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The total quasi-steady-state approximation for complex enzyme reactions

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Abstract

Biochemistry in general and enzyme kinetics in particular have been heavily influenced by the model of biochemical reactions known as Michaelis–Menten kinetics. 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)), which is valid when the enzyme concentration is sufficiently small. This condition is usually fulfilled for *in vitro* experiments, but often breaks down *in vivo*. The total QSSA (tQSSA), which is valid for a broader range of parameters covering both high and low enzyme concentrations, has been introduced in the last two decades. We extend the tQSSA to more complex reaction schemes, like fully competitive reactions, double phosphorylation, Goldbeter–Koshland switch and we show that for a very large range of parameters our tQSSA provides excellent fitting to the solutions of the full system, better than the sQSSA and the single reaction tQSSA. Finally, we discuss the need for a correct model formulation when doing "reverse engineering", which aims at finding unknown parameters by fitting the model to experimentally obtained data. We show that the estimated parameters are much closer to the real values when using the tQSSA rather than the sQSSA, which overestimates the parameter values greatly.

Keywords: Signal transduction; Enzyme kinetics; Reverse engineering

1. Introduction

One of the principal components of the mathematical approach to Systems Biology is the model of biochemical reactions set forth by Henri in 1901 [8–10] and Michaelis and Menten in 1913 [12], and further developed by Briggs and Haldane in 1925 [4]. This formulation considers a reaction where a substrate S binds to 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

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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 next 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 [18,19]. This condition is usually fulfilled for *in vitro* experiments, but often breaks down *in vivo* [21,20]. We refer to the next section for the mathematical formulation of scheme (1), and to [16] 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 [2]. 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) [3,22], which is valid for a broader range of parameters covering both high and low enzyme concentrations, have been introduced recently. Tzafriri [22] 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 MM 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., [2]). However, since these approximations cannot be expected to be valid *in vivo*, employing the tQSSA to these more complex situations would be preferable. Tzafriri and Edelman [23] studied the completely reversible enzyme reaction in terms of the tQSSA. We have recently derived the tQSSA for fully competitive reactions [13].

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.

We refer to [14] for further biological examples. We also discuss the use of reverse engineering as a tool of obtaining missing parameters, and show that the sQSSA can lead to wrong estimates, while the tQSSA finds estimates closer to the real values.

2. Theoretical background

We recall briefly the mathematical description of the sQSSA for (1), using the same symbols for the concentrations of the reactants. The reaction (1) can be described by the following system of nonlinear ordinary differential equations

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

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

with the initial conditions

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

and the conservation laws

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

Assuming that the complex is in a quasi-steady-state, i.e., $dC/dt \approx 0$, leads to [4,18,19]

$$\begin{aligned} &\frac{dS}{dt} \approx -\frac{V_{max} S}{K_M + S}, \quad S(0) = S_T, \\ &E(0) = E_T, \quad V_{max} = kE_T, \quad K_M = \frac{d+k}{a}. \end{aligned} \tag{6}$$

Here V_{max} is the maximal reaction rate and K_M is the Michaelis constant, identifying the substrate concentration giving the half-max reaction rate, i.e., K_M reflects the substrate affinity of the enzyme. This approximation is valid whenever [18,19]

$$\frac{E_T}{K_M + S_T} \ll 1, \tag{7}$$

i.e., at low enzyme concentrations.

The tQSSA [3,22] arises by introducing the total substrate

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

Assuming that the complex is in a quasi-steady-state yields the tQSSA

$$\frac{d\bar{S}}{dt} \approx -k\, C_{-}(\bar{S}), \quad \bar{S}(0) = S_T, \tag{8}$$

where

$$C_{-}(\bar{S}) = \frac{(E_T + K_M + \bar{S}) - \sqrt{(E_T + K_M + \bar{S})^2 - 4E_T\bar{S}}}{2}. \tag{9}$$

Tzafriri [22] showed that the tQSSA is valid whenever

$$\epsilon_{Tz} := \frac{K}{2S_T}\left(\frac{E_T + K_M + S_T}{\sqrt{(E_T + K_M + S_T)^2 - 4E_T S_T}} - 1\right) \ll 1, \qquad K = \frac{k}{a}, \tag{10}$$

and that this is always roughly valid in the sense that

$$\epsilon_{Tz} \leq \frac{K}{4K_M} \leq \frac{1}{4}. \tag{11}$$

The parameter K is known as the Van Slyke-Cullen constant. Tzafriri [22] expanded Eqs. (9) and (10) in terms of

$$r(\bar{S}) = \frac{4E_T\bar{S}}{(E_T + K_M + \bar{S})^2} < 1 \tag{12}$$

and assuming the validity of the tQSSA ($\epsilon_{Tz} \ll 1$) and $r \ll 1$, he found

$$\frac{d\bar{S}}{dt} \approx -\frac{V_{max}\bar{S}}{K_M + E_T + \bar{S}}, \quad \bar{S}(0) = S_T, \tag{13}$$

as a first order approximation to (8). This expression (13) is identical to the formula obtained by Borghans et al. [3] by means of a two point Padé approximant technique [1].

This approximation is valid at low enzyme concentrations (7) where it reduces to the MM expression (6), but holds moreover at low substrate concentrations $S_T \ll E_T + K_M$ [22]. We wish to highlight the fundamental fact that 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 with minimal effort.

3. Total quasi-steady-state approximation of the competitive system

In [13] we investigated the system

$$\begin{aligned} &S_1 + E \underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \overset{k_1}{\rightarrow} E + P_1, \\ &S_2 + E \underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \overset{k_2}{\rightarrow} E + P_2, \end{aligned} \tag{14}$$

which consists of two reactions catalyzed by the same enzyme, i.e., a system with competing substrates. It is governed by the coupled ODEs [15,18,17], $i = 1, 2$,

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

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

and the conservation laws

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

$$E_{\mathrm{T}} = E + C_1 + C_2. \tag{17}$$

The sQSSA of this system is [15,18]

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

which is valid when [17]

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

In [13], following [3], we introduced the total substrates

$$\bar{S}_i = S_i + C_i, \quad i = 1, 2, \tag{20}$$

and rewrote Eq. (15) in terms of these, obtaining the system of ODEs, $i = 1, 2$,

$$\frac{\mathrm{d}\bar{S}_i}{\mathrm{d}t} = -k_i C_i, \quad \bar{S}_i(0) = S_{i,\mathrm{T}}, \tag{21a}$$

$$\frac{\mathrm{d}C_i}{\mathrm{d}t} = a_i \left((E_{\mathrm{T}} - C_1 - C_2) \cdot (\bar{S}_i - C_i) - K_i^{\mathrm{M}} C_i \right), \quad C_i(0) = 0. \tag{21b}$$

where we have introduced the MM constants

$$K_i^{\mathrm{M}} = \frac{d_i + k_i}{a_i}.$$

We required $0 < C_i < \bar{S}_i$, $i = 1, 2$, because of (20), and applied the quasi-steady-state assumption [3,22],

$$\frac{\mathrm{d}C_i}{\mathrm{d}t} \approx 0, \quad i = 1, 2,$$

which is equivalent to the system

$$C_1 = E_{\mathrm{T}} - C_2 \left(1 + \frac{K_2^{\mathrm{M}}}{\bar{S}_2 - C_2} \right), \tag{22a}$$

$$C_2 = E_{\mathrm{T}} - C_1 \left(1 + \frac{K_1^{\mathrm{M}}}{\bar{S}_1 - C_1} \right). \tag{22b}$$

Then $C_i < E_T$, $i = 1, 2$, in agreement with (17).

Substituting (22b) into (22a) leads to the following equation in C_1

$$C_1 = E_T - \left(E_T - C_1(1 + \frac{K_1^M}{\bar{S}_1 - C_1})\right)\left(1 + \frac{K_2^M}{\bar{S}_2 - (E_T - C_1(1 + (K_1^M/(\bar{S}_1 - C_1))))}\right) \tag{23}$$

and C_2 can then be found from (22b).

Solving (23) is equivalent to finding roots of the third degree polynomial

$$\begin{aligned}\psi_1(C_1) \quad &= -(K_1^M - K_2^M)C_1^3 \\ &+ \left[(E_T + K_1^M + \bar{S}_1)(K_1^M - K_2^M) - (\bar{S}_1 K_2^M + \bar{S}_2 K_1^M)\right] C_1^2 \\ &+ \left[-E_T(K_1^M - K_2^M) + (\bar{S}_1 K_2^M + \bar{S}_2 K_1^M) + K_2^M(E_T + K_1^M)\right] \bar{S}_1 C_1 \\ &- E_T K_2^M \bar{S}_1^2.\end{aligned} \tag{24}$$

An analogous polynomial ψ_2 for C_2 can be found by interchanging the indexes 1 and 2 in (24), because of the symmetry of the system Eq. (21). Rearranging the terms, ψ_1 can also be written

$$\begin{aligned}\psi_1(C_1) = \quad & K_2^M(C_1 - E_T)\left(\bar{S}_1 - C_1\right)^2 \\ & + K_1^M C_1(C_1 + K_2^M + \bar{S}_2 - E_T)(\bar{S}_1 - C_1) + (K_1^M C_1)^2.\end{aligned} \tag{25}$$

We have shown the existence and uniqueness of biologically consistent roots of $\psi_1(C_1)$ and $\psi_2(C_2)$, which we will indicate with $C_i = C_i(\bar{S}_1, \bar{S}_2)$. We expect that after a short transient phase the complex concentrations 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

$$\frac{d\bar{S}_i}{dt} \approx -k_i C_i(\bar{S}_1, \bar{S}_2), \quad \bar{S}_i(0) = S_{i,T}. \tag{26}$$

Operating approximations for the two different regions of parameter space: $K_1^M \gg K_2^M$ and $K_1^M \ll K_2^M$ and matching them by means of a two point Padé approximant (TPPA) techniques, gives then

$$\frac{d\bar{S}_i}{dt} = -\frac{k_i E_T \bar{S}_i}{K_i^M(1 + \bar{S}_j/K_j^M) + \bar{S}_i + E_T}, \quad \bar{S}_i(0) = S_{i,T}, \quad j \neq i. \tag{27}$$

where $i = 1$, $j = 2$.

This formula reduces not only to the special case of identical affinities but also to the sQSSA (18) whenever this approximation holds as guaranteed by (19), and to the single reaction first order tQSSA (13) when $\bar{S}_j/K_j^M$ can be neglected.

Motivated by this and further encouraged by numerical simulations (see Fig. 1), we proposed the expression (27)(for $i = 1, 2$) as the general first order approximation to the tQSSA for fully competitive reactions.

Although not strictly theoretically founded, the above considerations using the TPPA can be seen as the motivation for the formula.

Our results are immediately applicable to, e.g., successive reactions catalyzed by the same enzyme, such as non-processive or distributive double phosphorylation or dephosphorylation processes, as seen, for example, in the MAPK cascade [5,6,24,11]. The reaction scheme can be seen as a special case of (14) with $P_1 = S_2$ and is summarized as

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

where it is usually assumed that at the beginning only S_1 is present. Fig. 2 shows that the results presented here are often a good approximation.

However, it should be remarked that our theoretical investigation of the validity of the tQSSA does not work in the case of successive reactions. The problem is that there is no S_2 at time $t = 0$, and hence the time scales cannot be found following [18] because the definition of the transient phase no longer holds.

Nevertheless, it seems like the conclusions concerning the validity of the first order approximation from above carries over to this scenario (compare the three panels of Fig. 1 with the panels of Fig. 2).

We will present the investigation of such reactions in another paper.

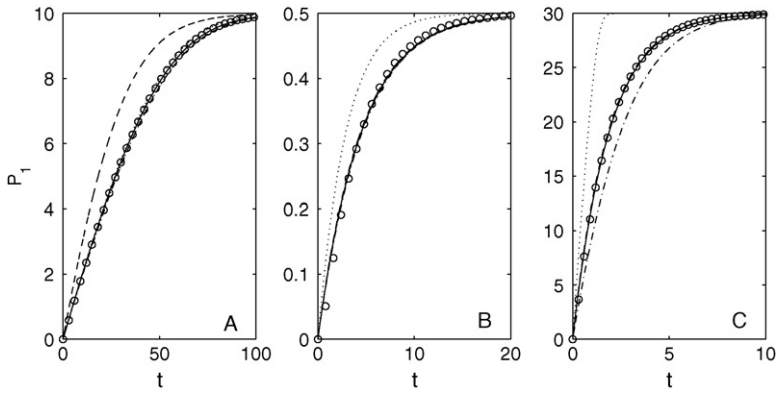

Fig. 1. QSSAs for the fully competitive reaction. The first order approximation (dash–dot curve) coincides with the competitive sQSSA (dotted curve) when it is valid (panel A), and with the single reaction tQSSA (dashed curve) when the competition is negligible (panel B). However, at high enzyme concentrations the single reaction tQSSA is often a better approximation than the first order tQSSA (panel C). Parameters are $a_1 = a_2 = 0.2$, $d_1 = d_2 = 1$, $k_1 = 0.6$, $k_2 = 0.5$, ($K_1^{\mathrm{M}} = 8$, $K_2^{\mathrm{M}} = 7.5$, $K = 3$). In A: $S_{1,\mathrm{T}} = S_{2,\mathrm{T}} = 10$, $E_{\mathrm{T}} = 1$. $\epsilon = 0.0038$. In B: $S_{1,\mathrm{T}} = S_{2,\mathrm{T}} = 0.5$, $E_{\mathrm{T}} = 5$. $\epsilon = 0.0832$. In C: $S_{1,\mathrm{T}} = S_{2,\mathrm{T}} = 30$, $E_{\mathrm{T}} = 100$. $\epsilon = 0.0219$. All units are arbitrary.

4. Total quasi-steady-state approximation of the Goldbeter–koshland switch

Goldbeter and Koshland [7] considered the following reaction

$$\begin{aligned} S + E_1 &\underset{d_1}{\overset{a_1}{\rightleftarrows}} C_1 \xrightarrow{k_1} E_1 + P, \\ P + E_2 &\underset{d_2}{\overset{a_2}{\rightleftarrows}} C_2 \xrightarrow{k_2} E_2 + S, \end{aligned} \tag{28}$$

which describes, for example, the cycle of phosphorylation and dephosphorylation of a substrate, by means of a kinase E_1 and a phosphatase E_2. 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 reaction is governed by the coupled ODEs

$$\frac{\mathrm{d}S}{\mathrm{d}t} = -a_1 E_1 \cdot S + d_1 C_1, \qquad S(0) = S_{\mathrm{T}}, \tag{29a}$$

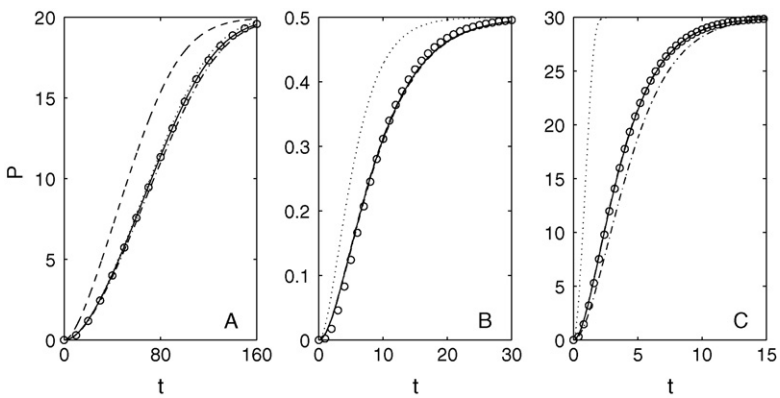

Fig. 2. Succesive reactions. The tQSSA estimates the development of the product of two successive reactions catalyzed by the same enzyme well, and the discussion of the validity of the sQSSA, the single reaction tQSSA and the first order tQSSA apparently carries over to this case. Legends and parameters are as in Fig. 1 except for the initial substrate concentrations, which are $S_{2,\mathrm{T}} = 0$ in all panels and: In A: $S_{1,\mathrm{T}} = 20$. In B: $S_{1,\mathrm{T}} = 0.5$. In C: $S_{1,\mathrm{T}} = 30$. All units are arbitrary.

$$\frac{dC_1}{dt} = a_1 E_1 \cdot S - (d_1 + k_1)C_1, \qquad C_1(0) = 0, \tag{29b}$$

$$\frac{dC_2}{dt} = a_2 E_2 \cdot P - (d_2 + k_2)C_2, \qquad C_2(0) = 0. \tag{29c}$$

and the conservation laws

$$S_T = S + C_1 + C_2 + P, \tag{30}$$

$$E_{i,T} = E_i + C_i \quad , \quad i = 1, 2. \tag{31}$$

The sQSSA of this system is given by setting $dC_1/dt \approx dC_2/dt \approx 0$ and neglecting the complex concentrations which yields

$$\frac{dS}{dt} = \frac{k_2 E_{2,T}(S_T - S)}{K_2^M + S_T - S} - \frac{k_1 E_{1,T} S}{K_2^M + S}, \qquad \bar{S}(0) = S_T. \tag{32}$$

Introducing the total substrates $\bar{S} = S + C_1$, $\bar{P} = P + C_2$, we rewrite the Eq. (29) in the following way:

$$\frac{d\bar{S}}{dt} = k_2 C_2 - k_1 C_1 = -\frac{d\bar{P}}{dt}, \qquad \bar{S}(0) = S_T, \tag{33a}$$

$$\frac{dC_1}{dt} = a_1(E_{1,T} - C_1) \cdot (\bar{S} - C_1) - (d_1 + k_1)C_1, \qquad C_1(0) = 0, \tag{33b}$$

$$\frac{dC_2}{dt} = a_2(S_T - \bar{S} - C_2) \cdot (E_{2,T} - C_2) - (d_2 + k_2)C_2, \qquad C_2(0) = 0. \tag{33c}$$

Assuming the tQSSA $dC_i/dt \approx 0$ and considering only the biologically significant roots C_i, we arrive at the following equation

$$\frac{d\bar{S}}{dt} \approx k_2 C_2^- - k_1 C_1^-, \qquad \bar{S}(0) = S_T \tag{34}$$

where

$$C_1^- = \frac{(\bar{S} + E_{1,T} + K_1^M) - \sqrt{(\bar{S} + E_{1,T} + K_1^M)^2 - 4\bar{S}E_{1,T}}}{2}, \tag{35}$$

$$C_2^- = \frac{(S_T - \bar{S} + E_{2,T} + K_2^M) - \sqrt{(S_T - \bar{S} + E_{2,T} + K_2^M)^2 - 4(S_T - \bar{S})E_{2,T}}}{2} \tag{36}$$

and $K_i^M = (d_i + k_i)/a_i$.

Formulas (35) and (36) show that, 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. In Fig. 3 we compare the simulations of the full system and of its sQSSA and tQSSA, and it is seen that the tQSSA is superior to the sQSSA.

The study of the sufficient conditions to guarantee the validity of the tQSSA, the time scales of the transient phases and a deeper comparison with the sQSSA are the subject of a paper in preparation.

5. Reverse engineering

Many biochemical systems, especially larger networks, are little understood, and many parameters are often unknown. A common method to obtain these parameters is to fit a mathematical model to experimentally obtained data, for example, by least square methods. This is often refered to as reverse engineering. However, a model formulated with the sQSSA cannot be expected to be a good representation of the full system as discussed in the previous sections. As for most other approaches, the use of this approximation produces excellent goodness of fit. But for the sQSSA such a good fit will necessarily correspond to parameter values far from the true ones. Indeed, inserting the true values in the sQSSA model does, in general, not approximate the full system. Instead, we expect that the tQSSA will reduce this problem consistently.

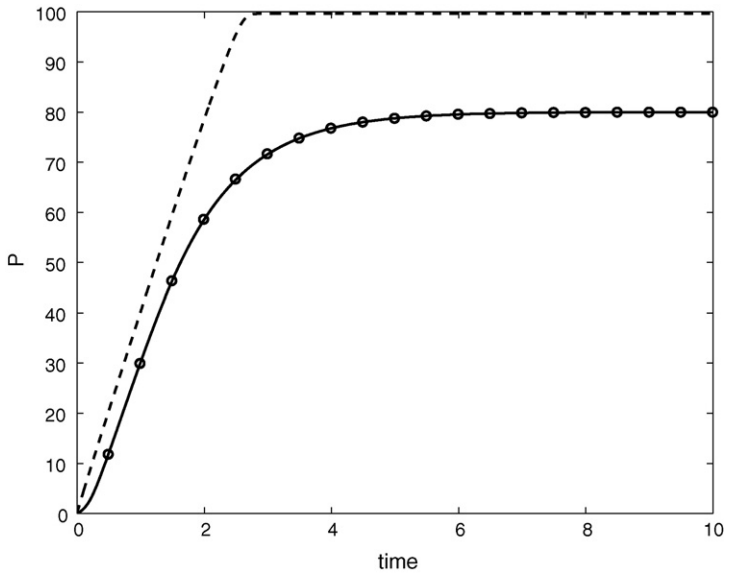

Fig. 3. The tQSSA estimates the development of the product P of the Goldbeter–Koshland switch well. Legends are: Full system (circles), sQSSA (dashed line) and tQSSA (full curve), where we show $P_{\mathrm{tQSSA}} := \bar{P} - C_2^-$ for a correct comparison for the tQSSA. The parameters are $S_{\mathrm{T}} = 100$, $E_{1,\mathrm{T}} = 50$, $E_{2,\mathrm{T}} = 10$, $a_1 = a_2 = 4$, $d_1 = d_2 = 5$, $k_1 = k_2 = 1$, all in arbitrary units.

To investigate this issue, we compare the results of parameter estimation using the two QSSA approaches for the simplest case of a single reaction (1), which concerns the evaluation of k and K_{M}. This was done using observations of the product P, which were generated by simulating the full system with the "true" parameter values $k = 0.6$ and $K_{\mathrm{M}} = 10$, extracting 50 points in time and adding noise from a normal distribution to each of these points. The

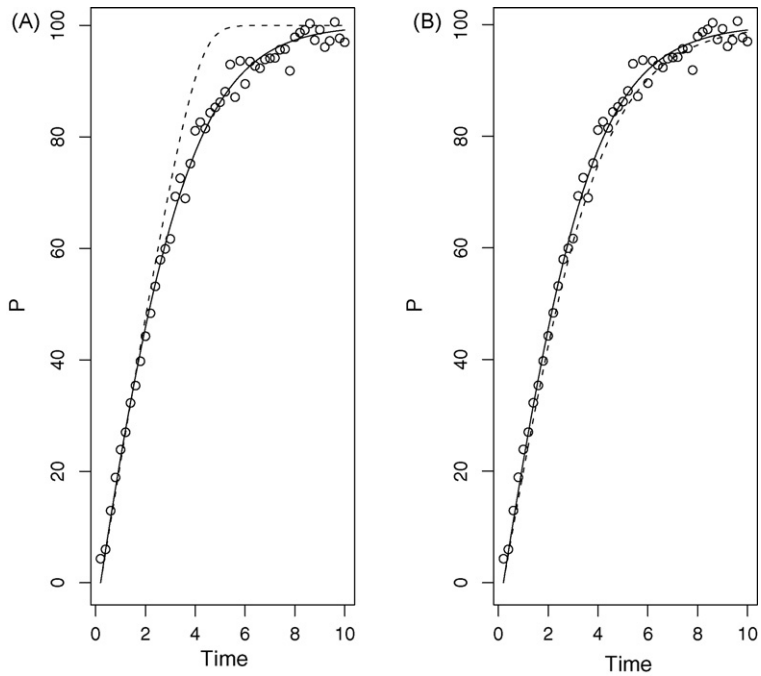

Fig. 4. Parameter estimation of k and K_{M} using perturbed observations from the full system with $k = 0.6$, $K_{\mathrm{M}} = 10$, $S_{\mathrm{T}} = 100$ and $E_{\mathrm{T}} = 50$ with noise from a $N(0, 2^2)$ distribution. Panels A and B show the fitting using the sQSSA and tQSSA, respectively. The generated data points are indicated by circles, the approximation using the true values is the dashed curved, while the fitted approximation is shown by the full curve. The obtained values are: Panel A: $k = 1.21 \pm 0.13$, $K_{\mathrm{M}} = 103.0 \pm 9.4$. Panel B: $k = 0.73 \pm 0.12$, $K_{\mathrm{M}} = 17.6 \pm 11.6$. All units are arbitrary.

noise was assumed to be normal with zero mean and standard deviation 2, corresponding to 2% of the total substrate concentration $S_T = 100$. The enzyme concentration was fairly high, $E_T = 50$. For both the sQSSA and the tQSSA we estimated the parameters k and K_M by optimizing the fit of each model to the observations. This was implemented using the "nls" package of the R program for the nonlinear least square estimates. An output of this procedure is shown in Fig. 4. The obtained values from the sQSSA (Fig. 4A) were $k = 1.21 \pm 0.13$ and $K_M = 103.0 \pm 9.4$, while the tQSSA (Fig. 4B) yielded $k = 0.73 \pm 0.12$ and $K_M = 17.6 \pm 11.6$. We note that when using the sQSSA the parameters are greatly overestimated, especially K_M which is an order of magnitude higher than the real value. On the other hand the estimation obtained from tQSSA is much nearer to the true values. This advantage of the tQSSA is clearly confirmed by the fact that the true values $k = 0.6$ and $K_M = 10$ fall inside one standard deviation from the corresponding estimated values, while the intervals estimated by the sQSSA do not include the true values. Indeed, they are very distant from these values. This is further confirmed by the 95% confidence intervals (0.998, 1.551) and (75.3, 147.4) for the k and K_M, respectively, in the sQSSA.

The wrong estimation by the sQSSA is related to the need to "move" the model with the true parameters (Fig. 4 A, dashed curve) in order to make it fit the data points as well as the full system. Since the model with the true parameters was not a good representation of the full system, we had to change the parameters a lot to obtain a good fit. This was not necessary for the tQSSA since already with the true parameters it was a good approximation of the full system, and hence, it fitted the data set well.

From the results we conclude that the confidence interval is important when obtaining parameters from "reverse engineering" rather than a single value. This is reflected by the large standard deviation for the tQSSA estimation of $K_M = 17.6 \pm 11.6$. However, even considering the confidence interval the sQSSA is far from the true value. We continue the study of reverse engineering in a future work.

6. Conclusions

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 [3,22], and later to increasingly more complex schemes such as reversible reactions [23] and fully competing systems [13].

We have here presented the application of the tQSSA to biologically realistic reactions, and shown that it is superior to the sQSSA in all the presented cases.

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).

From the considerations of the previous section it follows that the ability of the model to fit a certain data set cannot be used to test whether a certain approximation holds. Indeed, we found a good fit for the sQSSA even though it was known not to hold (Fig. 4A).

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. Again, the use of the tQSSA shows to be the most correct among all the known approximations.

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