Mathematical Biology

Introducing total substrates simplifies theoretical analysis at non-negligible enzyme concentrations: pseudo first-order kinetics and the loss of zero-order ultrasensitivity

Morten Gram Pedersen · Alberto Maria Bersani

Abstract   The Briggs–Haldane standard quasi-steady state approximation and the resulting rate expressions for enzyme driven biochemical reactions provide crucial theoretical insight compared to the full set of equations describing the reactions, mainly because it reduces the number of variables and equations. When the enzyme is in excess of the substrate, a significant amount of substrate can be bound in intermediate complexes, so-called substrate sequestration. The standard quasi-steady state approximation is known to fail under such conditions, a main reason being that it neglects these intermediate complexes. Introducing total substrates, i.e., the sums of substrates and intermediate complexes, provides a similar reduction of the number of variables to consider but without neglecting the contribution from intermediate complexes. The present theoretical study illustrates the usefulness of such simplifications for the understanding of biochemical reaction schemes. We show how introducing the total substrates allows a simple analytical treatment of the relevance of significant enzyme concentrations for pseudo first-order kinetics and reconciles two proposed criteria for the validity of the pseudo first-order approximation. In addition, we show how the loss of zero-order ultrasensitivity in covalent modification cycles can be analyzed, in particular that approaches such as metabolic control analysis are immediately applicable to scenarios described by the total substrates with enzyme concentrations higher than or comparable to the substrate concentrations. A simple criterion which excludes the possibility of zero-order ultrasensitivity is presented.

M. G. Pedersen (✉)
Department of Information Engineering, University of Padova,
Via Gradenigo 6/A, 35131 Padova, Italy
e-mail: pedersen@dei.unipd.it

A. M. Bersani
Department of Mathematical Methods and Models, "La Sapienza" University,
via A. Scarpa 16, 00161 Rome, Italy

1 Introduction

A major advance in the theoretical analysis of enzyme catalyzed reactions occurred with the model proposed by Henri (1901a,b, 1902) and further analyzed by Michaelis and Menten (1913) and Briggs and Haldane (1925) (reviewed in Schnell and Maini (2003)):

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \overset{k_2}{\longrightarrow} E + P. \tag{1}$$
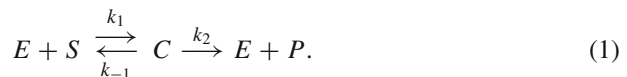
Here the enzyme E binds a substrate S to form an intermediate complex C, which can either dissociate or result in the formation of the product P and a free enzyme molecule. In the following we will use the same symbols for reactants and their concentrations. Moreover, we follow the usual assumptions saying that neither synthesis nor degradation of S and P occur. We denote by S_0 and E_0 the total substrate and enzyme concentrations, respectively. We acknowledge that the Michaelis–Menten reaction scheme might be a simplification of biochemical reactions since it neglects, for example, an intermediate EP complex (Lehninger 1975; Elliott and Elliott 1997; Fersht 1985), but since it is by far the most used description of enzyme catalyzed reactions (Goldbeter and Koshland 1981; Bisswanger 2002; Schnell and Maini 2003; Segel and Slemrod 1989), we focus our analysis on this scenario.

The simplification arising from the standard quasi-state approximation (sQSSA), yielding the well-known (Briggs and Haldane 1925) expression,

$$\frac{dS}{dt} = -\frac{k_2 E_0 S}{K_M + S}, \tag{2}$$

is due to the reduction of variables from two (usually S and C) to just one (S) variable. This equation was found already by Henri (1902), albeit with the equilibrium constant $K = k_{-1}/k_1$ replacing the Michaelis constant $K_M = (k_{-1} + k_2)/k_1$. From the formula one extracts immediately relevant biochemical information: saturation for high substrate concentrations, the affinity described by K_M, the proportional relation between enzyme concentration and reaction rate, etc.
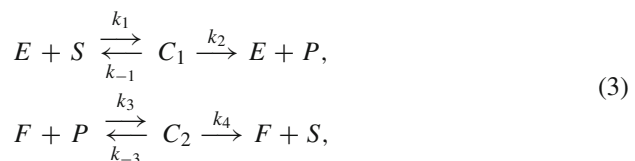
However, the sQSSA is invalid at enzyme concentrations higher than or comparable to the quantity $K_M + S_0$ (Segel and Slemrod 1989), a major reason being that the sQSSA neglects the intermediate enzyme-substrate complexes. Recently, the total quasi-steady state approximation (tQSSA) has been studied in order to treat such scenarios similarly to the standard quasi-steady state approach (Borghans et al. 1996; Tzafriri 2003). This work is based on the change-of-variables going back at least to Cha and Cha (1965), which consists in regarding the total substrate $\bar{S} = S + C$ in (1)

rather than the substrate S, and can be related to the seminal work by Laidler (1955), who used P instead of $\bar{S} = S_0 - P$. Tzafriri (2003) showed that the tQSSA is always roughly valid, and if $k_{-1} \gg k_2$, a fact that holds for many enzyme reactions (Tzafriri and Edelman 2007), the approximation is excellent for any combination of enzyme and substrate concentrations. The tQSSA has been extended to reversible reactions (Tzafriri and Edelman 2004), fully competitive reactions (Pedersen et al. 2007), stochastic kinetics (Tzafriri and Edelman 2007; MacNamara et al. 2008; Barik et al. 2008) as well as other more complex scenarios (Ciliberto et al. 2007; Pedersen et al. 2008a,b). The introduction of the total substrate yields simplifications similar to the sQSSA: it reduces the number of variables from two (S and C) to one ($\bar{S}$), but importantly without neglecting the contribution from the intermediate enzyme-substrate complexes, which can be significant at non-negligible enzyme concentrations compared to $K_M + S_0$. These scenarios are important to consider, since in vivo the enzyme concentration can be comparable to or higher than the substrate concentration and the Michaelis constant (Albe et al. 1990; Schnell and Maini 2003), especially in signal transduction networks such as the MAPK cascade (Blüthgen et al. 2006; Fujioka et al. 2006).

At high enzyme concentrations compared to the substrate concentrations, it is widely acknowledged that the reactions follow pseudo first-order kinetics (Silicio and Peterson 1961; Corbett 1972). Recently this belief was challenged and another criterion ($K_M \gg S_0$) for pseudo first-order kinetics was proposed (Schnell and Mendoza 2004). One aim of the present work is to reconcile these two criteria with the use of the change-of-variables to the total substrate. Pseudo first-order kinetics is of its own interest since it yields closed form solutions, which can be used to characterize the enzyme reaction completely, i.e., providing k_1, k_{-1} and k_2 of reaction (1), when fitted to experimental data.

Another case where it is important to consider the contribution from intermediate complexes, is the ubiquitous mechanism of covalent modification, such as phosphorylation and dephosphorylation of an enzyme. This mechanism, which provides the building blocks of the MAPK cascade, possesses ultrasensitivity, i.e., the system responds to a signal in an all-or-none way rather than a graded response, under the right conditions, as Goldbeter and Koshland (1981) showed more than 25 years ago.

The system 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

$$
E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C_1 \xrightarrow{k_2} E + P,
$$
$$
F + P \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} C_2 \xrightarrow{k_4} F + S,
$$

(3)

and it is assumed that the input signal is a variation in the relative total amount of (the active forms of) the enzymes E_0/F_0. This reaction mechanism was studied already by Cha and Cha (1965), but from another point of view. Later, several authors have
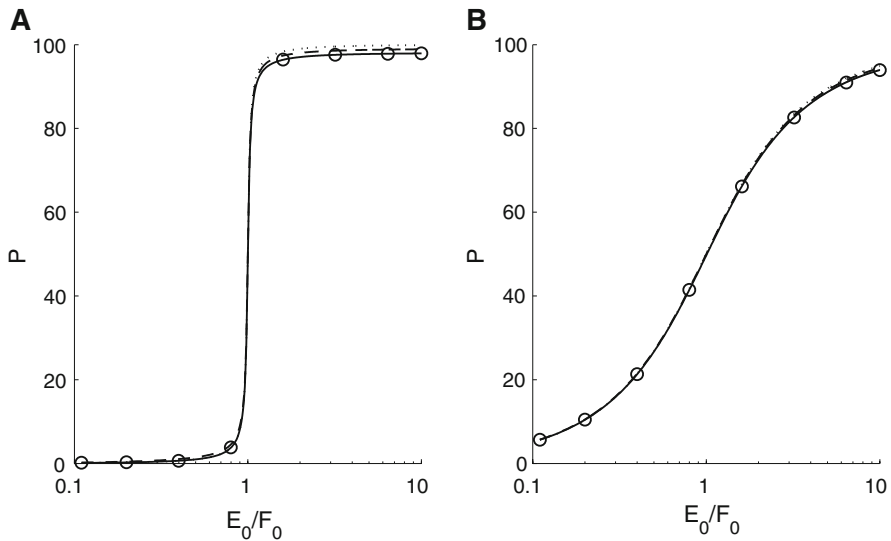
Fig. 1 Zero-order ultrasensitivity at low enzyme concentrations for system (3) as in Goldbeter and Koshland (1981) ($F_0 = 0.01 S_0$). **a** At $K_M = 0.01 S_0$ the system possesses zero-order ultrasensitivity. **b** At $K_M = S_0$ zero-order ultrasensitivity is lost. Both the sQSSA (*dotted curve*) and the tQSSA ($\bar{P}$ *dashed curve*, $P = \bar{P} - C_2^-(\bar{P})$ *full curve*) fit the solution from the full system (*circles*) well

reinvestigated ultrasensitivity in the Goldbeter–Koshland switch (see for example Berg et al. (2000); Blüthgen et al. (2006); Ciliberto et al. (2007)).

When the enzyme concentrations are low, e.g., $F_0, E_0 \ll S_0$, the complex concentrations are negligible, and thus we have approximately $S_0 = S + P$. For low K_M values, compared to S_0, the enzymes are saturated and we have approximately zero-order kinetics. The steady-state changes swiftly from $P \approx 0$ to $P \approx S_0$ as $\alpha = k_2 E_0 / (k_4 F_0)$ passes from below to above 1 (Fig. 1a). On the other hand, at high K_M values, the reactions are approximately first-order, and the system no longer possesses ultrasensitivity (Goldbeter and Koshland 1981). In fact, ultrasensitivity is lost already at K_M values comparable to the total substrate concentration S_0 (Fig. 1b).

However, even when $K_M \ll S_0$ but at non-negligible complex concentrations, the system is less sensitive to changes in the signal (Goldbeter and Koshland 1981; Blüthgen et al. 2006; Ciliberto et al. 2007) (Fig. 2). This happens at enzyme concentrations comparable to or higher than the substrate concentration, due to a large amount of substrate being bound in the complexes, so-called substrate sequestration (Blüthgen et al. 2006). Blüthgen et al. (2006) used metabolic control analysis (MCA) to study this scenario. MCA is a sensitivity method, and here the aim is to investigate how sensitive the output signal P is to a change in the input signal E_0. For this purpose new MCA formulas involving standard quasi-steady state expressions were developed (Blüthgen et al. 2006).

The present paper will show that the change-of-variables to the total substrates allows a simple analytical treatment of the relevance of significant enzyme concentrations for pseudo first-order kinetics and zero-order ultrasensitivity in system (3), and
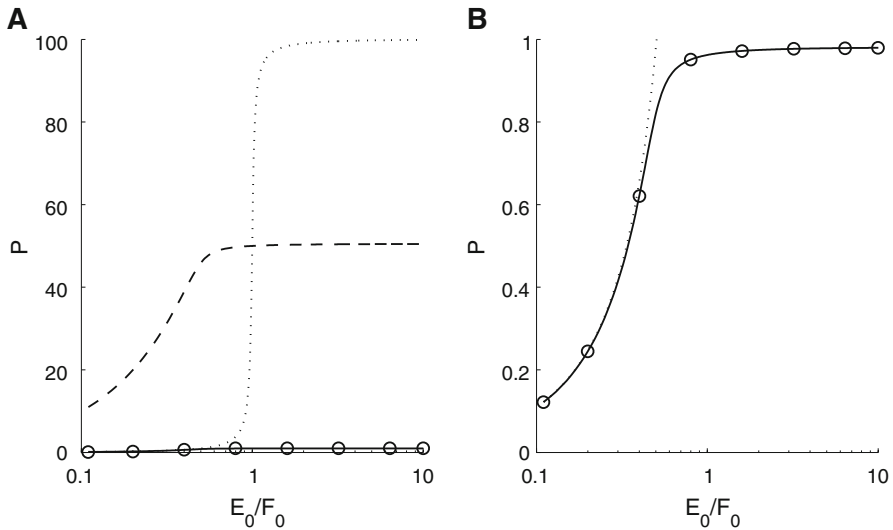
Fig. 2 Zero-order ultrasensitivity is lost at $S_0 = F_0 \gg K_M = 0.01 S_0$. Panel B shows a zoom of the lower part of panel A. The sQSSA (*dotted curve*) and $\bar{P}$ from tQSSA (*dashed curve*) no longer fit the solution from the full system (*circles*) well, due to a significant amount of complexes. However, $P = \bar{P} - C_2^-(\bar{P})$ from the tQSSA (*solid curve*) does fit well. Note that the sQSSA wrongly predicts zero-order ultrasensitivity, and that P and $\bar{P}$ have similar shapes

that approaches such as metabolic control analysis (MCA) are immediately applicable to scenarios with non-negligible concentrations described by total substrates. A simple criterion which excludes the possibility of zero-order ultrasensitivity is presented. The scope of this theoretical study is not to provide better ways of estimating kinetic parameters, which can be done using the full system of equations describing the reaction scheme (1). Rather, we wish to show that the introduction of the total substrates can yield theoretical insight, which is hard to obtain directly from the fundamental reactions, in much the same way the standard quasi-steady state approximation gives fundamental insight at negligible enzyme concentrations.

2 Results

2.1 Pseudo first-order kinetics

The rates of second order chemical reactions are often studied by so-called pseudo first-order kinetics by a set-up where one of the reactant concentrations is much larger than the other one, and therefore is approximately constant (Silicio and Peterson 1961; Corbett 1972). When the enzyme concentration remains effectively constant in the case of the Michaelis–Menten mechanism (1), the second order reaction $S + E \rightarrow C$ becomes mathematically equivalent to a first-order reaction, reducing

the Michaelis–Menten mechanism to

$$S \underset{k_{-1}}{\overset{k_\psi}{\rightleftarrows}} C \overset{k_2}{\longrightarrow} P, \tag{4}$$

where $k_\psi = k_1 E_0$ is a pseudo rate constant. The corresponding linear system of differential equations can be solved explicitly, and the solution can then be used to estimate the reaction constants k_1, k_{-1} and k_2 (Schnell and Mendoza 2004).

As discussed in Schnell and Mendoza (2004), the assumption underlying pseudo first-order kinetics is that almost all of the enzyme remains unbound, i.e.,

$$\max C(t)/E_0 \ll 1. \tag{5}$$

Schnell and Mendoza (2004) (see also Borghans et al. (1996)) showed that the trajectory of the system in the (S, C) phase plane describes a curve connecting $(S_0, 0)$ with $(0, 0)$ with a single local maximum (S_Q, C_Q), which can be found from $\frac{dC}{dS} = 0$. They derived $C_Q = E_0 S_Q/(K_M + S_Q)$ and using the estimate $S_Q < S_0$ with (5), they arrived at the following sufficient condition for pseudo first-order kinetics:

$$\frac{S_0}{K_M + S_0} \ll 1, \tag{6}$$

which is guaranteed by $S_0 \ll K_M$. However, at high enzyme concentrations compared to the substrate concentration we might expect that a significant amount of substrate is bound, so that the estimate $S_Q < S_0$ is very conservative. This would mean that although (6) is a sufficient condition it would be far from necessary.

A better estimate can be given as follows. Following (Cha and Cha 1965; Borghans et al. 1996) we introduce the total substrates $\bar{S} = S + C$. In the $(\bar{S}, C)$ phase plane the trajectories are described by

$$\frac{dC}{dt} = k_1 \left((E_0 - C)(\bar{S} - C) - K_M C \right), \tag{7}$$

$$\frac{d\bar{S}}{dt} = -k_2 C, \tag{8}$$

which yields (by formal division of (7) by (8))

$$0 = (E_0 - C)(\bar{S} - C) - \left(K_M - K \frac{dC}{d\bar{S}} \right) C, \tag{9}$$

where $K = k_2/k_1$. C has a single maximum, while $\bar{S}$ is constantly decreasing (Borghans et al. 1996). Thus the trajectories are again curves connecting $(S_0, 0)$ with $(0, 0)$. The maximum $(\bar{S}_Q, C_Q)$ can be found from $\frac{dC}{d\bar{S}} = 0$, i.e., using (9) in (7), from $\frac{dC}{dt} = 0$, which means at the tQSSA expression for the complex (Tzafriri 2003;

Laidler 1955)

$$C_Q = \frac{S_Q + E_0 + K_M - \sqrt{(S_Q + E_0 + K_M)^2 - 4S_Q E_0}}{2} \ . \tag{10}$$

At high enzyme concentrations and/or large K_M values, compared to the substrate concentration ($E_0 + K_M \gg S_0$) (Borghans et al. 1996; Tzafriri 2003), we get

$$C_Q \approx \frac{E_0 \bar{S}_Q}{E_0 + K_M + \bar{S}_Q} \leq \frac{E_0 S_0}{E_0 + K_M + S_0}. \tag{11}$$

Thus, using (5), the criterion for pseudo first-order kinetics reads

$$\frac{S_0}{E_0 + K_M + S_0} \ll 1, \tag{12}$$

which covers both expression (6), i.e., $S_0 \ll K_M$, as well as the often quoted situation of excess enzyme, $S_0 \ll E_0$. We can summarize these criteria as

$$S_0 \ll K_M + E_0.$$

Schnell and Mendoza (2004) showed an example with $S_0/E_0 = 1/10$ and $S_0/K_M = 10$, where pseudo first-order kinetics was a bad approximation to the full system (up to ~24% error on S), and argued that $E_0 \gg S_0$ is an insufficient condition, asserting, on the other hand, that $S_0 \ll K_M$ is a sufficient condition for the pseudo first-order approximation. However, when decreasing the ratio to $S_0/E_0 = 1/100$ while keeping $S_0/K_M = 10$, pseudo first-order kinetics is a good approximation to the full system (Fig. 3). This means that the condition $E_0 \gg S_0$ must be interpreted with much care, in the sense that the example in Schnell and Mendoza (2004) does not fulfill this criterion. Thus, (12) is a sufficient condition, in particular the often quoted $E_0 \gg S_0$ is sufficient for the validity of the pseudo first-order approximation.

We end this section by noting that pseudo first-order kinetics implies first-order kinetics when the complex C is in a quasi-steady state. When the system can be described by (4), the complex concentration follows

$$\begin{aligned} \frac{d\,C}{dt} &= k_\psi S - (k_{-1} + k_2)C \\ &= k_\psi \bar{S} - (k_\psi + k_{-1} + k_2)C, \end{aligned} \tag{13}$$

so if $\frac{d\,C}{dt} = 0$, then the complex concentration is proportional to both S and $\bar{S}$. Thus, also $\frac{d\,P}{dt} = k_2 C$ is proportional to S and $\bar{S}$, and we have first-order kinetics both with respect to the substrate and the total substrate.
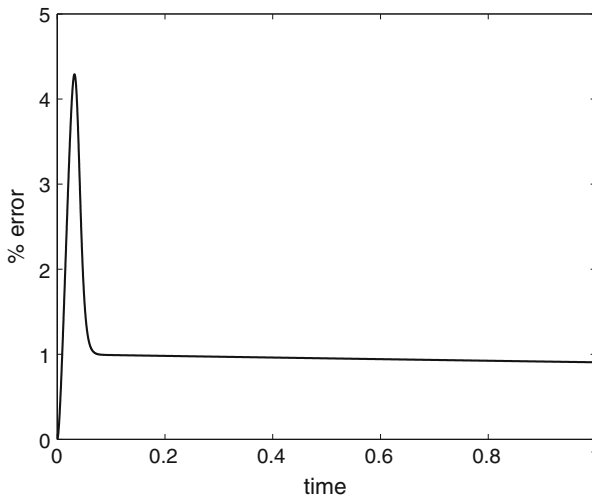
Fig. 3 The pseudo first-order approximation is valid at high enzyme concentrations. Only the relative error for S shown, since errors for C and P are smaller. Parameters are: $S_0 = 1$, $k_1 = 2$, $k_{-1} = k_2 = 0.1$, $E_0 = 100$, $(S_0/K_M = 10, S_0/E_0 = 1/100)$

2.2 First-order kinetics with substrate concentrations exceeding the K_M value implies enzyme excess in MAPK phosphatases

We now turn the picture around and ask what kind of information about the enzyme concentrations we can obtain from apparent first-order kinetics in the special case where the substrate concentration is much greater than the K_M value of the enzyme.

Zhao and Zhang (2001) found that the MAPK phosphatases can be described by the Michaelis–Menten scheme (1), and have K_M values on the order of tens of nM. Since MAPK is present at concentrations of $\sim$1 µM (Schoeberl et al. 2002; Fujioka et al. 2006), one would expect from the sQSSA formula (2) to observe saturation effects and hence approximately zero-order kinetics. However, Fujioka et al. (2006) found that dephosphorylation of MAPK was well described by first-order kinetics. This contradiction indicates that the sQSSA is invalid in this case. A likely explanation is that the criterion $E_0 \ll S_0 + K_M \approx S_0$ is violated, and hence that the enzyme, here the MAPK phosphatase, is present in concentrations comparable to or greater than the substrate concentration, i.e., at least at several hundred nM. Due to experimental difficulties, this conclusion awaits direct confirmation from measurements. For comparison, the MAPK kinase MEK is present at hundreds of nanomolar to micromolar concentrations in several cell types (Fujioka et al. 2006).

2.3 The Goldbeter–Koshland switch in terms of total substrates

As discussed in the previous section, covalent modification in the MAPK cascade occurs with non-negligible enzyme concentrations, for example for the phosphorylation and dephosphorylation of MAPK, where both MAPK kinases and phosphatases

are present in concentrations comparable to MAPK itself. In this section we illustrate how the use of total substrates can facilitate the analysis of the Goldbeter–Koshland switch under such conditions.

Since our aim is to illustrate the benefits of using the total substrates rather than investigating the covalent modification cycle in general, we have assumed for simplicity that the two reactions have the same Michaelis constant $K_M = \frac{k_{-1}+k_2}{k_1} = \frac{k_{-3}+k_4}{k_3}$. However, their maximal reaction rates, $V_{\max,1} = k_2 E_0$ and $V_{\max,2} = k_4 F_0$, are not necessarily equal. Moreover, the units are arbitrary, since any unit change does not affect our results.

We introduce the total substrates $\bar{S} = S + C_1$ and $\bar{P} = P + C_2 = S_T - \bar{S}$, and consider the steady-state scenario, which yields

$$k_2 C_1^- = k_4 C_2^-, \tag{14}$$

where the complex concentration are given by the tQSSA-expression ([Tzafriri 2003](); [Pedersen et al. 2008b]())

$$C_1^- = \frac{(\bar{S} + E_0 + K_M) - \sqrt{(\bar{S} + E_0 + K_M)^2 - 4\bar{S}E_0}}{2}, \tag{15}$$

$$C_2^- = \frac{(\bar{P} + F_0 + K_M) - \sqrt{(\bar{P} + F_0 + K_M)^2 - 4\bar{P}F_0}}{2}. \tag{16}$$

At this point it is important to note that we do not risk predicting ultrasensitivity with respect to $\bar{S}$ and $\bar{P}$ when there is no ultrasensitivity with respect to S and P and vice versa: If P switches rapidly from $P \approx S_0$ to another asymptotic value P_*, when the ratio E_0/F_0 changes, then $\bar{P}$ goes rapidly from $\bar{P} \approx S_0$ to $P_* + \frac{F_0 P_*}{K_M + P_*}$. Assume on the other hand that $\bar{P}$ switches from $\bar{P} \approx 0$ to $\bar{P} \approx S_0$. If also P goes rapidly from near zero to a non-negligible value P_*, then we have ultrasensitivity with respect to P as well. However, this fact is not obvious if $\bar{P} \approx S_0$ but $P/S_0 \ll 1$, and we must argue as follows. In this case $C_2 \approx S_0$. Note that from $\frac{dC_2}{dt} = 0$ it follows that $P = \frac{K_M C_2}{F_0 - C_2}$, so we have

$$1 \gg \frac{P}{S_0} = \frac{K_M C_2}{S_0 (F_0 - C_2)},$$

and hence $F_0 \gg C_2 \left(1 + \frac{K_M}{S_0}\right) \geq C_2$. Thus, in this case $P \approx \frac{K_M}{F_0} C_2$ is proportional to C_2, which switches from $C_2 \approx 0$ to $C_2 \approx S_0$, implying that the relative change in P, which goes from near 0 to a value close to $\frac{K_M}{F_0} S_0$, is comparable to the change in C_2. Hence, P shows the same switch-like behavior as $\bar{P}$ also in this case.

When $\max\{F_0, E_0\} \ll S_0 + K_M$ or $S_0 \ll \min\{E_0, F_0\} + K_M$ the above expressions can be approximated by [Tzafriri (2003)]()

$$C_1^- \approx \frac{E_0 (S_0 - \bar{P})}{E_0 + K_M + (S_0 - \bar{P})}, \quad C_2^- \approx \frac{F_0 \bar{P}}{F_0 + K_M + \bar{P}}, \tag{17}$$

such that at steady-state (14) we have

$$\frac{\alpha(S_0 - \bar{P})}{E_0 + K_M + (S_0 - \bar{P})} = \frac{\bar{P}}{F_0 + K_M + \bar{P}}, \tag{18}$$

where $\alpha = k_2 E_0/(k_4 F_0)$. Note that we have $\bar{P} \approx P$ for $\max\{E_0, F_0\} \ll S_0 + K_M$, and (18) reduces to the sQSSA expressions in this case. More interestingly, we see that it is not sufficient to have $K_M \ll S_0$ for ultrasensitivity to appear; the constraint $\max\{E_0, F_0\} \ll S_0$ is necessary (see also Ciliberto et al. (2007)). The reason is that the kinetics is no longer approximately zero-order when $E_0, F_0 \gg S_0 \gg K_M$. Indeed, it is seen from (17) that the reactions are approximately first-order for large enzyme concentrations compared to the substrate concentrations, see also Tzafriri (2003), Tzafriri and Edelman (2007) and the the final comment of Sect. 2.1.

The above expressions (17) can be invalid at moderately high enzyme concentrations compared to the substrate concentration, $E_0, F_0 \gtrapprox S_0$, which occurs for example if K_M is much larger than the enzyme and substrate concentrations. Since we expect any potential zero-order ultrasensitivity to appear at low K_M values compared to the concentrations of the involved chemical species, we investigate the case of $K_M \ll S_0 \lessapprox \min\{F_0, E_0\}$ rewriting (calculations for C_1^- are similar)

$$\begin{aligned}
C_2^- &= \bar{P} + \frac{(F_0 - \bar{P} + K_M) - \sqrt{(F_0 - \bar{P} + K_M)^2 + 4K_M\bar{P}}}{2} \\
&= \bar{P} + \frac{1}{2}(F_0 - \bar{P} + K_M)\left(1 - \sqrt{1 + \frac{4K_M\bar{P}}{(F_0 - \bar{P} + K_M)^2}}\right) \\
&\approx \bar{P} + \frac{1}{2}(F_0 - \bar{P} + K_M)\left[1 - \left(1 + \frac{1}{2}\frac{4K_M\bar{P}}{(F_0 - \bar{P} + K_M)^2}\right)\right] \\
&= \bar{P} - \frac{K_M\bar{P}}{F_0 - \bar{P} + K_M} \\
&= \bar{P}\frac{F_0 - \bar{P}}{F_0 - \bar{P} + K_M} \tag{19}
\end{aligned}$$

so that

$$C_1^- \approx \bar{S}\frac{E_0 - \bar{S}}{E_0 - \bar{S} + K_M}, \quad C_2^- \approx \bar{P}\frac{F_0 - \bar{P}}{F_0 - \bar{P} + K_M}, \tag{20}$$

where we have assumed $\frac{4K_M\bar{P}}{(F_0-\bar{P}+K_M)^2} \ll 1$ in order to substitute the square root with its linear approximation.

This assumption is satisfied if for example $F_0 \geq 2\bar{P}$, or less restrictive, $F_0 \geq 2S_0$. Note that the kinetics is almost first-order in this case, since from our assumptions $K_M \ll S_0 = 2S_0 - S_0 \leq F_0 - S_0 \leq F_0 - \bar{P}$, such that the fraction is close to 1. This merely says that almost all substrate will be bound to a high affinity enzyme present

at sufficiently high concentrations. Recently, Tzafriri and Edelman (2007) found a similar result in the limit of low K_M value.

In summary, we obtained by inspection of approximations to the expressions (15) and (16) that the reactions follow approximate first-order kinetics and zero-order ultrasensitivity is consequently lost in any of the following cases: high K_M values ($K_M \gg S_0$, (Schnell and Mendoza 2004)), high enzyme concentrations ($\min\{E_0, F_0\} \gg S_0$, (Blüthgen et al. 2006; Ciliberto et al. 2007)), or even moderately high enzyme concentrations but low K_M values ($K_M \ll S_0 \leq \frac{1}{2}\min\{E_0, F_0\}$). This latter scenario is likely to cover MAPK phosphatases, which show first-order kinetics (Fujioka et al. 2006), and have K_M values much lower than the substrate concentrations (Zhao and Zhang 2001; Fujioka et al. 2006).

2.4 Metabolic control analysis

To extend the above results even further, we must go into greater details when analyzing the expressions (15) and (16).

For this purpose we will use metabolic control analysis (Fell 1992), which links global properties of a system to local ones. Global properties are described by response coefficients, which describe the response of the entire system to changes in parameters. For example, the relative change in the concentration A in correspondence to a small change in the parameter p is described by the response coefficient $R_p^A = \frac{p}{A}\frac{\partial A}{\partial p}$. Local properties are described by the so-called elasticities, which describe the relative change of reaction rate v in response to a small change in the concentration of one of the biochemical species or parameters (B) involved in the reaction, and are defined as $\varepsilon_B^v = \frac{B}{v}\frac{\partial v}{\partial B}$.

Small and Fell (1990) developed metabolic control analysis for covalent modification systems with general rate expressions. Their approach is immediately applicable to our scenario with $\bar{S}$ and $\bar{P}$ being the modified quantities. Denote by $v_1 = k_2 C_1$ (respectively $v_2 = k_4 C_2$) the rate of formation of P (respectively S) from the reaction driven by the enzyme E (respectively F). The response coefficient of the modified substrate concentration ($\bar{P}$ for total substrates) to a change in the total enzyme concentration E_0 is given by Small and Fell (1990)

$$R_{E_0}^{\bar{P}} = \frac{\bar{S}}{\varepsilon_{\bar{P}}^{v_2}\bar{S} + \varepsilon_{\bar{S}}^{v_1}\bar{P}}. \tag{21}$$

$R_{E_0}^{\bar{P}} = 1$ corresponds to a linear response, while $R_{E_0}^{\bar{P}} > 1$ is needed for an ultrasensitive response. Thus, the elasticities must be close to zero, which happens when the rates v_1 and v_2 are nearly constant with respect to the substrates, i.e., in the zero-order regime.

As already observed, our goal is to illustrate the benefits of using the total substrates rather than investigating the covalent modification cycle in general. Thus let us in the following for simplicity assume that $k_2 = k_4$, as it was also done in Goldbeter and Koshland (1981), Blüthgen et al. (2006). We then use the fact that at steady state $v_1 = v_2$ such that

$$R_{E_0}^{\bar{P}} = \frac{\bar{S}}{\frac{\bar{P}}{v_2}\frac{\partial v_2}{\partial \bar{P}}\bar{S} + \frac{\bar{S}}{v_1}\frac{\partial v_1}{\partial \bar{S}}\bar{P}}$$

$$= \frac{v_2}{\bar{P}}\left(\frac{\partial v_2}{\partial \bar{P}} + \frac{\partial v_1}{\partial \bar{S}}\right)^{-1}$$

$$= \frac{C_2^-}{\bar{P}}\left(\frac{\partial C_2^-}{\partial \bar{P}} + \frac{\partial C_1^-}{\partial \bar{S}}\right)^{-1}$$

$$\leq \left(\frac{\partial C_2^-}{\partial \bar{P}} + \frac{\partial C_1^-}{\partial \bar{S}}\right)^{-1}. \tag{22}$$

Note that $R_{E_0}^{\bar{P}} \leq 1$ if

$$\min\left\{\frac{\partial C_1^-}{\partial \bar{S}}, \frac{\partial C_2^-}{\partial \bar{P}}\right\} \geq \frac{1}{2}, \tag{23}$$

and ultrasensitivity is then excluded.

Calculating

$$\frac{\partial C_2^-}{\partial \bar{P}} = \frac{1}{2}\left(1 + \frac{F_0 - (\bar{P} + K_M)}{\sqrt{(\bar{P} + F_0 + K_M)^2 - 4\bar{P}F_0}}\right), \tag{24}$$

it is readily shown that $\frac{\partial C_2^-}{\partial \bar{P}}$ is a decreasing function of $\bar{P}$ hence taking its minimum at the maximum allowable value of $\bar{P}$. Thus, to estimate an upper value of $R_{E_0}^{\bar{P}}$, we must find an upper bound for $\bar{P}$ (and $\bar{S}$, which similarly minimizes $\frac{\partial C_1^-}{\partial \bar{S}}$).

In the special case $E_0 = F_0$, such that $\bar{S} = \bar{P} = \frac{S_0}{2}$, we therefore find the following criterion, which excludes ultrasensitivity:

$$E_0 = F_0 \geq K_M + \frac{S_0}{2}. \tag{25}$$

For the more general case, but still assuming $k_2 = k_4$, we need some observations. At steady state $C_1 = C_2$, and hence we have

$$S_0 = \bar{P} + \bar{S} = 2C_2 + P + S, \tag{26}$$

which implies $C_2 \leq \frac{S_0}{2}$. Moreover either $\bar{P} \geq \frac{S_0}{2} \geq \bar{S}$ or $\bar{S} \geq \frac{S_0}{2} \geq \bar{P}$. In the following we assume the former condition, calculations for the second case being identical. Motivated by the above considerations and in particular by condition (23), we make the assumption

$$\min\{E_0, F_0\} \geq K_M + \frac{S_0}{2}. \tag{27}$$

which is the natural generalization of criterion (25), from which we immediately see

$$\frac{\partial C_1^-}{\partial \bar{S}} \geq \frac{1}{2},$$ (28)

since $\bar{S} \leq \frac{S_0}{2}$. In addition, using the fact that C_2^- is an increasing function of both F_0 and $\bar{P}$ and recalling that $F_0 \geq K_M + \frac{S_0}{2}$ and $\bar{P} \geq \frac{S_0}{2}$, we obtain

$$\frac{S_0}{2} \geq C_2 \geq \frac{1}{2} \left[2 \left(K_M + \frac{S_0}{2} \right) - \sqrt{4 \left(K_M + \frac{S_0}{2} \right)^2 - 4 \left(K_M + \frac{S_0}{2} \right) \frac{S_0}{2}} \right]$$

$$= \frac{S_0}{2} + K_M - \sqrt{K_M}\sqrt{K_M + \frac{S_0}{2}}.$$ (29)

Thus, using $\bar{S} \geq C_1 = C_2$,

$$\bar{P} = S_0 - \bar{S} \leq S_0 - C_2 \leq \frac{S_0}{2} + \sqrt{K_M}\sqrt{K_M + \frac{S_0}{2}} - K_M.$$ (30)

From (24) it follows that $\frac{\partial C_1^-}{\partial \bar{S}} \geq \frac{1}{2}$ for $F_0 \geq \bar{P} + K_M$. Hence, if we require

$$\min\{E_0, F_0\} \geq \frac{S_0}{2} + \sqrt{K_M}\sqrt{K_M + \frac{S_0}{2}},$$ (31)

we see that not only does (27), and therefore also (28) and (30), hold, but in addition we find from (24), (30) and (31) that

$$\frac{\partial C_2^-}{\partial \bar{P}} \geq \frac{1}{2},$$ (32)

and therefore, from (22), (23) and (28), that $R_{E_0}^{\bar{P}} \leq 1$.

The relation (31) is an important criterion for determining the lack of ultrasensitivity in covalent modification cycles, under the assumption of $k_2 = k_4$. In the limit $K_M/S_0 \to 0$, we see that a sufficient condition to exclude ultrasensitivity is $\min\{E_0, F_0\} > \frac{S_0}{2}$, which corresponds well to the region ($E_0/F_0 > 0.5$) where the curve is nearly horizontal in Fig. 2. However, (31) is only a sufficient condition for determining the lack of ultrasensitivity; the violation of (31) does not guarantee that $R_{E_0}^{\bar{P}} > 1$ nor ultrasensitivity, as seen from the example $F_0 \approx E_0 \ll S_0 = K_M$, which clearly violates (31) but does not have ultrasensitive behavior (Goldbeter and Koshland 1981) (Fig. 1b).

3 Discussion

A significant part of the success of the quasi-steady state approximations lies in the theoretical insight and simplification that they provide compared to the full set of equations describing the enzymatic reactions. This reduction was crucial for many decades before the easy access to computer simulations that characterize modern Systems Biology. The entry of computers has in some sense foregone the total quasi-steady state approximation, which can seem complicated to work with. Nonetheless, theoretical analysis can give insight that can be hard to obtain from computer simulations alone, as reflected in recent papers also on the topics discussed here (Blüthgen et al. 2006; Schnell and Mendoza 2004; Miller and Beard 2008).

As shown here, the change-of-variables underlying the total quasi-steady state approximation (tQSSA) reduces the number of variables under study and thereby simplifies theoretical reasoning. In contrast to the standard quasi-steady state approximation (sQSSA), this simplification is done without neglecting the enzyme-substrate complex concentrations (included in the total substrate $\bar{S}$), which are non-negligible at enzyme concentrations comparable to or higher than the substrate concentrations and Michaelis constants. Moreover, this approach has the advantage that it directly allows comparison to previous results obtained using the sQSSA. Thus, decades of obtained knowledge and methods can be extended to intermediate and high enzyme concentrations. We showed how introducing the total substrates directly yields the fact that at high enzyme concentrations the kinetics becomes pseudo first-order. Moreover, the change-of-variables allowed us to reconcile previous criteria in the relation (12), or equivalently, $S_0 \ll K_M + E_0$. We noted, in agreement with Tzafriri (2003), that this expression implies first-order kinetics when the complex is in a quasi-steady state, and in particular, at steady state. As a consequence, zero-order ultrasensitivity is lost in the covalent modification system under these conditions.

The conclusion was further supported by applying previously existing formulas from metabolic control analysis (Small and Fell 1990) to the total substrates description. Metabolic control analysis can also be used on the full system of equations (Blüthgen et al. 2006), but a new formula is necessary in this case. Blüthgen et al. (2006) found such a formula expressed in terms of the elasticities of the sQSSA reaction term. However, since the sQSSA is known not to hold at high enzyme concentrations, it can be questioned how to interpret these elasticities, and therefore the formula, under such conditions. The use of the total substrates sidesteps this issue. In the previous section we found the simple relation (31), which excludes the possibility of ultrasensitivity.

Goldbeter and Koshland (1981) suggested that ultrasensitivity could be rescued at high enzyme concentrations by assuming that not only P but also the complex C_2 is active. However, we considered directly the total substrate $\bar{P} = P + C_2$, and we found that the response coefficient $R_{E_0}^{\bar{P}} < 1$ for sufficiently high enzyme concentrations as stated in (31). Therefore, ultrasensitivity is excluded in this case in agreement with Blüthgen et al. (2006).

As pointed out by Blüthgen et al. (2006), the lack of ultrasensitivity at enzyme concentration higher than or similar to the substrate concentrations, a scenario expected

to be of importance in vivo, means that the cells must possess other means to create a steep signal-response curve, for example signaling cascades. We (Pedersen et al. 2008a) and others (Blüthgen et al. 2006) have shown that high enzyme concentrations can modify the response of the MAPK cascade greatly, and make an otherwise oscillatory response (Kholodenko 2000) non-oscillatory.

The usefulness of the tQSSA and its underlying assumptions for theoretical considerations is starting to appear in literature. Recently, Ciliberto et al. (2007) and Gomez-Uribe et al. (2007) used the first-order tQSSA (17) for a theoretical investigation of covalent modification in line with the treatment presented here. However, (17) does not hold at comparable enzyme and substrate concentrations (Tzafriri 2003) as noted by Ciliberto et al. (2007). The present paper thus extends those results. As argued in Sect. 2.2 this scenario of comparable enzyme and substrate concentrations occurs for example for phosphorylation and dephosphorylation of MAPK.

Dynamic computer simulations are easily done for the full set of reactions and equations, but theoretical analysis as presented here is greatly aided by the reduction resulting from the introduction of the total substrates and the tQSSA, in the same way that the sQSSA simplifies the analysis at low enzyme concentrations. Such simplifications also help parameter estimation; however the use of an invalid approximation, e.g., the sQSSA at high enzyme concentrations, can lead to incorrectly estimated parameters (Schnell and Maini 2003). We have previously shown that the tQSSA can help overcoming this problem (Pedersen et al. 2008b).

Summing up, we outline a strategy for choosing a suitable set of variables and simplification for a theoretical investigation of a reaction network composed of Michaelis–Menten schemes of the form (1):

- Is the criterion $E_0 \ll S_0 + K_M$ satisfied? Then the sQSSA is generally applicable, and traditional approaches and analyses are valid.
- Otherwise, change variables to the total substrates ($\bar{S} = S + C$). This change can for example give better estimates of the maximum complex concentrations, as shown in our study of pseudo first-order kinetics in Sect. 2.1. If studying a steady-state situation, such as the present investigation of ultrasensitivity, approaches such as metabolic control analysis (MCA) might be immediately applicable. This is true for reaction networks, such as covalent modification cycles (Small and Fell 1990), where MCA formulas either exist or can be derived for general rate expressions.
- If studying dynamic phenomena, the tQSSA might be applicable. Tzafriri (2003) found conditions guaranteeing the validity of the tQSSA of (1). The tQSSA for a few other simple schemes has also been thoroughly investigated (Tzafriri and Edelman 2004; Pedersen et al. 2007), but for more general reaction schemes a careful analysis of its validity has still not been carried out. We mention in passing that to our knowledge, also the conditions guaranteeing the validity of the sQSSA for general reaction networks are poorly understood. In such cases it might be convenient to simulate and analyze the full system of equations derived from mass-action.

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