the basis of the model using the sQSSA, since this model predicts oscillations in a rather wide parameter range (Table 1). However, this could encourage a waste of resources searching for an appropriate pharmaceutical compound, since the drug would have to be very finely

tuned and, hence, difficult to find, because the full system has a very narrow parameter range yielding oscillations. Thus, one might be better off looking for a drug acting elsewhere in the network.

Since the tQSSA, although superior to the sQSSA, also does not always work, one could suggest to use the alternative of simulating each step of the reaction by means of the full system of ODEs, which means describing every reaction in terms of two equations, and facing three instead of two parameters for every reaction, as it has been done for example for the MAPK cascade [4]. However, more equations would mean, especially for larger systems, that this approach quickly would become computer expensive.

A more serious problem is the fact that the three rate constants (a, d and k in (1)) are usually unknown, while finding the QSSA parameters K_M and V_{max} (or $k_{cat} = V_{max}/E_T$) is a standard procedure in biochemistry. Thus, the reduction obtained from the QSSA is in this sense an advantage compared to the full system. We could in any case rebuild the parameters a, d, k starting from the MM parameters, but as shown in Appendix B, we then introduce a degree freedom. Bhalla and Iyengar [2] try to overcome this problem supposing that $d = 4k$, but this hypothesis seems to us a bit arbitrary without any strong experimental support, as already remarked by the authors. However, we have applied this assumption through out this work when modeling the full system.

The validity of the tQSSA depends on the precise values of a, d and k as stated for example in (13) for the case of a single reaction: The smaller the ratio K/K_M, i.e., the larger the ratio d/k, the better the approximation. However, for any choice with a large ratio d/k, the tQSSA holds. A similar result holds for fully competitive reactions [23]. This is consistent with the choice of $d = 4k$ and supported by the fact that for many enzymes the parameter d is much greater than k [44, 45].

From a theoretical point of view, the application of the tQSSA in this way makes the actual parameter values of a, d and k less important. When we *a priori* know that

the system can be well-approximated by the tQSSA, all the possible choices of a, d, k will give approximations near each other, and hence, near the true solution, assuming that the true parameters are such that the tQSSA is valid. This can be used in cases where only the parameters K_M and V_{max} are available, the sQSSA is known not to hold, and only a very complicated tQSSA, too complicated to implement effectively on a computer, exists. One can then choose any relation between a, d and k giving the correct values for K_M and V_{max}, check that the tQSSA holds using a theoretically founded validity criteria, and then do the simpler implementation of the full system of equations.

Related to the above, but from another point of view, is the lack of reliable experimental data about the kinetic constants of the intracellular biochemical reactions, including K_M and V_{max} values. To reconstruct these missing parameter values, some authors rely on the so-called reverse engineering (or inverse problem). The classical approach to reverse engineering is based on least square techniques with the aim to find the set of parameters that gives the best fitting curve, i.e., the curve passing "as close as possible" to the experimental data. This is done searching for the global minimum of a function of as many variables as there are unknown parameters. To find the global minimum of these functions is in general far from trivial, for example due to the risk of finding only local, not global, minima. Furthermore the uniqueness of this minimum cannot, in general, be guaranteed; several sets of parameters could give the global minimum. This is the question of *a priori identifiability* [46].

As shown in the present work, the misuse of the sQSSA can lead to large quantitative and qualitative errors. However, even when the sQSSA is not a good approximation of the system, we can still find parameters for which the sQSSA does fit the data (the full system), by minimizing, e.g., the least square error. This would inevitably lead to wrongly estimated parameters, since the original ones did not provide a good approximation.

From these considerations it follows that the ability of the model to fit a certain data set can not be used to test whether a certain approximation holds. Applying reverse

engineering for the sQSSA, without any *a priori* examination of its validity, one could argue that the (mis)use of the sQSSA causes no problems, since we obtain a good fit anyway. However, one would prefer to have a model that works under many different conditions, not only in a certain experimental setting. If fitting the sQSSA model to the data yields wrong estimates of the parameters, then it is likely that the predicted behavior using these parameters would be far from the true behavior. The same would be true if the model was later used as a subsystem of an enlarged model. For example, the estimation of the Michaelis or inhibition constant relying on a wrong model formulation could be crucial as seen in the following example.

Assume that all the parameters except the inhibition constant K_I were known for the model illustrated by Fig. 4. If we had a data set for this model showing stable behavior, according to Table 1, using the sQSSA we would estimate a value of K_I greater than 3.02, even though the true value of K_I could be between 0.18 and 3.02. Assume now that we obtain a drug capable of lowering the K_I value according to some known mechanisms, and that we decide to administrate the drug to lower K_I with the aim to let the system oscillate. Believing that the sQSSA estimated K_I is the true value, we would apply a certain amount of the drug in order to get below the threshold value at $K_I = 3.02$. But the actual value of K_I could be completely different from the wrongly estimated one and such that the drug administration, though lowering K_I, would leave the system stable.

Similar problems can be expected to occur in metabolic control analysis [47–49], which is used to find the steps in the network that controls some output behavior, e.g., the concentration of a certain biochemical species. It seems likely that an invalid sQSSA model might predict that a certain step is the most important, while the full system or the corresponding tQSSA model finds that step to be less important. In the light of applications for the pharmaceutical industry, this could lead to a waste of money and energy focusing on an apparently sensitive target, which then turns out to be unimportant or, viceversa, the neglect of an important target that apparently seems unimportant.

In conclusion, we have shown that the use of the classical MM approach (sQSSA) should be done with much care, since it can lead to both quantitative and qualitative errors. This has further impact on techniques such as reverse engineering and metabolic control analysis. Finding approximations improving the sQSSA for complex reactions such as successive reactions, open systems, loops such as the Goldbeter-Koshland switch [50], feedback systems etc., and investigating their validity, should be of great interest for further investigations and simulations of such reactions *in vivo*, where the MM description can be expected to break down.

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Methods

We compare various approximation schemes of the time concentration development of the chemical species involved in the reactions. This is done by numerically solving the system of ordinary differential equations (ODEs) derived from the reaction scheme using the methods and approximations within the various approaches. To illustrate this idea we use the example of an isolated reaction

$$E + S \underset{d}{\overset{a}{\rightleftarrows}} C \xrightarrow{k} E + P. \tag{5}$$

The fundamental step is modeling all of the intermediate steps 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. We refer to this as the full system. For (5) the equations are

$$\frac{dS}{dt} = -a(E_T - C)S + d\ C, \tag{6a}$$

$$\frac{dC}{dt} = a(E_T - C)S - (d + k)C\ . \tag{6b}$$

with the initial conditions

$$S(0) = S_T, \quad C(0) = 0, \tag{7}$$

and the conservation laws

$$E + C = E_T, \quad S + C + P = S_T. \tag{8}$$

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 [12, 14, 19]. Let us observe that this system (6) 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.

The next, well-known and widely used step is that of the Henri-Michaelis-Menten-Briggs-Haldane approximation [9–15]. It leads to an ODE for each substrate while the complexes are assumed to be in a quasi-steady state (i.e., $\frac{dC}{dt} \approx 0$). See e.g. [19] for a general introduction to this approach. We stress here that this is an approximation to the full system, and that (for (5)) it is only valid at low enzyme concentrations, i.e., $E_T \ll S_T + K_M$ [14, 15]. We refer to this as the standard quasi-steady state approximation (sQSSA). For (5) it is given by

$$\begin{aligned} \frac{d\,S}{dt} &\approx -\frac{V_{max} S}{K_M + S}, \quad S(0) = S_T, \\ E(0) = E_T, \quad V_{max} &= k\,E_T, \quad K_M = \frac{d+k}{a}. \end{aligned} \tag{9}$$

When we have more than one reaction in the system we denote the MM constant for reaction i by K_i^M, and the reaction constants by a_i, d_i and k_i.

As mentioned in the introduction, *in vivo* we cannot in general assume a low enzyme concentration and hence, the MM approximation can not be expected to hold. A recent approach to resolve this problem is that of the total quasi-steady state assumption

(tQSSA). It was introduced by Borghans et al. [20] and refined by Tzafriri [21] for isolated reactions. We have recently extended it to fully competitive reactions [23]. The tQSSA [20, 21] arises by introducing the total substrate

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

and assuming that the complex is in a quasi-steady state as for the sQSSA. For (5) it gives [21]

$$\frac{d\,\bar{S}}{dt} \approx\ -k\,C_-(\bar{S}), \quad \bar{S}(0) = S_T, \tag{10}$$

where

$$C_-(\bar{S}) = \frac{(E_T + K_M + \bar{S}) - \sqrt{(E_T + K_M + \bar{S})^2 - 4E_T\bar{S}}}{2}. \tag{11}$$

Numerical integration of (10) easily gives the time behavior of $\bar{S}, C$ (by (11)) and S (by the relation $S = \bar{S} - C$).

Tzafriri [21] showed that the tQSSA (10) is valid whenever

$$\epsilon_{Tz} := \frac{K}{2S_T}\left(\frac{E_T + K_M + S_T}{\sqrt{(E_T + K_M + S_T)^2 - 4E_TS_T}} - 1\right) \ll 1, \quad \text{where} \quad K = \frac{k}{a}, \tag{12}$$

and that this is always roughly valid in the sense that

$$\epsilon_{Tz} \leq \frac{K}{4K_M} \leq \frac{1}{4}. \tag{13}$$

This means that for *any* combination of parameters and initial conditions (10) is a decent approximation to the full system (6). The parameter K is known as the Van Slyke-Cullen constant.

As a first order approximation to (10), Tzafriri [21] found the expression, obtained originally in [20] by different techniques,

$$\frac{d\,\bar{S}}{dt} \approx\ -\frac{V_{max}\bar{S}}{K_M + E_T + \bar{S}}, \quad \bar{S}(0) = S_T. \tag{14}$$

This approximation is valid at low enzyme concentrations $E_T \ll S_T + K_M$, where it reduces to the MM expression (9), but holds moreover at low substrate concentrations $S_T \ll E_T + K_M$ [21]. Thus, with minimal effort performing the substitutions

of S by $\bar{S}$ and of K_M by $K_M + E_T$ one obtains a significantly improved MM-like approximation, without any need of more advances mathematics.

We refer to the Appendix for the full system of differential equations describing the different networks investigated in the Results section.

The parameter values used for the MAPK cascade are given in figure captions or Appendix C (online material), Table 1. As shown in Appendix B, when going from experimentally obtained (MM) parameters (V_{max}, K_M) to the full system, there is one degree of freedom with respect to the choice of parameters (a, d, k). We have used the constraint proposed in [2, supplementary, on-line material] that $d = 4k$. This is consistent with the fact that for many enzymes the parameter d is much greater than k [44].

A The tQSSA for fully competitive enzyme reactions

The system (2) is governed by the coupled ODEs [14, 27, 51], $i = 1, 2$,

$$\frac{d\,S_i}{dt} = -a_i E \cdot S_i + d_i C_i, \qquad S_i(0) = S_{i,T}, \tag{15a}$$

$$\frac{d\,C_i}{dt} = a_i (E \cdot S_i - K_i^M C_i), \qquad C_i(0) = 0, \qquad K_i^M = \frac{d_i + k_i}{a_i}. \tag{15b}$$

and the conservation laws

$$S_{i,T} = S_i + C_i + P_i, \qquad i = 1, 2, \tag{16}$$

$$E_T = E + C_1 + C_2. \tag{17}$$

The sQSSA of this system is [14, 51]

$$\frac{d\,S_i}{dt} \approx -\frac{k_i E_T S_i}{K_i^M (1 + S_j/K_j^M) + S_i}\ , \quad S_i(0) = S_{i,T}, \qquad i = 1, 2, \quad j \neq i, \tag{18}$$

which is valid when [27]

$$\frac{E_T}{K_i^M (1 + S_{j,T}/K_j^M) + S_{i,T}} \ll 1\ , \qquad i = 1, 2, \quad j \neq i. \tag{19}$$

As in the non-competitive case, it says that the sQSSA holds at low enzyme concentrations.

We have improved these results [23], applying the tQSSA to both reactions and showing that the tQSSA is given by finding C_1 as the unique biologically acceptable root ($0 < C_1 < \min\{E_T, \bar{S}_1\}$) of the third degree polynomial

$$\begin{aligned}\psi_1(C_1) = &-(K_1^M - K_2^M)C_1^3 \\ &+ \big[(E_T + K_1^M + \bar{S}_1)(K_1^M - K_2^M) - (\bar{S}_1 K_2^M + \bar{S}_2 K_1^M)\big]C_1^2 \\ &+ \big[- E_T(K_1^M - K_2^M) + (\bar{S}_1 K_2^M + \bar{S}_2 K_1^M) + K_2^M(E_T + K_1^M)\big]\bar{S}_1 C_1 \\ &- E_T K_2^M \bar{S}_1^2,\end{aligned} \tag{20}$$

and similarly finding C_2 as the root in the polynomial ψ_2 obtained by interchanging the indices 1 and 2 in (20). After a short transient phase the complex concentrations are assumed to equal the quasi steady-state concentrations, $C_i = C_i(\bar{S}_1, \bar{S}_2)$, given by the roots in the respective polynomials as discussed above. Then the evolution of the system can be studied by means of the tQSSA

$$\frac{d\,\bar{S}_i}{dt} \approx -k_i C_i(\bar{S}_1, \bar{S}_2), \qquad \bar{S}_i(0) = S_{i,T}. \tag{21}$$

This approach extends both the sQSSA for competitive reactions (18) as well as the tQSSA for isolated reactions (10) as shown in [23].

B Relationship among the kinetic parameters

While every reaction is characterized by three constant rates (a, d, k), its QSSA works with only two parameters: V_{max} and K_M. Since $K_M = \frac{k+d}{a}$ and $V_{max} = kE_T$, we have

$$k = \frac{V_{max}}{E_T}, \qquad a = \frac{k+d}{K_M} = \frac{V_{max} + dE_T}{E_T K_M}. \tag{22}$$

Posing $d = 4k$ [2, on-line material] we uniquely obtain the values of a, d and k from K_M and V_{max} as

$$k = \frac{V_{max}}{E_T}, \qquad d = \frac{4V_{max}}{E_T}, \qquad a = \frac{5V_{max}}{E_T K_M}. \tag{23}$$

In general, posing $d = \alpha k$ we have

$$k = \frac{V_{max}}{E_T}, \qquad d = \frac{\alpha V_{max}}{E_T}, \qquad a = \frac{k+d}{K_M} = \frac{(1+\alpha)V_{max}}{E_T K_M}, \tag{24}$$

with a freedom degree, related to the value of α. Consequently, it is possible to vary the triplet (a, d, k) obtaining the same pair (V_{max}, K_M).

However, the different choices of (a, d, k) could produce significantly different outputs, and thus predict completely different behavior in the solutions of the full system and of its QSSAs, respectively.

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List of Tables

Method	Oscillations
Full system	$K_I < 0.18$
Competitive tQSSA	$K_I < 0.86$
Competitive sQSSA	$K_I < 3.02$

Table 1: Regions of oscillations for the MAPK cascade with feedback expressed by the inhibition constant K_I.

List of Figures

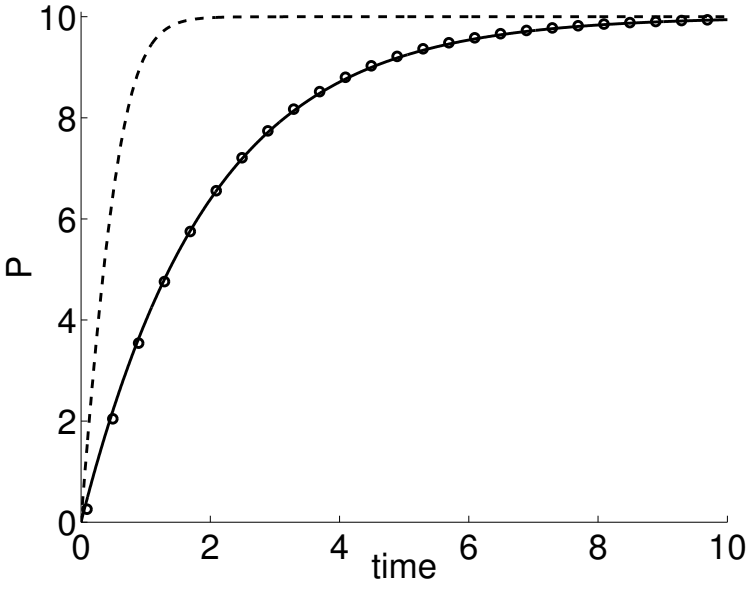

Figure 1: Temporal evolution of the product P at high enzyme concentrations for the single reaction (1). In this case, the solution of the full system (circles) is badly approximated by the MM approximation (sQSSA, dashed curve), while the tQSSA (full curve) estimates the behavior very well. Parameters are $k = 0.6, K_M = 8, E(0) = E_T = 50$, and $S(0) = S_T = 10$, all in arbitrary units.

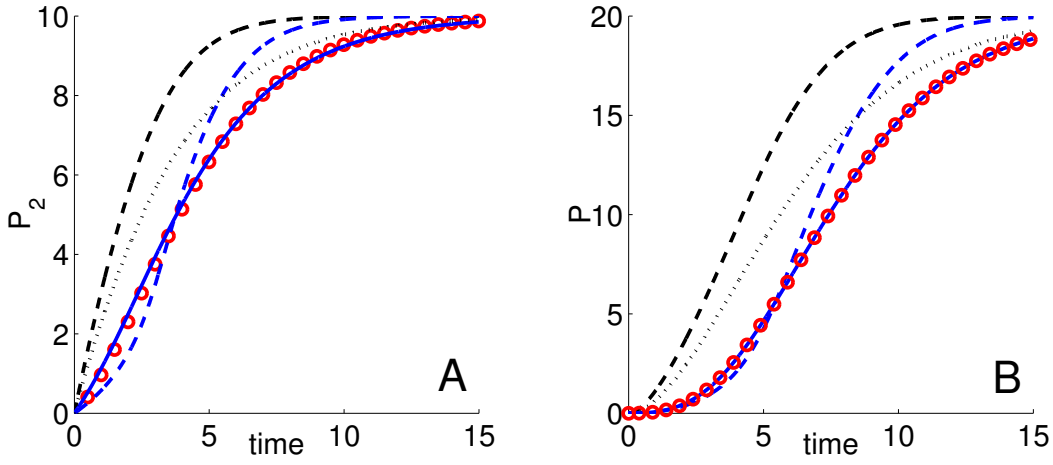

Figure 2: Competitive systems. Panel A: A simulation of competing substrates (scheme (2)). Panel B: A simulation of two successive reactions catalyzed by the same enzyme (scheme (3)). In both cases the full system (red circles) is estimated very well by the competitive tQSSA (blue, full curve), while the competitive sQSSA (blue, dashed curve) as well as the non-competitive sQSSA (black, dashed curve) and tQSSA (black, dotted curve) do not fit. The parameters are in both panels: $k_1 = 0.5, k_2 = 0.6, K_1^M = 0.75, K_2^M = 8, E_{tot} = 10$, and $S_1(0) = S_2(0) = 10$ in panel A, $S_1(0) = 20, S_2(0) = 0$ in panel B. All units are arbitrary.

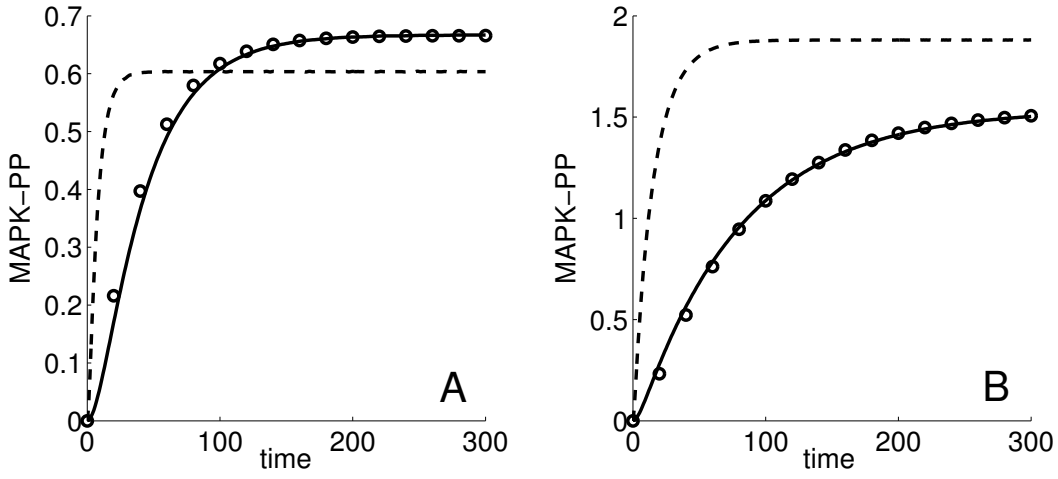

Figure 3: Phosphorylation and dephosphorylation. The sQSSA (dashed line) leads to a wrong estimation of both the transient behavior as well as steady state levels, while the tQSSA (full line) fits well, for the double phosphorylation/dephosphorylation (4) modelled with MM kinetics. The full system is shown as circles. Parameters: $[\text{MAPK}]_T = 500$ (panel A), $[\text{MAPK}]_T = 50$ (panel B), $[\text{MKP}]_T = 100, [\text{MAPKK}]_T = 50,\ K_1^M = 50,\ K_2^M = 500,\ K_3^M = 22,\ K_4^M = 18,$ $k_1 = 0.01,\ k_2 = 15,\ k_3 = 0.084,\ k_4 = 0.06.$ (Concentrations and time in arbitrary units, but for consistence with [32] one can think of nM and seconds).

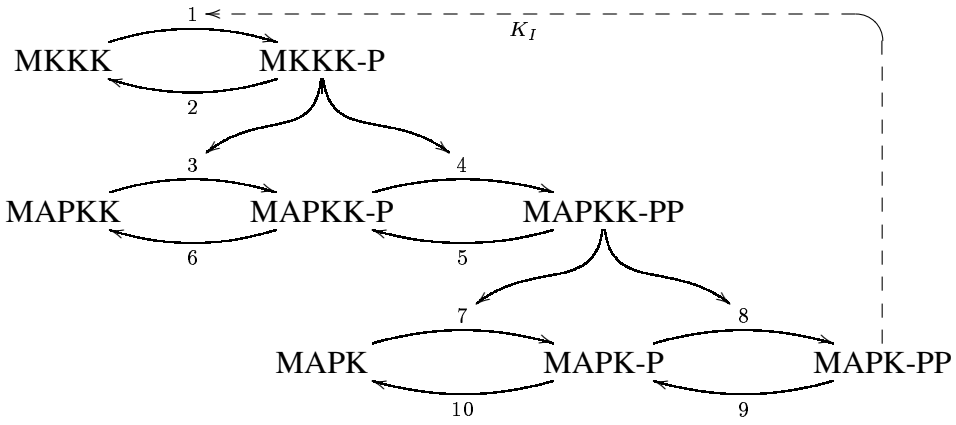

Figure 4: The MAPK cascade. The diagram is based on [3]. Each of the reactions is assumed to follow MM kinetics, but there are competitive reactions since MKKK-P catalyzes both reactions 3 and 4 and MAPKK-PP catalyzes both reactions 7 and 8. Similarly, reactions 5 and 6 are assumed to compete for a phosphatase, and both reactions 9 and 10 to be catalyzed by another phosphastase (MKP in (4)). The phosphatases are not shown for clarity of the figure. The dashed line indicates inhibition of reaction 1 by MAPK-PP as in [3]. However, we assume that this inhibition is competitive. *M*-P and *M*-PP represent, respectively, monophosphorylated and diphosphorylated *M*, where *M* is either MKKK, MAPKK or MAPK.

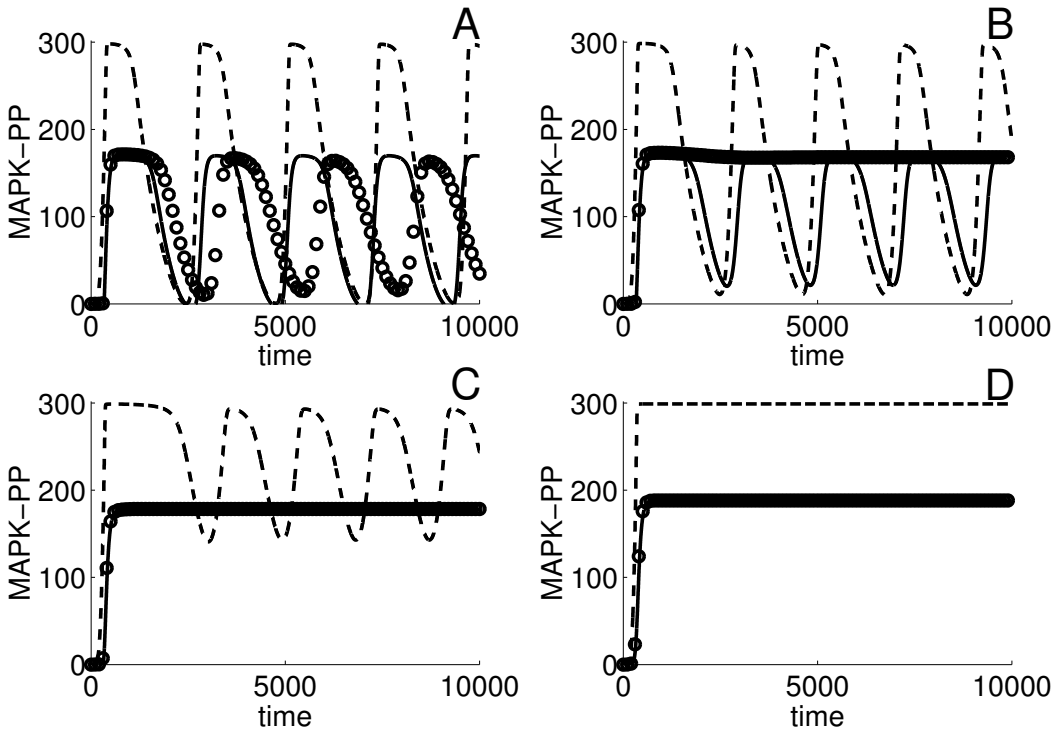

Figure 5: Simulations of the MAPK cascade with feedback as in Fig. 4. The computed MAPK-PP concentration is shown following the legends in Fig. 3. The values of the inhibition constant are as follows: Panel A: $K_I = 0.1$. Panel B: $K_I = 0.5$. Panel C: $K_I = 2.5$. Panel D: $K_I = 20$. At low values of K_I (panel A), all the three schemes, full system (circles), tQSSA (full curve) and sQSSA (dashed curve), produce oscillations, but the tQSSA follows the solution much better than the sQSSA, especially with respect to the amplitude of the oscillations. In panel C, although the MAPK-PP modelled by the full system (circles) almost immediately reaches a steady-state, the sQSSA (dashed curve) shows oscillations. On the other hand, the full system is followed very well by the tQSSA (full curve). However, this is not always the case, since the tQSSA can also predict oscillations when the full system is stable (panel B). Finally, at high values of K_I all the three approaches go to a steady state, but the sQSSA overestimates the MAPK-PP level significantly (panel D).

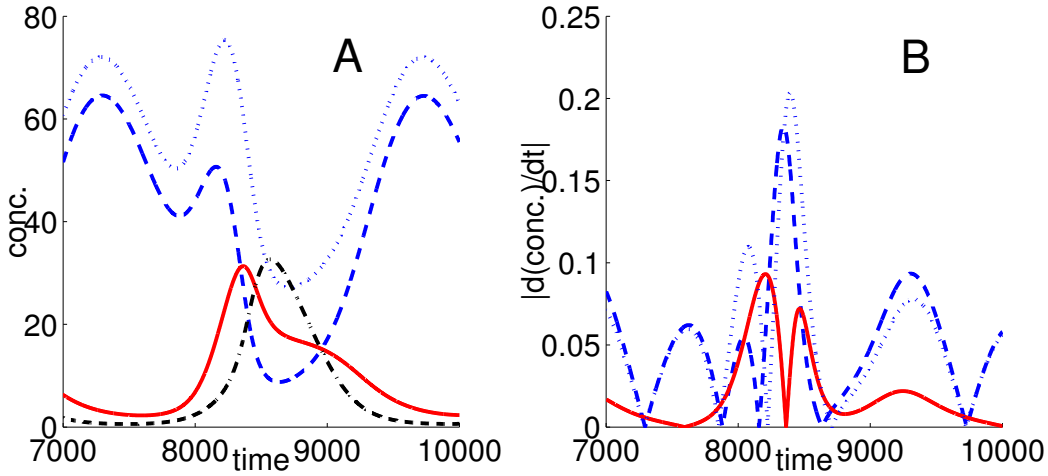

Figure 6: Non-neglectible and non-constant complex concentrations in the MAPK cascade. In the full system describing the MAPK cascade with feedback, the complex (MAPK-P)–(MAPKK-PP) is neither negligible (panel A) nor approximately constant (panel B). Panel A shows the concentrations of the complex (MAPK-P)–(MAPKK-PP) (red, full curve), MAPK-P (blue, dashed curve), "total MAPK-P" ($\overline{\text{MAPK-P}}$; blue, dotted curve) and MAPKK-PP (black, dash-dot curve) during the last part of the simulation of the full system from figure 5B. Panel B shows the absolute value of the time derivative of the complex, MAPK-P and $\overline{\text{MAPK-P}}$ (same legends as in panel A).