A kinetic analysis of coupled sequential enzyme reactions

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Abstract As a case study, we consider a coupled enzyme assay of sequential enzyme reactions obeying the Michaelis–Menten reaction mechanism. The sequential reaction consists of a single-substrate, single enzyme non-observable reaction followed by another single-substrate, single enzyme observable reaction (indicator reaction). In this assay, the product of the non-observable reaction becomes the substrate of the indicator reaction. A mathematical analysis of the reaction kinetics is performed, and it is found that after an initial fast transient, the sequential reaction is described by a pair of interacting Michaelis–Menten equations. Timescales that approximate the respective lengths of the indicator and non-observable reactions, as well as conditions for the validity of the Michaelis–Menten equations are derived. The theory can be extended to deal with more complex sequences of enzyme catalyzed reactions.

Keywords Coupled enzyme assay · sequential enzymes · slow manifold · timescale separation · singular perturbation analysis · initial rate experiments · reactant stationary approximation · Schnell–Mendoza equation

This work is partially supported by the University of Michigan Protein Folding Diseases Initiative.

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1 Introduction

Catalyzed chemical reactions are often carried experimentally as a pair consisting of an initial non-observable reaction, followed by an indicator reaction. The latter (indicator) reaction is used to detect (monitor) the preliminary non-observable reaction (Cornish-Bowden, 2012). We consider a coupled reaction that consists of non-observable component in which a substrate $S_1$ and an enzyme $E_2$ reversibly bind to form a complex $C_1$ that in turn irreversibly releases substrate $S_2$. The second substrate $S_2$ then binds with a second enzyme $E_2$ to form a product $P$:

$$S_1 + E_1 \xrightleftharpoons[k_{-1}]{k_1} C_1 \rightarrow E_1 + S_2$$  \hspace{1cm} (1)

$$S_2 + E_2 \xrightleftharpoons[k_{-3}]{k_3} C_2 \rightarrow E_2 + P$$  \hspace{1cm} (2)

Both enzyme reactions obey a Michaelis–Menten (MM) mechanism of enzyme action (Schnell and Maini, 2003; Schnell, 2014).

Couple enzyme reactions generally exhibit a lag or transient time (McClure, 1969; Bergmeyer, 1965; Barwell and Hess, 1970; Cleland, 1979; Easterby, 1973), which is effectively the length of time it takes before measurable formation rates of $P$ become experimentally detectable. From the chemical kinetics point of view, the lag time is essentially the amount of time it takes to reach the quasi-steady-state (QSS) period and the initialization of product synthesis (Shoffner and Schnell, 2016; Eilertsen and Schnell, 2018). Therefore, when the quasi-steady-state assumption (QSSA) is applied to the sequential enzyme reaction (1)-(2), it has generally been assumed that the lag time coincides with the initial transient timescale during which the intermediate complex $C_2$ is accumulating until it reaches its maximum value (McClure, 1969; Easterby, 1973, 1981).

Most of the theory concerning sequential enzyme reactions and other coupled enzyme assays has concentrated on the derivation of mathematical expressions to estimate the lag times (Easterby, 1981; Brooks et al, 1984b; Barwell and Hess, 1970; Brooks and Suelter, 1986). While the rate of formation of $P$ is generally small prior to a significant accumulation in $C_2$, previous theoretical work (Easterby, 1973, 1981; Brooks et al, 1984b) assumed that the onset of the QSS always corresponds to onset of $C_2$ having reached its critical maximum value and therefore the steady-state production of $P$ does not occur until $C_2$ reaches this critical threshold value. To derive lag time expressions, numerous assumptions have been made to the dynamical behavior of the coupled enzyme assays. Most work has assumed that the sequential enzyme catalyzed reactions follow first-order kinetics (McClure, 1969; Barwell and Hess, 1970; Hart, 1970; Goldman and Katchalski, 1971; Easterby, 1973), which limit the validity of
the lag time expressions, and practical usage of the time course expressions of the reactions (Schnell and Mendoza, 2004; Pedersen and Bersani, 2010). Other studies rely on using single substrate, single enzyme MM equations for the non-observable and indicator reactions, but these expressions are simplified to pseudo-first order by assuming that the enzyme assays are performed at substrate concentrations lower than their Michaelis constants (Storer et al, 1974; Cleland, 1979). Brooks et al (1984a,b) showed that MM equations for uncoupled reactions can adequately describe coupled sequential enzyme assay if the three conditions are met: (i) the rate of the non-observable enzyme is constant, (ii) the coupled enzyme reactions are irreversible, and (iii) the indicator reaction has a short-live intermediate with a concentration smaller or equal to its Michaelis constant.

The aforementioned studies do not provide general principles that can be used to ascertain the possible use of the first-order kinetics or the QSSA to the sequential enzyme reaction. They do not present a clear criterion for the validity of the expressions derived, nor do they carry out a mathematical analysis to derive expressions to measure the steady state kinetics using initial rate or time course experiments.

Here we analyze the sequential enzyme reaction (1)-(2) using scaling methods and singular perturbation theory. In particular, We are able to derive timescale expressions to estimate the initial fast transients and the QSS periods of the non-observable (1) and indicator (2) reactions. These scales allow us to derive a pair of interacting MM equations

\[
\dot{s}_1 = -\frac{V_1}{K_{M_1} + s_1} s_1 \\
\dot{s}_2 = -\frac{V_1}{K_{M_1} + s_1} s_1 + \frac{V_2}{K_{M_2} + s_2} s_2,
\]

governing the state-state kinetics of the sequential enzyme reaction (1)-(2). Rigorous mathematical statements regarding the validity of the above MM equations are derived. We will show that as long as the reactant-stationary assumption (RSA) (Hanson and Schnell, 2008; Schnell, 2014) holds, the above equations describe the reaction dynamics during the QSS period. We conclude with a discussion of our results, as well as possible a description of the experimental applicability of the interacting MM equations to estimate the kinetic parameters of the sequential enzyme reaction (1)-(2) via initial rate experiments for the indicator substrate and product.
2 Derivation of mass-action rate expressions

The mass-action equations governing the sequential enzyme reaction (1)-(2) are

\[ \dot{s}_1 = -k_1 s_1 e_1 + k_{-1} c_1 \]  
\[ \dot{c}_1 = k_1 e_1 s_1 - (k_{-1} + k_2) c_1 \]  
\[ \dot{e}_1 = -k_1 s_1 e_1 + (k_{-1} + k_2) c_1 \]  
\[ \dot{s}_2 = -k_3 s_2 e_2 + k_2 c_1 + k_{-3} c_2 \]  
\[ \dot{c}_2 = k_3 s_2 e_2 - (k_{-3} + k_4) c_2 \]  
\[ \dot{e}_2 = -k_3 s_2 e_2 + (k_{-3} + k_4) c_2 \]  
\[ \dot{p} = k_4 c_2, \]

where \( s_1, c_1, e_1, s_2, c_2, e_2, p, \) denote the concentrations of \( S_1, C_1, E_1, S_2, C_2, E_2 \) and \( P \) respectively. The system (3) has the following initial conditions

\[ (s_1, c_1, s_2, c_2, e_2, p)(t = 0) = (s_0^1, 0, e_0^1, 0, 0, e_0^2, 0), \]

and obeys three conservation laws

\[ e_1 + c_1 = e_0^1 \]
\[ e_2 + c_2 = e_0^2 \]
\[ s_1 + s_2 + c_2 + p = s_0^1. \]

In the above expressions, \( s_0^1 \) is the initial non-observable substrate concentration, \( e_0^1 \) is the initial non-observable enzyme concentration, and \( e_0^2 \) is the initial indicator enzyme concentration. Utilizing (5a-5c) to reduce (3), yields the following rate expressions for \( s_1, c_1, s_2 \) and \( c_2 \):

\[ \dot{s}_1 = -k_1 (e_0^1 - c_1) s_1 + k_{-1} c_1 \]  
\[ \dot{c}_1 = k_1 (e_0^1 - c_1) s_1 - (k_{-1} + k_2) c_1 \]  
\[ \dot{s}_2 = -k_3 (e_0^2 - c_2) s_2 + k_2 c_1 + k_{-3} c_2 \]  
\[ \dot{c}_2 = k_3 (e_0^2 - c_2) s_2 - (k_{-3} + k_4) c_2. \]

We remark that not only are the equations (6a)-(6b) that model the non-observable reaction autonomous and independent from the equations (6c)-(6d) that model the indicator reaction, but they also describe a single-substrate, single-enzyme catalyzed reaction describing the MM mechanism. Consequently, the equations for the non-observable reaction can be reduced to a differential-algebraic system. The system (6a)-(6b) exhibits a slow manifold, \( M \), which describes geometrically the enzyme catalyzed reaction (Roussel and Fraser, 1990).
3 Mathematical analysis and validity of rate equation for the non-observable substrate depletion

Primarily due to the seminal works of Segel and co-workers (Segel, 1988; Segel and Slemrod, 1989) and Schnell and co-workers (Schnell and Mendoza, 1997; Schnell and Maini, 2003; Hanson and Schnell, 2008; Schnell, 2014), we understand the general principles that can be used to derived the MM equation for the single-substrate, single enzyme catalyzed reaction following the MM mechanism (1). The reaction is assumed to be separated in two phases: an initial fast transient followed by a QSS period. Briefly, after an initial fast transient, the complex $c_1$ reaches a QSS, in which the velocity of its formation is more or less equal to the velocity of its depletion, making $\dot{c}_1 \sim 0$ for the duration of the QSS phase. Setting $\dot{c}_1$ to 0 in (6b) yields the following approximation for $c_1$ in the QSS

$$c_1 = \frac{e_0^1}{K_{M_1} + s_1},$$  

(7)

where $K_{M_1} = (k_{-1} + k_2)/k_1$ is the Michaelis constant. Substitution of (7) into (6a) yields

$$\dot{s}_1 = -\frac{V_1}{K_{M_1} + s_1}s_1,$$

(8)

where $V_1$ ($V_1 = k_2 e_0^1$) denotes the limiting velocity of the non-observable reaction. Equation (8) is the MM equation. Moreover, an exact solution to (8) was derived by Schnell and Mendoza (1997)

$$s_1 = K_{M_1} W [\sigma_1 \exp(\sigma_1 - \eta_1 t)],$$

(9)

where “$W$” is the Lambert-$W$ function, and $\sigma_1$ and $\eta_1$ are constants:

$$\sigma_1 = \frac{s_0^1}{K_{M_1}}, \quad \eta_1 = \frac{V_1}{K_{M_1}}.$$  

(10)

Biochemically, $\sigma_1$ is the specific non-observable substrate concentration, and $\eta_1$ is the reciprocal of specificity time for the non-observable reaction. The validity in the reduction of (6a)-(6b) to the differential-algebraic system (7)-(8) is dependent not only on the intrinsic microscopic rate constants $k_{-1}, k_1$ and the catalytic constant $k_2$, but also on the initial substrate and enzyme concentrations $s_0^1, e_0^1$. First, it is generally assumed that $s_1 \approx s_0^1$ during the initial buildup $c_1$. This is known as the RSA (Hanson and Schnell, 2008; Schnell, 2014). The timescale over which the buildup of $c_1$ occurs is given by $t_{c_1}$, and was first estimated by Segel (1988) to be

$$t_{c_1} = \frac{1}{k_1 (K_{M_1} + s_0^1)}.$$  

(11)

If the depletion of substrate $s_1$ is negligible over this timescale, then, Segel (1988) proposed that

$$\varepsilon \equiv \max \frac{\dot{s}_1}{s_1^0} \cdot t_{c_1} = \frac{e_0^1}{K_{M_1} + s_0^1} \ll 1.$$  

(12)
must hold. This inequality (12) is the condition for the RSA, and is the general criteria for the validity of the MM equation for in vitro experimental assays. Under the RSA, the evolution of \( s_1 \) is approximately

\[
s_1 = \begin{cases} 
  s_1^0, & t < t_{c_1} \\
  K_{M_1} W [\sigma_1 \exp(\sigma_1 t - \eta_1 t)], & t \geq t_{c_1}
\end{cases}
\]  

with the approximation (13) getting better as \( \varepsilon \to 0 \). The substrate \( s_1 \) will be depleted in the timescale \( t_{s_1} \), which provides an estimate for the duration of the non-observable reaction (see Fig. 1). The depletion timescale \( t_{s_1} \) was estimated by Segel (1988) as

\[
t_{s_1} = \frac{K_{M_1} + s_1^0}{V_1}.
\]  

4 Mathematical analysis and validity of rate equation for the indicator substrate depletion

Theoreticians have shown that MM equation can be derived using singular perturbation analysis (Heineken et al, 1967b,a; Klonowski, 1983; Seshadri and Fritsch, 1980; Schauer and Heinrich, 1989; Goussis, 2012; Goeke et al, 2017). Central to the singular perturbation analysis is the selection of appropriate timescales, and scaling of the variables (Segel, 1972; Segel and Slemrod, 1989). To derive an a rate expression for the indicator reaction, and the necessary conditions for its validity, we will use singular perturbation analysis.

4.1 Estimation of critical timescales for the monitor reaction

We start by estimating an initial fast transient timescale for a significant change in the concentration of \( c_2 \), \( t_{c_2} \), and a timescale for a significant depletion of the indicator substrate, \( s_2 \), \( t_{s_2} \). Note that after \( t_{c_2} \), \( c_2 \) is QSS, and the indicator reaction will be completed briefly after \( t_{s_2} \). To derive these timescales, we follow the mathematical formalism first proposed by Rice (1960), which has been recently reviewed by Shoffner and Schnell (2017). Starting with the fast timescale characterized by \( t_{c_2} \), we take

\[
t_{c_2} = \left| \frac{\partial v_f}{\partial s_2} - \frac{\partial v_d}{\partial s_2} \right|^{-1},
\]  

where \( v_f \) is the velocity of formation of \( c_2 \)

\[
v_f = k_3 e_2^0 s_2,
\]  

and \( v_d \) is the velocity of depletion of \( c_2 \)

\[
v_d = c_2 k_3 (s_2 + K_{M_2}).
\]
In the above expression, \( K_{M_2} = (k_{-3} + k_3)/k_3 \) is the Michaelis constant for the indicator reaction. Substitution of (16) and (17) into (15) yields

\[
t_{c_2} = \frac{1}{k_3(K_{M_2} + s_2)}.
\]

We choose \( s_2 = s_1^0 \) to minimize this timescale, hence we acquire

\[
t_{c_2} = \frac{1}{k_3(K_{M_2} + s_1^0)}
\]

as our final estimate of the fast timescale of the indicator reaction. Next, we derive an estimate for \( t_{s_2} \). Again, per the formalism of Rice (1960), we have

\[
t_{s_2} = \left| v_f \left( \frac{\partial v_f}{\partial t} \right)^{-1} \right|,
\]

which yields

\[
t_{s_2} = \left| \frac{K_{M_2} + s_2}{(1 + K_{M_2}/s_2)k_2 \varepsilon s_1 - k_4 e_2^0} \right|.
\]

To maximize the \( t_{s_2} \) scale, we choose \( s_1 = 0 \) and \( s_2 = s_1^0 \) in (21) which gives us the following estimate for \( t_{s_2} \):

\[
t_{s_2} = \frac{K_{M_2} + s_1^0}{V_2}
\]

where \( V_2 (V_2 = k_4 e_2^0) \) is the limiting velocity of the indicator reaction. The timescale \( t_{s_2} \) of (22) does give a rough estimate of the time to completion of the indicator reaction, and numerical simulations confirm this estimate (see Fig. 1). A final word of caution is in order. Since the indicator reaction cannot completed faster than the non-observable reaction, it is more appropriate to define \( t_{s_2} \) as

\[
t_{s_2} = \max\{t_{s_2}, t_{s_1}\}.
\]

4.2 Scaling analysis: Derivation of the coupled MM equations

With the critical timescales \( t_{c_2} \) and \( t_{s_2} \) now estimated, we proceed through scaling analysis to derive the necessary conditions that allow for a model reduction of the mass action equations (6c)-(6d). Following Segel (1972), we introduce dimensionless variables with scales that provide an estimate to the variables’ maximum order of magnitude:

\[
\tilde{s}_1 = s_1 / s_1^0, \quad \tilde{s}_2 = s_2 / s_1^0, \quad \tilde{c}_1 = \frac{K_{M_1} + s_1^0}{e_1^0 s_1^0} c_1, \quad \tilde{c}_2 = \frac{K_{M_2} + s_1^0}{e_2^0 s_1^0} c_2.
\]
Fig. 1 The timescales $t_s$ and $t_s^2$ provide estimates for the duration of the non-observable and indicator reactions. Parameter values for numerical solutions are: $s_0^1 = 180$, $e_0^1 = 1$, $e_0^2 = 1$, $k_1 = 1$, $k_2 = 10$, $k_{-1} = 10$, $k_3 = 1$, $k_4 = 0.01$ and $k_{-3} = 10$ (units have been omitted). The concentrations have been scaled, $s_2 = s_2/s_0^1$ and $s_1 = s_1/s_0^1$ and the time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 - 1/\ln(t + e)$.

and the dimensionless time $T = t/t_s^2$ into equations (6c)-(6d). This leads to the following scaled system of differential equations:

\begin{align*}
-\dot{\hat{s}}_2 &= (1 + \kappa_2)(1 + \sigma_2) \left[ \left( \frac{\sigma_2}{1 + \sigma_2} \right) \hat{c}_2 - 1 \right] \dot{\hat{s}}_2 - \frac{\alpha}{1 + \sigma_2} \hat{c}_2 - \delta^{-1} \hat{c}_1 \tag{25a}
\end{align*}

\begin{align*}
\lambda \hat{c}_2 &= (1 + \kappa_2)(1 + \sigma_2) \left[ \left( 1 - \frac{\sigma_2}{1 + \sigma_2} \right) \hat{s}_2 - \frac{1}{1 + \sigma_2} \right], \tag{25b}
\end{align*}

where $\kappa_2, \sigma_2, \alpha, \delta$, and $\lambda$ are constants:

\begin{align*}
\kappa_2 &= \frac{k_{-3}}{k_4}, & \sigma_2 &= \frac{s_0^1}{K_{M_2}}, & \alpha &= \frac{\kappa_2}{1 + \kappa_2}, & \delta &= \frac{t_{s_1}}{t_{s_2}}, & \lambda &= \frac{e_0^2}{K_{M_2} + s_0^1}. \tag{26}
\end{align*}

If $0 < \lambda \ll 1$, then the scaling (25a)-(25b) indicates that the indicator reaction is a singularly-perturbed problem with respect to $T$. As a result, the complex $c_2$ is approximately

\begin{align*}
c_2 \approx s_2 \frac{e_0^2}{K_{M_2} + s_2}. \tag{27}
\end{align*}

on $T$. Substitution of (27) and (7) into (6c) gives the following expression for $s_2$ during the QSS period

\begin{align*}
\dot{s}_2 &= \frac{V_1}{K_{M_1} + s_1} \frac{V_2}{K_{M_2} + s_2 s_2}. \tag{28}
\end{align*}

Combining (28) with (8) yields a set of coupled MM equations that describe the complete reaction during the QSS phase of the coupled sequential enzyme
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\[ \dot{s}_1 = -\frac{V_1}{K_{M_1} + s_1} s_1 \]  
\[ \dot{s}_2 = \frac{V_1}{K_{M_1} + s_1} s_1 - \frac{V_2}{K_{M_2} + s_2} s_2 \]

It is possible to solve the MM equation for the substrate \( s_2 \) asymptotically. However, there is no obvious closed-form solution of (29b) that is homologous to the Schnell-Mendoza equation (13) due to the nonlinear terms in (29b). The advantage of the MM equations (29a)–(29b) over the mass-action equations (6a)–(6d) resides in the fact that the model equations have been reduced from a four-dimensional autonomous system to a two-dimensional autonomous system. Moreover, a reduction in the number of parameters also follows from the model reduction since the MM equations contain four parameters \( K_{M_1}, V_1, K_{M_2}, V_2 \) compared to the original six, \( k_1, k_{-1}, k_2, k_3, k_{-3} \) and \( k_4 \).

4.3 Conditions for the validity of the coupled MM equations for the sequential enzyme reaction

The condition for the validity of (29a) is well established; namely, that \( 0 < \varepsilon \ll 1 \), which, as mentioned in the Section 3, is also known as the RSA. It is clear from inspection of (29b) that the RSA is also, in conjunction with the condition \( 0 < \lambda \ll 1 \), is a necessary condition for the validity of (29b). Thus, per the scaling analysis, it is imperative that

\[ \max\{\varepsilon, \lambda\} \ll 1 \]  

holds in order to apply (29a)-(29b). However, the inequality (30) may not be sufficient to guarantee the validity of (29b). The onset of the validity of (27) can be determined from the general theory of singular perturbations. With \( \lambda = 0 \), equations (6c) and (6d) reduce to the differential-algebraic system

\[ \dot{s}_2 = -k_3(e_2^0 - c_2)s_2 + k_2c_1 + k_{-3}c_2 \]  
\[ c_2 = \frac{e_2^0}{K_{M_2} + s_2} s_2. \]

It is straightforward to show that the critical manifold \( \mathcal{M}_0 \)

\[ \mathcal{M}_0 = \{(s_1, c_1, s_2, c_2) \in \mathcal{P}|c_2 = \frac{e_2^0}{K_{M_2} + s_2} s_2 = 0\} \]

is hyperbolic (\( \mathcal{P} \) denotes the phase-space of system of equations, and is defined as \( \mathcal{P} \equiv \{(s_1, c_1, s_2, c_2) \in \mathbb{R}^4|s_1 \geq 0, c_1 \geq 0, s_2 \geq 0, c_2 \geq 0\} \)). Thus, if \( 0 < \lambda \ll 1 \), there will exist manifolds \( \mathcal{M}_\lambda \), such that

\[ \mathcal{M}_\lambda = \mathcal{M}_0 + \mathcal{O}(\lambda). \]
Moreover, experimental initial conditions (i.e., $s_1(0) = s_1^0, c_1(0) = 0, s_2(0) = 0, c_2(0) = 0, e_1(0) = e_1^0, e_2(0) = e_2^0$) lie on the critical manifold $M_0$. This implies that when $t = 0$, the approximation

$$c_2 = \frac{e_2^0}{K_M + s_2}$$

is valid provided $\lambda \ll 1$. Combining the result (34) with (6c) yields

$$\dot{s}_2 = -\frac{V_2}{K_M + s_2} s_2 + k_2 c_1 + O(\lambda), \quad 0 \leq t < t_{c_1}$$

(35a)

$$c_2 = \frac{e_2^0}{K_M + s_2} s_2 + O(\lambda), \quad t \geq 0.$$  

(35b)

Since (29b) is not valid until $t \geq t_{c_1}$, we must supply (29b) with a boundary condition at $t = t_{c_1}$. To obtain an initial condition for (35a)-(35b), we can consider $s_2$ to be a slow variable during the initial fast transient over $t_{c_1}$. This implies that indicator substrate cannot accumulate significantly over the course of $t_{c_1}$, which is equivalent to a RSA for the indicator reaction. Thus, we require that

$$\max v_f \cdot t_{c_1} \ll s_1^0,$$

(36)

where $\max v_f$ denotes the maximum velocity of formation of $s_2$. From (6c) we have

$$\max v_f = \frac{k_3 c_2^0 s_1^0}{K_M + s_1^0} + k_{-3} \frac{e_2^0}{K_M + s_1^0} + k_2 \frac{e_1^0}{K_M + s_1^0}.$$  

(37)

Multiplying (37) by $t_{c_1}$ and dividing by $s_1^0$ yields,

$$\frac{\max v_f \cdot t_{c_1}}{s_1^0} = \delta_\lambda \left[ \kappa_2 + (1 + \kappa_2) \sigma_2 \right] + \delta_\varepsilon$$

(38)

where $\delta_\lambda = t_{c_1} / s_1$ and $\delta_\varepsilon = t_{c_1} / s_1$. It follows from (38) that

$$\delta_\varepsilon \ll 1$$

(39a)

$$\delta_\lambda \ll \frac{1}{\kappa_2 + (1 + \kappa_2) \sigma_2}$$

(39b)

The resulting equations (39a)-(39b) constitute the first RSA for the indicator reaction, and provide conditions for the validity of the initial condition $s_2(t_{c_1}) = 0$. Notice that (39) designates separation in the timescales that respectively define the initial fast transient and QSS regime of the non-observable reaction. Timescale separation entails

$$\varepsilon \ll \left( 1 + \frac{K_S}{K} \right) (1 + \sigma_1),$$

(40)

where $K_S = k_{-1}/k_1$ and $K = k_2/k_1$. Note that the above conditions is satisfied under the RSA for the non-observable reaction (i.e., is $\varepsilon \ll 1$).
There are additional RSAs for the indicator substrate. We also require that $s_2$ be a slow variable over $t_{c_2}$. Rescaling (6c) with respect to $\hat{s}_2, \hat{c}_2, \hat{c}_1$ and $t^* = t/t_{c_2}$ yields

$$\frac{d\hat{s}_2}{dt^*} = -\lambda \left( 1 - \frac{\sigma_2}{1 + \sigma_2} \right) \hat{s}_2 + \frac{\lambda \alpha}{1 + \sigma_2} \hat{c}_2 + \delta_\nu \hat{c}_1,$$

where $\delta_\nu = t_{c_2}/t_{s_1}$. As a result of the scaling analysis, it is clear that $s_2$ will also be a slow variable on the $t_{c_2}$ timescale as long as:

$$\lambda \ll 1 \quad (42a)$$
$$\delta_\nu \ll 1 \quad (42b)$$

In addition, we will require that $c_2$ be a fast variable over both depletion timescales. Rescaling (25b) with respect to $\tau = t/t_{s_1}$ yields,

$$\frac{\lambda d\hat{c}_2}{d\tau} = \delta(1 + \kappa_2)(1 + \sigma_2) \left[ \left( 1 - \frac{\sigma_2}{1 + \sigma_2} \right) \hat{s}_2 - \frac{1}{1 + \sigma_2} \hat{c}_2 \right]. \quad (43)$$

The scaling implies

$$\lambda \ll \delta(1 + \kappa_2)(1 + \sigma_2) \quad (44)$$

where $\delta = t_{s_1}/t_{s_2}$. Collectively, the inequalities (44),(42a) and (42b) define the second and third RSA conditions for $s_2$.

5 Comparison of analytical approximations to the numerical solutions

We are now ready to compare the numerical solutions of the mass action equations (6a) and (6c) with the analytical approximations (29a)-(29b). Both solutions are in good in quantitative agreement when the simple necessary criteria (30) for the validity of the RSA for the non-observable and indicator reaction hold (see Figs. 2(a)) and 2(b)).

From the context of singular perturbation theory, the QSS period is achieved when both $\hat{c}_1 \approx 0$ and $\hat{c}_2 \approx 0$. From a phase-space point of view, this occurs when trajectories are arbitrarily close to the slow manifold $M_{c,\lambda}$, which is the intersection of the surfaces $\mathcal{T}$ and $\Omega$:

$$\mathcal{T} = \{(s_1, c_1, s_2, c_2) \in \mathcal{P} | (s_1, c_1) \in \mathcal{M}_c \} \quad (45a)$$
$$\mathcal{M}_c = \mathcal{T} \cap \mathcal{M}_\lambda \quad (45b)$$

where $\delta = t_{s_1}/t_{s_2}$. Collectively, the inequalities (44),(42a) and (42b) define the second and third RSA conditions for $s_2$. 

Fig. 2 Comparison of the numerical solutions to the mass-action equations of (6a)-(6d) (labeled “s1” and “s2”) versus the numerical solutions of the MM equations of (29a)-(29b) (labeled “sMM1” and “sMM2.” Time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 − 1/\ln(t+\varepsilon)$. In both cases, the concentrations s1 and s2 have been scaled as $s_1/s_1^0$ and $s_2/s_1^0$. With $M_\varepsilon$ and $M_\lambda$ denoting the one-dimensional slow-manifolds:

\[
M_\varepsilon = \frac{c_1^0}{K_{M_1} + s_1} s_1 + \mathcal{O}(\varepsilon) \tag{46a}
\]

\[
M_\lambda = \frac{c_2^0}{K_{M_2} + s_2} s_2 + \mathcal{O}(\lambda). \tag{46b}
\]

$M_\varepsilon$ and $M_\lambda$ are the projections of $\Upsilon$ and $\Omega$ onto the respective subsets $\mathcal{P}_1 = (s_1, c_1, 0, 0)$ and $\mathcal{P}_2 = (0, 0, s_2, c_2)$.
Fig. 3 When initial conditions are experimental and \( \lambda \ll 1 \), the numerical solution lies very close to the critical manifold \( \mathcal{M}_\lambda \) for all time. This particular solution was computed with \( s_1^0 = 2000, s_2^0 = 0, c_1^0 = 1, c_2^0 = 1, c_3^0 = 0, c_4^0 = 0 \) and \( k_1 = 1, k_2 = 10, k_{-1} = 10, k_3 = 1, k_{-3} = 10 \), and \( k_4 = 10 \). The red solid line corresponds to \( \approx \mathcal{M}_\lambda \) and the diamonds are numerical solutions of the indicator reaction.

The typical experimental test tube initial conditions (i.e., \( s_2^0 = 0 \) and \( c_2^0 = 0 \)) lie on \( \Omega \) since the points \((s_1, c_1, 0, 0)\) are contained in both the critical manifold \( \mathcal{M}_0 \) and \( \Omega \):

\[
(s_1, c_1, 0, 0) \in \mathcal{M}_0 \cap \Omega \tag{47}
\]

Therefore, \( c_2 \) is in the QSS phase for the duration of the reaction, and the initial fast transient occurs as the phase-space trajectory’s approaches \( \Upsilon \). \( c_1 \) will reach QSS period when \( t = t_{c_1} \), at which time the phase-space trajectory is, and remains, arbitrarily close to \( \Upsilon \) (see, Fig. 3).

From a numerical perspective, since experimental test tube initial conditions imply that \( c_2 \) reaches the QSS period, it holds that the numerical solution of the mass-action equations (6a)-(6d) should satisfy

\[
c_2 = \frac{e_2^0}{K_{M_2} + s_2} + \mathcal{O}(\lambda) \tag{48}
\]

for all time. Furthermore, if initial conditions are not experimental (i.e., if \( s_2^0 > 0 \) and \( c_2^0 > 0 \)), then \( t_{c_2} \) should give an accurate estimate of the time it takes the trajectory to reach \( \Omega \), or, chemically, the time it takes for \( c_2 \) to reach the QSS period. Both of the results are easily verified numerically (see Figs. 4(a)-4(b)).

6 Discussion

When an enzyme catalyzed reaction of primary interest cannot be observed experimentally, enzymologists monitor the substrate depletion or product accumulation by linking the reaction catalyzed by the primary enzyme to one or
more enzyme-catalyzed reactions. If the linked reaction can be observed experimentally, then it is theoretically possible to investigate the kinetic behavior of the non-observable reaction of primary interest. As a case study, we carried out a kinetic analysis of the coupled sequential enzyme reaction (1)–(2) in this paper. The two most important results of our analysis are:

I. The derivation of a simple rate equation to monitor the substrate depletion of the indicator reaction in the form of an interacting system of MM equations:

\begin{align*}
\dot{s}_1 &= -\frac{V_1}{K_{M_1} + s_1} s_1 \\
\dot{s}_2 &= \frac{V_1}{K_{M_1} + s_1} - \frac{V_2}{K_{M_2} + s_2} s_2.
\end{align*}

II. The derivation of criteria for the validity of the above equations:

\begin{align*}
\lambda &\equiv \frac{e_2}{K_{M_2} + s_1} \ll \min\{1, \delta\} \\
\varepsilon &\equiv \frac{e_1}{K_{M_1} + s_1} \ll 1.
\end{align*}

Our numerical simulations show, and as our analysis explains, this system of interacting MM equations provide an excellent approximation to the QSS phase of the sequential enzyme reaction (1)–(2) when the RSA is valid. This requires setting up initial experimental conditions, where \( s_1^0 \) is in excess relative to both \( e_1^0 \) and \( e_2^0 \). The RSA criteria can also be valid when \( s_1^0 \approx e_1^0 \approx e_2^0 \) as long as the initial concentrations of enzymes are small compared to the Michaelis constants.
Additionally, we can derived a rate expression for the product accumulation by substituting the expression for $c_2$ during the QSS phase, Eq. (27), into (5c), that is

$$\dot{p} = \frac{V_2}{K_{M_2} + s_2 s_2}. \quad (49)$$

Our phase plane analysis of the invariant manifold shows that (49) provides a good approximation to $p$ for the duration of the reaction under experimental test tube conditions as long as the RSA holds. Interestingly, the rate of $P$ accumulation of the sequential enzyme reaction does not exhibits a lag time, though the $p$ accumulation is subject to a lag time in the time course of the sequential reaction. The time lag for the $p$ accumulation is governed by

$$t_{c_2} = \frac{1}{k_3(K_{M_2} + s_1^0)}.$$

This is a novel and more rigorous expression for the lag time of the sequential enzyme catalyzed reaction, which does not rely on linear approximations to the complex nonlinear dynamics reported in previous studies (McClure, 1969; Barwell and Hess, 1970; Hart, 1970; Goldman and Katchalski, 1971; Easterby, 1973, 1981; Brooks et al, 1984b; Barwell and Hess, 1970; Storer et al, 1974; Cleland, 1979; Brooks et al, 1984a,b).

A more thorough examination of the system of interacting MM equations is necessary to design steady-state experimental procedures for the estimation of the enzyme kinetic parameters of the non-observable reaction from the measurement of the indicator substrate depletion and product accumulation. Our analysis suggests that previous approaches relying on lag time studies can be ineffective to estimate non-observable reaction parameters for the coupled sequential enzyme reaction (1)–(2). One possibility is to estimate the kinetic parameters of this reaction in two phases. First, the kinetic parameters of the indicator reaction, $K_{M_2}$ and $V_2$, can be estimated using (49) through initial rate experiments of the product accumulation. Then, by fixing $K_{M_2}$ and $V_2$ in (29b), we can use this rate equation to estimate the parameters of the non-observable reaction, $K_{M_1}$ and $V_1$, through through initial rate experiments of the indicator substrate depletion. The development of standard-based approaches to measure kinetics parameters is a complex inverse problem as the conditions for the validity of mathematical approximations to described chemical reactions do not guarantee an accurate estimation of the kinetic parameters (Stroberg and Schnell, 2016, 2017).

The results of this paper were obtained by scaling and singular perturbation analysis, which can provide a procedure to estimate higher order corrections to estimate the error of approximations. Our approach can be extended to a variety of coupled enzyme catalyzed assays, including sequential enzyme catalyzed reactions with more reaction steps.
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