Theory of the reactant-stationary kinetics for zymogen activation coupled to an enzyme catalyzed reaction

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Abstract

A theoretical analysis is performed on the nonlinear ordinary differential equations that govern the dynamics of a coupled enzyme catalyzed reaction. The reaction consists of a primary non-observable zymogen activation reaction that it is coupled to an indicator (observable) reaction, where the product of the first reaction is the enzyme of the indicator reaction. Both reactions are governed by the Michaelis–Menten reaction mechanism. Using singular perturbation methods, we derive asymptotic solutions that are valid under the quasi-steady-state and reactant-stationary assumptions. In particular, we obtain closed form solutions that are analogous to the Schnell–Mendoza equation for Michaelis–Menten type reactions. These closed-form solutions approximate the evolution of the observable reaction and provide the mathematical link necessary to measure the enzyme activity of the non-observable reaction. Conditions for the validity of the asymptotic solutions are also derived and demonstrate that these asymptotic expressions are applicable under the reactant-stationary kinetics.

Keywords: Coupled enzyme assay, time course experiments, timescale separation analysis, singular perturbation analysis, Schnell–Mendoza equation, zymogen activation

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1. Introduction

Many enzyme catalyzed reactions that occur in physiological processes require an activation step, in which a precursor of a zymogen (inactive enzyme precursor or pro-enzyme) is converted to an active enzyme. This process, known generally as zymogen activation [1], is typically the first step in a cascade of coupled enzyme catalyzed reactions since the newly activated enzyme is then free to either (1) bind with a substrate to yield a product through an enzyme catalyzed reaction or (2) activate an additional enzyme [2]. The activation step of the zymogen is itself an enzyme catalyzed reaction, since the inactive enzyme precursor is activated by an active enzyme. The active enzyme can be generated by enzyme-catalyzed proteolysis or enzyme activation by phosphorylation [3]. For example, the digestive enzyme trypsin, which is the active form of trypsinogen, is activated by the enzyme enterokinase; trypsin can then bind with trypsinogen to convert remaining trypsinogen into trypsin [2]. Likewise, plasminogen is activated by streptokinase to form plasmin (enzyme), which then binds with and degrades fibrin (substrate) to break down clots in blood coagulation [4]. Regardless of the reactants, the preliminary zymogen activation step coupled with another secondary enzyme-catalyzed reaction can be expressed with the following reaction mechanism (1)–(2)

\[
E_1 + E_i^2 \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} C_1 \rightarrow E_1 + E_2
\]

in which the primary enzyme, \(E_1\), reacts with the zymogen \(E_i^2\) to form an intermediate complex \(C_1\) following the Michaelis–Menten (MM) [5] reaction mechanism. The product of the primary reaction is thus the activated form of the secondary enzyme \(E_2\). In the secondary reaction the substrate (S) binds with the enzyme (\(E_2\)) which forms a complex (\(C_2\)) and synthesizes the product
(P) in a catalytic reaction step

\[
E_2 + S \xrightleftharpoons[k_{-3}]{k_3} C_2 \xrightarrow{k_4} P + E_2 .
\]

In the above chemical pathways, \(k_1, k_{-1}, k_3, k_{-3}\) are microscopic rate constants, and \(k_2, k_4\) are catalytic constants.

The mass action equations governing (1)–(2) are inherently nonlinear. However, much of the literature in which reactions of this form are analyzed mathematically invokes the assumption of pseudo-first-order (PFO) kinetics \([6, 1, 7, 4, 2]\). Of course, the PFO kinetic models have the mathematical advantage of being linear; thus, closed-form solutions are easily obtained through the use of Laplace transforms. To date however, a nonlinear analysis of zymogen activation in a coupled enzyme assay has not been performed.

Another interesting aspect of coupled enzyme catalyzed reactions with a zymogen activation step (1)–(2) is the quantification of the catalytic conversion of zymogen in vitro. If the activation step (1) is not detectable experimentally (i.e., non-observable), the secondary reaction step (2) is selected to be an easily observable reaction in the enzyme kinetic assay. This is done with the goal of measuring the enzyme activity of the non-observable reaction by analyzing the progress curves of the secondary observable reaction. In this case, the secondary reaction step (2) is known as the indicator reaction. Traditionally, coupled enzyme assays are designed so that the product of the non-observable reaction is a substrate for the secondary enzyme in the indicator reaction (see \([8]\) for specific applications). This type of assay is well-studied \([9, 10, 11, 12]\) and is known as a coupled (or auxiliary) enzyme assay or coupled sequential enzyme assay.

Zymogen activation coupled to an enzyme catalyzed reaction (1)–(2) occurs naturally in coagulation cascades \([13]\). As a distinct example, the activation of
protein C (PC) by thrombin (T) follows a reaction consistent with (1):

$$T + PC \rightleftharpoons TPC \rightarrow T + APC$$

(3)

where “APC” denotes the activated form of PC. In the experimental assay, the activated enzyme APC then catalyzes a substrate (S). Assuming S is specific to APC and does not bind with T, the secondary observable reaction follows the form of (2)

$$APC + S \rightleftharpoons SAPC \rightarrow APC + P .$$

(4)

Experimentally, the kinetics of the non-observable reaction is measured by decoupling the analysis of progress curves by adding excessive concentrations of the primary enzyme, thus making the first reaction PFO [13]. However, it has been demonstrated that excessive concentrations of the initial enzyme $E_1$ is not sufficient to guarantee the validity of PFO kinetic models. What is necessary is that initial concentration of zymogem for (1) be much less than the Michaelis constant of the primary reaction [14]. Thus, from an experimental point of view, it is difficult to ensure the validity of the PFO model when the Michaelis constant is unknown and consequently to be determined.

It is well-established that under appropriate experimental conditions the MM reaction mechanism

$$E + S \rightleftharpoons C \rightarrow P + E .$$

(5)

will obey quasi-steady-state (QSS) kinetics, and the rate of substrate depletion for the reaction is described by the MM equation

$$\dot{s} = -\frac{V}{K_M + s} s.$$
where $s$ is the concentration of $S$, $K_M = (k_− + k_{\text{cat}})/k$ is the Michaelis constant, and $V = k_{\text{cat}}c_0$ is limiting rate of the reaction [5], which is dependent on both the catalytic constant $k_{\text{cat}}$ and the initial concentration of $E$ (the initial concentration of $E$ is denoted as $e_0$). Note that the zymogen activation step in [1]-[2] is a single enzyme, single substrate reaction, where the substrate is effectively the zymogen.

Of great interest to both theoreticians [15, 16] and experimentalists is the estimation of the constants $K_M$ and $V$ from the so-called inverse problem. The inverse problem is carried out in two stages: First, experimental data is produced in the form of a progress curve for either $s$ or $p$ (we have used lower case letters to denote the concentrations of $S$ and $P$ respectively). Second, the experimental data is then used to estimate both $K_M$ and $V$ by optimally fitting the model [6] through the utilization of either a deterministic (i.e., such as Levenburg-Marquardt) or a stochastic (Markov Chain Monte Carlo) algorithm. In general, one seeks to estimate kinetic constants with an expression that contains the fewest number of parameters, which is why the MM equation is more attractive than the complete set of mass action equations. The MM equation is what is known as a reduced model, and it is reduced in the sense that it contains fewer variables ($s$ versus $s$ and $c$) and fewer parameters ($K_M$ and $V$ versus $k, k_-$ and $k_{\text{cat}}$).

The inverse problem presents a unique challenge for both experimentalists and theorists in coupled enzyme assays like [1]-[2]. First, the parameters that govern the enzyme activity of the non-observable reaction must somehow be determined from the indicator reaction, since progress curves from a typical in vitro laboratory experiment can only be generated for the indicator reaction. Second, a reduced model for the zymogen activation coupled to an enzyme catalyzed reaction [1]-[2] must be developed. The reduced model should: (1) decrease the number of variables, and (2) lessen the number of parameters needed to describe the time course of the complete zymogen activation coupled to an enzyme catalyzed reaction reaction.
1.1. Goals of this paper

For the single enzyme, single substrate MM reaction mechanism \(\text{(5)}\), the MM equation is the result of a model reduction method known as slow manifold projection. The validity of the MM equation resides under the assumption that the single-enzyme, single-substrate reaction \(\text{(5)}\) has two intrinsic timescales. The first timescale is very short, and accounts for the rapid accumulation of the complex \(C\). The second timescale is very long, and gives a rough measure of the time it takes for the completion of the reaction. Respectively, these timescales are known as fast and slow timescales. If these timescales are inherently present within \(\text{(5)}\), then the dimensionless mass action equations that model \(\text{(5)}\) can be written in the form

\[
\dot{s} = f_1(s, c) \\
\varepsilon \dot{c} = f_2(s, c),
\]

where \(\varepsilon\) is very small (i.e., \(\varepsilon \ll 1\)), and is proportional to the ratio of the fast timescale to the slow timescale. Differential equations in the form of \(\text{(7)}\) are called singularly perturbed differential equations, and they are ubiquitous in mathematical chemistry \[17, 18, 19\] and biology \[20\]. Thus, central to deriving a reduced model for the zymogen activation coupled to an enzyme catalyzed reaction (using slow manifold projection) is the estimation of the slow and fast timescales for the non-observable reaction. This is challenging for coupled reactions, since the time to completion of the indicator reaction can occur before, after, or at approximately the same time as the non-observable reaction. Furthermore, it is unlikely that the relative speeds and completion time of the non-observable reaction will be known. Thus, there is a need derive a reduced model that is general enough so that its validity is certain regardless of which reaction is fastest. Finally, the most desirable reduced model will be one in which a closed form solution is obtainable so that the reduced model may be expressed as an explicit function of time. This will eliminate the need to generate explicit progress curves for substrate depletion of the primary reaction since the time course of substrate is unknown in coupled enzyme assays.
1.2. Structure of this paper

As mentioned previously, the theoretical reduction analysis of zymogen activation reactions has been limited to PFO kinetics models. Such models have limited validity in time course experiments \[14\], and the aim of this work is first and foremost to take a necessary “first step” in the nonlinear analysis of such reactions. First, we will introduce proper scaling techniques that can be employed in a general methodology to more complicated reaction. In Section 3, we will show how to estimate timescales based on these scaling methods, as well as how to formulate reduced model from the analysis of these timescales (Section 4). The reduced model is analogous to [7], and admits closed-form solutions in the form of a Schnell–Mendoza equation \[21\]. Conditions for the validity of the reduced model will be established, and timescale estimates will be derived. In addition, we will exploit the geometry of the mathematical structure \[22, 23\] in extreme situations when the speeds of the reactions are significantly disparate. This will allow us to “simplify” the reduced model and obtain asymptotic solutions that are in some ways easier in form than both the general reduced model and the system of mass action equations. Finally, in Section 7 we conclude with a brief discussion of the results and their relevance in possible future work involving the inverse problem.

2. Derivation of the governing equations for the zymogen activation coupled to an enzyme catalyzed reaction

Let us consider the zymogen activation coupled to an enzyme catalyzed reaction \(1 \rightarrow 2\). In reaction \(1\), the zymogen \(E_2\) is effectively a substrate. To distinguish between substrates and enzymes in \(1 \rightarrow 2\), we will change notation by replacing \(E_2\) with \(S_1\) in \(1\), and \(S\) with \(S_2\) in \(2\). Under this notation, applying the law of mass action to zymogen activation coupled to an enzyme...
catalyzed reaction yields seven rate equations

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

where lowercase letters represent concentrations of the corresponding uppercase species. Typically, laboratory enzyme assays present the following initial conditions

\[(e_1, s_1, c_1, e_2, s_2, c_2, p) \big|_{t=0} = (e_1^0, s_1^0, 0, 0, s_2^0, 0, 0).\]  

(9)

By examining the system of rate equations (8), the zymogen activation coupled to an enzyme catalyzed reaction obeys three conservation laws:

\[
\begin{align*}
e_1 (t) + c_1 (t) &= e_1^0, \\
s_1 (t) + c_1 (t) + c_2 (t) + e_2 (t) &= s_1^0, \\
s_2 (t) + c_2 (t) + p (t) &= s_2^0.
\end{align*}
\]

(10a) (10b) (10c)

Mathematically speaking, the solution trajectory to (8) must lie on the intersection of the hyperplanes defined in (10), which means the original seven-dimensional problem can be reduced to a four-dimensional problem. Using (10a) and (10b) to decouple the enzyme concentrations, the redundancies in the system (8) are eliminated to yield

\[
\begin{align*}
\dot{s}_1 &= -k_1 (e_1^0 - c_1) s_1 + k_{-1} c_1 \\
\dot{c}_1 &= k_1 (e_1^0 - c_1) s_1 - (k_{-1} + k_2)c_1 \\
\dot{s}_2 &= -k_3 (s_1^0 - s_1 - c_1 - c_2)s_2 + k_{-3} c_2 \\
\dot{c}_2 &= k_3 (s_1^0 - s_1 - c_1 - c_2)s_2 - (k_{-3} + k_4)c_2,
\end{align*}
\]

(11a) (11b) (11c) (11d)
where $e_1(t)$, $e_2(t)$ and $p(t)$ are readily calculated once $s_1(t)$, $c_1(t)$, $s_2(t)$ and $c_2(t)$ are known.

3. Rate expressions for the non-observable enzyme catalyzed reaction

The rate equations (11a)–(11b) are uncoupled from (11c)–(11d). These rate equations have the same structure to those of the single substrate, single enzyme reaction following the MM mechanism. Therefore, it is possible to derive rate equations to model the zymogen activation coupled to an enzyme catalyzed reaction, and estimate its kinetic parameters using the general theory of the reactant-stationary assumption (RSA, [24]). The rate equations for the non-observable reaction are identical to those of the single substrate, single enzyme reaction following the MM mechanisms.

3.1. Review of the single substrate, single enzyme MM reaction

Revisiting the analysis for the single-substrate, single-enzyme reaction, it has long been established that there there can be a rapid buildup of $c_1$ during an initial fast transient of the non-observable reaction. After this rapid buildup (where the rate of depletion of $c_1$ approximately equals its rate of formation) $c_1$ is assumed to be in a QSS

$$c_1' \approx 0 \quad \text{for} \quad t > t_{c_1}. \quad (12)$$

The timescale $t_{c_1}$ is the time associated with the initial transient buildup of $c_1$

$$t_{c_1} = \frac{1}{k_1(K_{M_1} + s_1^0)}.$$ \hspace{1cm} (13)

In the above equation, $K_{M_1} = (k_{-1} + k_2)/k_1$ is the Michaelis constant for the zymogen activation step \(\text{I}\). The quasi-steady-state assumption (QSSA, [12]), in combination with (11a)–(11b), leads to the derivation of the well-known rate expressions

$$c_1 = \frac{e_1^0}{K_{M_1} + s_1} s_1 \quad (14a)$$
$$s_1 = -\frac{V_1}{K_{M_1} + s_1} s_1. \quad (14b)$$
In (14b), $V_1 = k_2 e_1^0$ is the limiting rate of the zymogen reaction. Note that the mass action equations (11a)–(11b) are reduced to a differential-algebraic equation systems with one single differential equation for $s_1$ in (14a)–(14b).

Since equations (14a) and (14b) are only valid after the initial transient, $t_{c_1}$, it is necessary to define a boundary condition for $s_1$ at $t = t_{c_1}$. This is equivalent to the initial experimental condition for the initial rate or time course experiments. To find this condition, it can be assumed that there is a negligible decrease in $s_1$ during the initial transient. This is known as the RSA, and is expressed as

$$s_1(t < t_{c_1}) \approx s_1^0.$$ (15)

The RSA provides an initial condition for (11a) under the variable transformation $\hat{t} \mapsto t - t_{c_1}$. The mathematical expression (14b) is the MM equation, and the system (14a)–(14b) governs the dynamics of the substrate $s_1$ and complex $c_1$ of the non-observable reaction under the QSS and RSA. The explicit closed-form solution of (14b), with the initial condition (15), is known as the Schnell–Mendoza equation [21], and is written in terms of the Lambert-$W$ function

$$s_1(\hat{t}) = K_{M_1} W \left[ \sigma_1 \exp(\sigma_1 - \eta_1 \hat{t}) \right], \quad \sigma_1 = \frac{s_1^0}{K_{M_1}}, \quad \eta_1 = \frac{V_1}{K_{M_1}}.$$ (16)

From the perspective of asymptotic theory, Schnell and Mendoza [21] have provided a piecewise solution for the MM reaction in terms of a fast transient solution for $s_1$, valid for $t \leq t_{c_1}$, as well as a QSS solution for $s_1$, valid for $t > t_{c_1}$:

$$s_1 = s_1^0, \quad t \leq t_{c_1} \quad \text{(17a)}$$

$$s_1 = K_{M_1} W \left[ \sigma_1 \exp(\sigma_1 - \eta_1 \hat{t}) \right], \quad t > t_{c_1} \quad \text{(17b)}$$

From the earlier work of Segel [25], we have a fast solution, valid when $t \leq t_{c_1}$,
for the complex $c_1$, as well as a QSS solution which is valid for $t > t_{c_1}$

\[ c_1 \simeq \tilde{c}_1 [1 - \exp(-t/t_{c_1})] \quad \text{with} \quad \tilde{c}_1 = \frac{c_1^0}{K_{M_1} + s_1^0}, \quad t < t_{c_1}, \quad (18a) \]

\[ c_1 \simeq \frac{c_1^0}{K_{M_1} + s_1}, \quad t \geq t_{c_1}. \quad (18b) \]

Collectively, equations (17a) and (18b) constitute an asymptotic solution that serves as an accurate approximation to the full time course of (11), provided the appropriate qualifiers (i.e., the RSA and the QSSA) are obeyed.

In addition to the timescale $t_{c_1}$, which quantifies the length of the initial fast transient (build-up of $c_1$), the time it takes for the majority of the substrate $s_1$ to deplete is given by $t_{s_1}$. Although there are several methods for estimating the significant timescales of chemical reactions [26], we employ the heuristic method proposed by Segel [25], and approximate the depletion time to be effectively the total depletion of $s_1$ (the total depletion is $s_1^0$) divided by the maximum rate of substrate of depletion after $t_{c_1}$:

\[ t_{s_1} = \frac{\Delta s_1}{\max_{0 \leq t} |s_1|} = \frac{K_{M_1} + s_1^0}{V_1}. \quad (19) \]

Generally speaking, $t_{s_1}$ is a reasonable measure of how long it takes for the non-observable reaction to complete.

3.2. Geometrical picture of the enzyme catalyzed reaction, and conditions for the validity of asymptotic solutions of the rate equations

While the asymptotic solutions are useful in that they can be employed to make certain predictions about the behavior of the reaction, asymptotic theory fails to yield a visual or geometric understanding of the dynamical behavior of the zymogen activation coupled to an enzyme catalyzed reaction. To paint a complete picture of the mathematical structure behind the reaction mechanism [1]–[2], we turn to dynamical systems theory, and analyze this problem from phase space. From this perspective, after the initial buildup of $c_1$, the phase
space trajectory of the non-observable reaction $[11a] - [11b]$ hugs a slow manifold, $\mathcal{M}_e$, and is asymptotic to $\mathcal{M}_e$ in the approach to equilibrium. The time it takes for the trajectory to reach the slow manifold is approximately $t_{c1}$, while the time it takes for the trajectory to equilibrium is approximated by $t_{s1}$. The condition for the validity of the asymptotic solution resides in how well the $c_1$-nullcline approximates the slow manifold, $\mathcal{M}_e$, and also how straight the phase space trajectory is in its approach to the slow manifold during the initial fast transient. The former of these conditions is known as the QSSA, and the latter is of course the geometrical interpretation of the RSA. We note that, chemically speaking, if the trajectory is close the slow manifold $\mathcal{M}_e$, then the complex $C_1$ is assumed to be in a QSS and the difference between the rate of $C_2$ depletion is approximately equal to the rate $C_2$ formation. It was originally proposed that the QSSA was valid if $t_{c1} \ll t_{s1}$

$$\frac{1}{k_1(K_{M_1} + s_1^0)} \ll \frac{K_{M_1} + s_1^0}{V_1}. \quad (20)$$

In other words, it was assumed that the $c_1$-nullcline should be considered a good approximation to the slow manifold $\mathcal{M}_e$ if the timescale accounting for the build-up of $c_1$ was small compared to the timescale accounting for the duration of the reaction.

As for the validity of the RSA, Segel [27] proposed that one could assume little change in $s_1$ (an almost straight phase space trajectory towards the slow manifold) if the depletion of $s_1$ over the timescale $t_{c1}$ is minimal:

$$\max_{t \geq 0} |\dot{s}_1| \cdot t_{c1} \ll s_1^0. \quad (21)$$

Since $|\dot{s}_1| \leq s_1^0 e_1^0$, the strict inequality given in (21) translates to

$$\frac{e_1^0}{K_{M_1} + s_1^0} \equiv \varepsilon \ll 1. \quad (22)$$

Through scaling analysis, Segel [25] went on to show that the RSA determines single-handedly the validity of the asymptotic solutions (17) and (18). Introducing the dimensionless variables $\hat{s}_1 = s_1/s_1^0$ and $\hat{c}_1 = c_1/c_1$, Segel and
Slemrod [27] demonstrated that, with respect to the dimensionless timescale \( \tau = t/t_c \), equations (11a)-(11a) scale as

\[
\frac{d\hat{s}_1}{d\tau} = \varepsilon \left[-\hat{s}_1 + \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 + \frac{\kappa_1(1 + \kappa_1)^{-1}}{\sigma_1 + 1} \hat{c}_1\right]
\]

(23a)

\[
\frac{d\hat{c}_1}{d\tau} = \hat{s}_1 - \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 - \frac{1}{\sigma_1 + 1} \hat{c}_1,
\]

(23b)

where \( \kappa_1 = k_{-1}/k_2 \) and \( \varepsilon = e_1^0/(k_{M_1} + s_1^0) \). In contrast, under the timescale \( T = t/t_{s_1} \), (11a)-(11a) become:

\[
\frac{d\hat{s}_1}{dT} = (\kappa_1 + 1)(\sigma_1 + 1) \left[-\hat{s}_1 + \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 + \frac{\kappa_1(1 + \kappa_1)^{-1}}{\sigma_1 + 1} \hat{c}_1\right]
\]

(24a)

\[
\varepsilon \frac{d\hat{c}_1}{dT} = (\kappa_1 + 1)(\sigma_1 + 1) \left[\hat{s}_1 - \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 - \frac{1}{\sigma_1 + 1} \hat{c}_1\right].
\]

(24b)

Thus, it is apparent from the dimensionless equations (23)-(24b) that if \( \varepsilon \ll 1 \), then not only will the RSA hold, but the QSSA (which assumes that the \( c_1 \)-nullcline is a good approximation to \( M_\varepsilon \)) also holds. In fact the RSA (i.e., \( \varepsilon \ll 1 \)) is more restrictive than separation of timescales. After some algebraic calculations, the separation of timescales \( (t_{c_1}/t_{s_1} \ll 1) \) can be written as:

\[
\frac{e_1^0}{K_{M_1} + s_1^0} \ll \left(1 + \frac{K_S}{K_1}\right) \left(1 + \frac{s_1^0}{K_{M_1}}\right),
\]

(25)

where \( K_{S_1} = k_{-1}/k_1 \), and \( K_1 = k_2/k_1 \). For the RSA to be valid, the condition

\[
\frac{e_1^0}{K_{M_1} + s_1^0} \ll \left(1 + \frac{s_1^0}{K_{M_1}}\right),
\]

(26)

must be satisfied, which is more stringent than condition (25), and hence dictates the conditions under which equation (14b) or (16) can be applied. For this reason, it is nowadays considered that MM expressions are valid under the RSA (see Figures 1a and 1b) rather than the QSSA [28].

### 3.3. Scaling analysis of the indicator reaction

The scaling analysis of the indicator reaction requires knowledge of fast and slow timescales, as well as knowledge of reasonable upper and lower bounds
Figure 1: Geometrical picture of the single-substrate, single-enzyme non observable reaction (1) representing the zymogen activation step. (a) Phase space dynamics with $e_1^0 = 10, k_1 = 1, k_2 = 1$ and $k_{-1} = 1$. (b) Phase space dynamics with $e_1^0 = 1, s_1^0 = 78, k_1 = 1, k_2 = 5$ and $k_{-1} = 1$. As $\varepsilon \to 0$, the accumulation of $c_1$ is more rapid, and the $c_1$-nullcline (dashed red curve) becomes a better approximation to the slow manifold, $M_\varepsilon$, which is the thick black curve. The slow manifold curve is a graphical representation of the steady-state kinetic rate equation. The thin black curves are trajectories (numerical solutions of the mass action equations (11)) starting from different initial conditions, and represent the fast-transient kinetics of the reaction.

of $s_2$ and $c_2$. We will start by trying to estimate a slow timescale for the indicator reaction. An accurate slow timescale should give us a reasonable estimation of the completion time for the indicator reaction. In the case of the
zymogen activation coupled to an enzyme catalyzed reaction, the completion of
the indicator reaction can be faster, as fast, or slower than the non-observable
reaction. For the non-observable reaction, the slow timescale is expressed in
terms of the initial quantities \( s^0_1 \) and \( e^0_1 \), and the Michaelis constant \( K_{M_1} \):

\[
t_{s_1} = \frac{K_{M_1} + s^0_1}{V_1}.
\]  

(27)

The quantity \( e^0_1 \) is the total amount of enzyme for the non-observable reaction.

The construction of a homologous slow timescale for the indicator reaction is
problematic in that the total amount of available enzyme \( e^A_2 \),

\[
e^A_2(t) = s^0_1 - s_1 - c_1,
\]  

(28)
is a time-dependent quantity. If we start by assuming the QSSA is valid, then
the mass action equations reduce to

\[
c_2 \simeq \frac{e^A_2(t)}{K_{M_2} + s_2},
\]  

(29a)

\[
s_2 \simeq -\frac{V_2(t)}{K_{M_2} + s_2},
\]  

(29b)

where \( V_2(t) \equiv k_4 e^A_2(t) \). The general solution to (29b) is given in terms of a
Lambert-\( W \) function

\[
s_2 = K_{M_2} \text{W} \left[ \sigma_2 e^{\sigma_2 - \int_0^t V_2(s) \, ds} / K_{M_2} \right],
\]  

(30)

where “\( s \)” has been employed as a dummy variable and \( \sigma_2 \equiv s^0_2 / K_{M_2} \). We
will employ a mean-field approach to derive a slow (depletion) timescale for the
indicator reaction. Let us first assume that we know the slow timescale for the
indicator reaction, and denote this timescale as \( T_{s_2} \). Then, the mean available
enzyme over the time course of the indicator reaction, which we will denote as
\( \langle e^A_2 \rangle \), is given by

\[
\langle e^A_2 \rangle = \frac{1}{T_{s_2}} \int_0^{T_{s_2}} e^A_2(t) \, dt.
\]  

(31)

If the completion of the indicator reaction occurs long before the completion
of the non-observable reaction, then we expect that \( \langle e^A_2 \rangle \ll s^0_1 \). In contrast, if
the completion of the indicator reaction occurs long after the completion of the non-observable reaction, then we expect $\langle e^A_2 \rangle \approx s^0_1$. In any case, we can define the slow timescale as

$$T_{s_2} = \frac{K_{M_2} + s^0_2}{k_4 \langle e^A_2 \rangle},$$  \hspace{1cm} (32)$$

which should yield a reasonable estimate for the slow timescale if the depletion of $s_2$ is influenced by a slow manifold. Note that $K_{M_2} = (k_{-3} + k_4)/k_3$ is the Michaelis constant of the indicator reaction.

Next, we want to scale the mass action equations that model the indicator reaction with respect to the quantities $\hat{T} = t/\hat{t}$, $s^0_2$, and $\max(\bar{e}^A_2)$, where $\max(\bar{e}^A_2)$ is the maximum amount of $e^A_2$ over the course of the indicator reaction:

$$\max(\bar{e}^A_2) \equiv \max_{t \leq T_{s_2}} (s^0_1 - s_1 - c_1).$$  \hspace{1cm} (33)$$

Utilizing $\max(\bar{e}^A_2)$ as an upper bound on the available enzyme dictates a natural scaling of $c_2$

$$c_2 \leq \frac{\max(\bar{e}^A_2)}{K_{M_2} + s^0_2} = \hat{c}_2.$$  \hspace{1cm} (34)$$

Scaling the mass action equations with respect to the following dimensionless variables,

$$\bar{s}_2 s^0_2 = s_2, \quad \bar{c}_2 \hat{c}_2 = c_2, \quad \bar{e}^A_2 \max(\bar{e}^A_2) = e^A_2, \quad \hat{T}\hat{t} = t,$$

where $\hat{t}$ denotes an arbitrary timescale. Substitution of these quantities into the mass action equation yields

$$\frac{d\bar{s}_2}{d\hat{t}} = \frac{\max(\bar{e}^A_2)}{\langle \bar{e}^A_2 \rangle} \frac{\hat{t}}{T_{s_2}} (1 + \kappa_2)(1 + \sigma_2) \left[ \left( \frac{\sigma_2}{1 + \sigma_2} \bar{e}^A_2 - \bar{e}^A_2 \right) \bar{s}_2 + \frac{\alpha}{1 + \sigma_2} \bar{c}_2 \right].$$  \hspace{1cm} (36a)$$

$$\frac{\lambda d\bar{c}_2}{d\hat{t}} = \frac{\max(\bar{e}^A_2)}{\langle \bar{e}^A_2 \rangle} \frac{\hat{t}}{T_{s_2}} (1 + \kappa_2)(1 + \sigma_2) \left[ \left( \frac{\sigma_2}{1 + \sigma_2} \bar{e}^A_2 - \bar{e}^A_2 \right) \bar{s}_2 - \frac{1}{1 + \sigma_2} \bar{c}_2 \right].$$  \hspace{1cm} (36b)$$

In the above expressions, the dimensionless quantities $\sigma_2, \kappa_2$ and $\alpha$ are:

$$\sigma_2 \equiv s^0_2/K_{M_2}, \quad \kappa_2 \equiv k_{-3}/k_4, \quad \alpha \equiv \kappa_2/(1 + \kappa_2).$$  \hspace{1cm} (37)$$
The parameter $\lambda$, defined as

$$\lambda \equiv \max(e^A_2) \frac{1}{K_M + s_2^0}$$

is unique in that if it is sufficiently small, then it mathematically characterizes the indicator reaction as a singularly perturbed differential equation for which model reduction is possible through means of projecting onto the slow manifold $\mathcal{M}_\lambda$.

### 4. Asymptotic analysis of the zymogen activation coupled to an enzyme catalyzed reaction system

Now that we have a good idea as to how the mass action equations of the indicator reaction scale, we want to try and find closed-form asymptotic solutions to the mass action equations or, at the very least, try and reduce the dimension of the mass action differential equations. The exact form of the scaled mass action equations will depend on the slow timescales of both the observable and non-observable indicator reactions. Thus, given that the respective slow timescale of the indicator and non-observable reactions are $T_s$ and $t_{s_1}$, we will analyze

$$\frac{d\tilde{s}_2}{dT} = \frac{\max(e^A_2)\left(1 + \kappa_2\right)}{\left(e^A_2\right)\delta_S} \left[ \left( \frac{\sigma_2}{\sigma_2 + 1} \tilde{e}_2 - \tilde{e}_2 \right) \tilde{s}_2 + \frac{\alpha}{\sigma_2 + 1} \tilde{e}_2 \right]$$

$$\lambda \frac{d\tilde{e}_2}{dT} = \frac{\max(e^A_2)\left(1 + \kappa_2\right)}{\left(e^A_2\right)\delta_S} \left[ \left( \tilde{e}_2 - \frac{\sigma_2}{\sigma_2 + 1} \tilde{e}_2 \right) \tilde{s}_2 - \frac{1}{\sigma_2 + 1} \tilde{e}_2 \right],$$

where $\delta_S$ is the ratio of the substrate depletion timescales, $\delta_S = T_{s_2}/t_{s_1}$, and $T = t/t_{s_1}$. Based on the scaling given in (39a) and (39b), we will derive an estimate for $T_{s_2}$ as well as solutions for three particular cases, which are defined by the scale of $\delta_S$: (i) **Case 1**: the indicator reaction is faster than the non-observable reaction ($\delta_S \ll 1$), **Case 2**: the indicator reaction is roughly the same speed as the non-observable reaction ($\delta_S \approx 1$), and **Case 3**: the indicator reaction is much slower than the non-observable reaction ($\delta_S \gg 1$).
4.1. Case 1: The indicator reaction is faster than the non-observable reaction \((\delta_S \ll 1)\)

If the indicator reaction is fast, and \(\delta_S \ll 1\), then the dominant slow timescale is \(t_{s_1}\), and thus the completion of the non-observable reaction will occur long after the completion of the indicator reaction. To start the analysis, we will rescale the mass action equations that govern the non-observable reaction with respect to \(\hat{T} = t/T_{s_2}\):

\[
\frac{d\hat{s}_1}{d\hat{T}} = \delta_S(1 + \kappa_1)(1 + \sigma_1) \left[ -\hat{s}_1 + \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 + \frac{\kappa_1(1 + \kappa_1)^{-1}}{\sigma_1 + 1} \hat{c}_1 \right] \quad (40a)
\]

\[
\varepsilon \frac{d\hat{c}_1}{d\hat{T}} = \delta_S(1 + \kappa_1)(1 + \sigma_1) \left[ \hat{s}_1 - \frac{\sigma_1}{\sigma_1 + 1} \hat{c}_1 \hat{s}_1 - \frac{1}{\sigma_1 + 1} \hat{c}_1 \right]. \quad (40b)
\]

By inspection of \((40a)\), if \(\delta_S \ll 1\), then \(s_1\) will be a slow variable over the \(T_{s_2}\) timescale, and thus we will expect \(s_1\) to be essentially constant over the time course of the indicator reaction. In addition, let us assume that \(T_{s_2} \gg t_{c_1}\), in which case \(c_1\) will be on the order of its maximum value on the \(T_{s_2}\) timescale. Combining these observations leads to the approximation

\[
s_1 \simeq s_1^0, \quad t \leq T_{s_2} \quad (41a)
\]

\[
c_1 \simeq \varepsilon s_1^0, \quad t \leq T_{s_2} \quad (41b)
\]

for the non-observable reaction over the timescale \(T_{s_2}\). Equations \((41a)\) and \((41b)\) seem to suggest that \(e_2^A \ll 1\) over the \(T_{s_2}\) timescale. Furthermore, since the changes in \(s_1\) and \(c_1\) are comparatively minimal when \(t_{c_1} \leq t \leq T_{s_2}\), the production of \(e_2^A\) is effectively constant over the \(T_{s_2}\) timescale

\[
\dot{e}_2^A \approx \varepsilon k_2 s_1^0 \equiv \varpi. \quad (42)
\]

Integration of \((42)\) yields the following approximation of \(e_2^A\) on the \(T_{s_2}\) timescale

\[
e_2^A \approx \int_0^t \varpi \, du = \varpi t, \quad (43)
\]
where $u$ is a dummy variable. The approximate average value $\langle e_2^A \rangle$ on $T_{s_2}$ is easily obtainable through straightforward integration

$$
\langle e_2^A \rangle = \frac{\varpi}{T_{s_2}} \int_0^{T_{s_2}} t \, dt = \frac{1}{2} T_{s_2} \varpi,
$$

and inserting (44) into (32) yields the following estimate for $T_{s_2}$:

$$
T_{s_2} = \sqrt{\frac{2(K_M + s_2^0)}{k_4 \varpi}} \equiv T_{s_2}^*.
$$

We can write (45) in a slightly more convenient form. Defining the limiting slow timescale $t_{s_2}^*$ as

$$
t_{s_2}^* = \frac{K_M + s_2^0}{V_2},
$$

allows us to express $T_{s_2}^*$ as

$$
T_{s_2}^* = \sqrt{2t_{s_1}t_{s_2}^*},
$$

(47)

Note that $V_2 = k_4 s_1^0$ is defined as the limiting rate of the indicator reaction.

$T_{s_2}^*$ should provide an accurate estimate for total completion time of the indicator reaction as long as the non-observable reaction is comparatively slow.

For a generic (and linear) dynamical system of the form

$$
\dot{x} = -ax, \quad x(0) = x_0,
$$

(48)

the depletion or characteristic timescale is $1/a$, and thus we look for a timescale that is indicative of the time it takes for the initial quantity (i.e., $x_0$ in the context of (48)) to deplete to an amount that is less than or equal to $x_0/e$.

Following suit from the linear theory, we will consider the timescale $T_{s_2}^*$ to be a sufficient depletion timescale as long as

$$
s_2(T_{s_2}^*) \leq \frac{s_2^0}{e} \approx 0.37 s_2^0.
$$

(49)

Numerical solutions of the mass action equations confirm the validity of the timescale $T_{s_2}^*$ when the indicator reaction is much faster than the non-observable reaction provided $t_{c_1} \ll T_{s_2}^*$ (see Figures 2a and 2b).
Figure 2: The accuracy of the timescale $T_{s2}^*$ when the indicator reaction (2) is fast ($\delta_S \ll 1$). The solid black curves numerical solutions to the mass action equations of the complete reaction \[1\]. The dashed line marks the timescale $T_{s2}^*$ and the dotted line represents the quantity $1/e$. (a) The constants (without units) used in the numerical simulation are: $c_1^0 = 1$, $s_1^0 = 100, k_1 = 1, k_2 = 1$ and $k_{-1} = 1$. $s_2^0 = 10$, $k_3 = 10, k_4 = 100$ and $k_{-3} = 10$. (b) The constants (without units) used in the numerical simulation are: $c_1^0 = 1, s_1^0 = 100, k_1 = 1, k_2 = 1$ and $k_{-1} = 1$. $s_2^0 = 100$, $k_3 = 10, k_4 = 100$ and $k_{-3} = 10$. In both cases, we see that the timescale $T_{s2}^*$ yields an accurate approximation to the completion time of the indicator reaction. Time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 - 1/\ln(t + e)$.

Next, we develop an asymptotic solution to the mass action equations that will be valid when: (1) $T_{s2}^*$ is an accurate and precise depletion timescale, (2) the concentrations $s_1$ and $c_1$ remain on the order of their maximum values ($s_1^0$
and \( \varepsilon s_1^0 \) respectively) for the duration of the indicator reaction, and (3) the fast timescale \( t_{c_1} \) is negligibly short. To begin, let us assume that the initial concentration \( s_2^0 \) is large enough so that

\[
\max_{t \leq T_{s_2}^*} (e_2^A) \ll s_2^0,
\]

in which case we can assume \( \lambda \ll 1 \). Then, from Tikhonov’s theorem, and due to the existence of the slow manifold \( \mathcal{M}_\lambda \), we have

\[
c_2 \simeq \frac{e_2^A}{K_{M_2} + s_2} s_2
\]

as a leading order approximation. Insertion of this approximation into the mass action equation for \( s_2 \) yields

\[
\dot{s}_2 \simeq -\frac{k_4 e_2^A}{K_{M_2} + s_2} s_2.
\]

Substitution of \( e_2^A \approx \omega t \) into (52) gives us

\[
\dot{s}_2 \simeq -\frac{k_4 \omega t}{K_{M_2} + s_2} s_2
\]

as our final asymptotic approximation to the differential equations governing the temporal depletion of \( s_2 \). Equation (53) has a closed-form solution in the form of the Schnell–Mendoza equation

\[
s_2 = K_{M_2} W \left[ \sigma_2 \exp \left( \sigma_2 - \frac{k_4 \omega t^2}{2K_{M_2}} \right) \right],
\]

and provides an accurate approximation to the mass action model (see Figures 3a and 3b).

4.2. Case 2: The indicator reaction is roughly the same speed as the non-observable reaction (\( \delta \approx 1 \))

It is instinctive, in the case that the non-observable reaction and the indicator reaction both complete at roughly the same time, to use either slow timescale,
Figure 3: The leading order asymptotic solution of the substrate concentration for the indicator reaction matches the numerical solution when the indicator reaction is faster than the non-observable reaction \( \delta S \ll 1 \). The solid black curves numerical solutions to the mass action equations of the complete reaction \( \Phi \) and the broken red curves are numerical solutions to the asymptotic differential equation \( \Phi \). (a) The constants (without units) used in the numerical simulation are: \( e_1^0 = 1, s_1^0 = 100, k_1 = 1, k_2 = 1 \) and \( k_{-1} = 1 \). \( S_2^0 = 10, k_3 = 10, k_4 = 100 \) and \( k_{-3} = 10 \). (b) The constants (without units) used in the numerical simulation are: \( e_3^0 = 1, s_1^0 = 100, k_1 = 1, k_2 = 1 \) and \( k_{-1} = 1 \). \( S_2^0 = 100, k_3 = 10, k_4 = 100 \) and \( k_{-3} = 10 \). Time has been mapped to the \( t_\infty \) scale: \( t_\infty(t) = 1 - 1/\ln(t + e) \).

\( t_{s_1} \) or \( T_{s_2} \), as the depletion timescale for the complete reaction. Of course, given
our earlier definition of the timescale $T_{s_2}$

$$T_{s_2} = \frac{K_{M_2} + s_2^0}{k_4 \langle e_A^2 \rangle}, \quad (55)$$

we can formulate a nonlinear algebraic equation that will allow us to compute an estimate for the depletion timescale when the reactions are equivalent in speed. First,

$$\langle e_A^2 \rangle = \frac{1}{T_{s_2}} \int_0^{T_{s_2}} (s_1^0 - s_1 - c_1) \, dt, \quad (56)$$

and thus we see that $T_{s_2}$ should satisfy

$$\int_0^{T_{s_2}} (s_1^0 - s_1 - c_1) \, dt = \frac{K_{M_2} + s_2^0}{k_4}. \quad (57)$$

Second, under the RSA, the concentration $c_1$ is expressible (algebraically) in terms of $s_1$, and therefore

$$\int_0^{T_{s_2}} (s_1^0 - s_1 - c_1) \, dt \approx \int_0^{T_{s_2}} \frac{(K_{M_1} + s_1) \Delta s_1 - e_1^0 s_1}{K_{M_1} + s_1} \, dt, \quad (58)$$

where $\Delta s_1 = s_1^0 - s_1$ (the timescale $t_{c_1}$ has been assumed to be negligibly small and hence left out of the integrand, although it is straightforward to include this term). Third, the definite integral on the right hand side of (58) is straightforward to compute analytically; evaluating it will yield a nonlinear equation in terms of the variable $T_{s_2}$, and the solution to (57) can be approximated numerically. Using the average $\langle e_A^2 \rangle$ provides an accurate estimate of the slow (depletion) timescale (see Figure 4).

From a practical point of view, the utility in estimating $T_{s_2}$ numerically is rather minimal. The objective here will be to construct a criteria from which a reduced model can be be extracted from the mass action equations that will be valid without any a priori knowledge of the intrinsic timescales of the indicator reaction (or the non-observable reaction). To do this, let us first revisit the
Figure 4: The averaging method for the estimation of the depletion timescale $T_{s_2}$ for the indicator substrate is still valid when the non-observable and indicator reactions occur at roughly the same speed ($\delta_S \approx 1$). The solid black curve is the numerically-computed depletion curve of $s_2$ and the dotted/dashed black curve is the numerically-integrated depletion curve of $s_1$. In this numerical simulation $k_3 = 1, k_4 = 1, k_{-3} = 10, s_0^0 = 70$, and $k_1 = 10, k_2 = 15, k_{-1} = 1, e_1^0 = 1$ and $s_1^0 = 70$. Both substrates have been scaled as $s_2/s_0^2$ and $s_1/s_0^1$. Time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 - 1/\ln(t+\epsilon)$.

Bearing in mind the assumption $\delta_S \approx 1$, it is sufficient (but not necessary) to bound $\lambda$ in order to assemble a dynamical model that can be reduced (asymptotically) through slow manifold projection. The upper bound on $\lambda$, which we denote as $\lambda_{\text{max}}$, is

$$\lambda \leq \lambda_{\text{max}} \equiv \frac{s_1^0}{K_{M_2} + s_2^0}. \quad (60)$$

The parameter $\lambda_{\text{max}}$ is the natural small parameter when the indicator is very slow. Furthermore, if the non-observable reaction completes very quickly relative to the non-observable reaction, and $\delta_S \ll 1$, then the average available
enzyme should be on the order of $s_1^0$:

$$
\langle e_2^A \rangle = \frac{1}{T_{s_2}} \int_0^{T_{s_2}} e_2^A \, dt \approx s_1^0.
$$

(61)

Thus, if $s_2^0 \gg s_1^0$, then the approximation

$$
\dot{s}_2 \approx -\frac{k_4 e_2^A}{K_{M_2} + s_2} s_2^0
$$

(62)

will be valid regardless of the relative speeds of the reactions when $\lambda_{\text{max}} \ll 1$.

Furthermore, (62) admits a closed-form solution using separation of variables that consists of composite Lambert-$W$ functions (we do not present this expression here, although we remark that it is straightforward, albeit somewhat tedious to derive). Under the RSA, we obtain

$$
\dot{s}_2 \approx -\left(\frac{(K_{M_1} + s_1) \Delta s_1 - e_1^0 s_1}{K_{M_1} + s_1} \right) \left(\frac{k_4}{K_{M_2} + s_2}\right) s_2^0
$$

(63)

as the final form of our reduced differential equation for $\dot{s}_2$.

4.3. Case 3: The indicator reaction is much slower than the non-observable reaction ($\delta_S \gg 1$)

We now consider the case when $\delta_S \gg 1$, and the completion of the non-observable occurs much sooner than the completion of the indicator reaction. As mentioned in the previous subsection, a very slow indicator reaction suggests that $s_2$ will be slow over the timescale $t_{s_1}$. Consequently, we can approximate $s_2$ as

$$
s_2 = s_2^0, \quad t < t_{s_1}.
$$

(64)

Furthermore, because the non-observable reaction has effectively completed when $t = t_{s_1}$, we can approximate $\Delta s_1 = s_1^0$ when $t \geq t_{s_1}$, in which case

$$
\dot{s}_2 \approx -\frac{k_4 s_1^0}{K_{M_2} + s_2} s_2, \quad t \geq t_{s_1}.
$$

(65)

Equation (65) can be integrated directly to yield a Schnell–Mendoza equation for $s_2$:

$$
s_2 = K_{M_2} W [\sigma_2 \exp(\sigma_2 - \eta_2(t))], \quad t \geq t_{s_1}.
$$

(66)
The validity of the approximate solution (64) can be established by the mathematical formulation of the RSA for the indicator reaction. If \( s_2 \approx s_2^0 \) over the interval \([0, t_{s_1}]\), then
\[
\max_{t \leq t_{s_1}} |\dot{s}_2| \cdot t_{s_1} \ll s_2^0.
\] (67)

The inequality given in (67) translates to
\[
\delta_S \gg (\sigma_2 + 1)(\kappa_2 + 1),
\] (68)
with \( \max\dot{s}_2 = k_3 s_1^0 s_2^0 \). In the case of a slow indicator reaction, we expect that \( T_{s_2} = t_{s_2}^* \). Thus, we have a RSA that is pertinent to the indicator reaction
\[
\frac{V_1}{V_2} \gg \frac{K_{M_1}}{K_{M_2}}(1 + \sigma_1)(1 + \kappa_2),
\] (69)
and establishes a region of validity for the solution to the mass action equations during the initial build-up of \( c_2 \) when \( t \leq t_{s_1} \). Equation (69) is analogous to the term used to measure the strength of fully competitive enzyme reactions with alternative substrates [29, 30]. Numerical simulations (see Figure 5) confirm the validity of \( t_{s_2}^* \) and (65).

5. Interpretation of fast timescales of the indicator reaction

Up until this point, we have not mentioned the equivalent of a fast timescale that is pertinent to the indicator reaction. In the case of the non-observable reaction, the fast timescale corresponds to the time it takes the reaction to reach QSS. However, based on our scaling analysis, we have demonstrated that a QSSA can be imposed over the \( T_{s_2} \) timescale reaction as long as \( \lambda \ll 1 \). At first glance, it would seem that the kinetics of the indicator reaction omit the influence of a fast timescale. This is however false. To derive the fast timescale, we will assume that initial conditions are not experimental, and that the indicator reaction is equipped with a non-trivial amount of complex \( c_2^0 \) at the start of the reaction. Furthermore, we will assume that the substrate, \( s_2 \) does not deplete significantly over the duration of the fast timescale. Denoting
Figure 5: Validity of the timescale $t^*_{c_2}$ and the reduced ordinary differential equation given by (65) for the substrate depletion of the indicator reaction when the indicator reaction is much slower than the non-observable reaction ($\delta_S \gg 1$). The solid black curve in the numerical solution to the mass action equations (8) and the solid red curve corresponds to the numerical solution to (65) extended to $t \geq 0$. In this numerical simulation $k_3 = 0.1, k_4 = 1, k_{-3} = 10, s^0_2 = 10000$, and $k_1 = 25, k_2 = 100, k_{-1} = 1, e^1_1 = 1$ and $s^0_1 = 100$. The respective values of $\lambda_{\text{max}}$ and $\delta_S$ are $\approx 0.009$ and $\approx 0.01$. Time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 - 1/\ln(t + 1)$. The fast timescale as $t_{c_2}$, the mass action equation can be linearized:

$$\dot{c}_2 = k_3(e^4_2 - c_2)s^0_2 - (k_{-3} + k_4)s^0_2, \quad t \leq t_{c_2} \quad (70a)$$

$$c_2(0) = c^0_2. \quad (70b)$$

The solution to the linear equation (70) is given by Duhamel’s Principle

$$c_2(t) = \exp(-\mu t)c^0_2 + \int_0^t \exp(\mu(s - t))e^4_2(s) \, ds, \quad \mu \equiv k_3(K_{M_2} + s^0_2), \quad (71)$$

from which the naturally occurring characteristic timescale is $1/\mu$:

$$t_{c_2} = \frac{1}{k_3(K_{M_2} + s^0_2)}. \quad (72)$$

Since typical experimental initial conditions start on the $c_2$-nullcline, we turn to scaling to provide a biochemical interpretation of the timescale $t_{c_2}$. Defining
$T^* = t/t_{c_2}$, we obtain:

$$
\frac{d\bar{s}_2}{dT^*} = \lambda \left[ \left( \frac{\sigma_2}{1 + \sigma_2} \bar{c}_2 - \bar{e}_2 \right) \bar{s}_2 + \frac{\alpha}{1 + \sigma_2} \bar{e}_2 \right],
$$

(73a)

$$
\frac{d\bar{c}_2}{dT^*} = \left( \bar{e}_2^A - \frac{\sigma_2}{1 + \sigma_2} \bar{c}_2 \right) \bar{s}_2 - \frac{1}{1 + \sigma_2} \bar{e}_2.
$$

(73b)

We see from the scaling that $t_{c_2}$ defines a stagnation timescale when experimental initial conditions are prescribed. If the timescale $t_{c_2}$ is short, then we expect the indicator reaction to be effectively stationary over $t_{c_2}$. This is because $s_2$ scales as a slow variable over $t_{c_2}$ and the phase space trajectory should stay near the $c_2$-nullcline over short timescales, and thus $\dot{c}_2 \approx 0$ when $t \leq t_{c_2}$. Thus, if $t_{c_2}$ is small (i.e., $t_{c_2} \ll \min\{t_{s_2}, t_{s_1}\}$), then the fast timescale of the indicator reaction defines translates to a scale over which the indicator reaction exhibits a “slow response”. In fact, any timescale $t^*$ such that

$$
\frac{t}{\bar{e}_2} \ll \min\{t_{s_1}, T_{s_2}\}
$$

defines a “fast, stagnation timescale”.

The relationship between $\lambda, t_{c_2}$ and $T_{s_2}$ is now evident. The ratio of fast and slow timescales is bounded above by $\lambda$

$$
\frac{t_{c_2}}{T_{s_2}} < \lambda.
$$

(75)

The strict inequality follows from the fact that

$$
\frac{t_{c_2}}{T_{s_2}} = \frac{\bar{\lambda}}{(1 + \sigma_2)(1 + \kappa_2)},
$$

(76)

where $\bar{\lambda}$ is given by

$$
\bar{\lambda} = \frac{\langle e_2^A \rangle}{K_{M_2} + s_0^f}.
$$

(77)

Furthermore, since $\langle e_2^A \rangle \leq \max(e_2^A)$, we have that

$$
\bar{\lambda} \leq \lambda
$$

(78)

from which (75) follows.
To explore the relationship between the QSSA and the RSA, we note that the parameter $\lambda_{\text{max}}$ is easily derived using Segel’s heuristic approach [25]:

$$\max |\dot{s}_2| \cdot t_{c_2} \ll s_2^0 \rightarrow \lambda_{\text{max}} \ll 1.$$  

(79)

Since it is clear that

$$\bar{\lambda} \leq \lambda \leq \lambda_{\text{max}},$$  

(80)

it follows that the RSA (i.e., $\lambda_{\text{max}} \ll 1$) ensures separation of fast and slow timescales. Consequently, the RSA for the indicator reaction implies the QSSA and is thus a universal qualifier for the validity of the reduced model over the timescale $T_s^2$.

In addition, it is important to note that $c_2$ may or may not accumulate in QSS. We rescale the mass action equation for $c_2$ with respect to $T = t/t_s$ to determine how $c_2$ accumulates:

$$\lambda \frac{d \bar{c}_2}{dT} = \frac{\max(e_2^A)(1 + \kappa_2)(1 + \sigma_2)}{\langle e_2^A \rangle} \frac{1}{\delta_S} \left[ \left( \bar{e}_2^A - \frac{\sigma_2}{1 + \sigma_2} \right) \bar{s}_2 - \frac{1}{1 + \sigma_2} \bar{c}_2 \right].$$  

(81)

From the previous section we have that

$$\lambda \cdot \frac{\langle e_2^A \rangle}{\max(e_2^A)} \cdot \frac{1}{(1 + \kappa_2)(1 + \sigma_2)} \cdot \delta_S = \frac{t_{c_2}}{t_{s_1}},$$  

(82)

and consequently we see that $c_2$ accumulates in QSS when $t_{c_2}/t_{s_1} \ll 1$. We remark this is equivalent to demanding that $\max e_2^A \cdot t_{c_2} \ll s_1^0$, where $\max e_2^A = k_2 \varepsilon s_1^0$.

6. Estimation of lag times

Under the QSSA, enzyme catalyzed reactions usually express a lag time. The lag time is normally defined as the time it takes for the rate of product generation to reach its maximum (steady-state) value. This coincides with the time it takes for $c_2$ to reach its maximum value, and is straightforward to calculate under the limiting circumstances. In this section, we calculate the lag time under the assumption that the indicator reaction is extremely fast and extremely slow.
6.1. Estimation of the lag time for fast indicator reactions

Let us start by considering the case when the indicator reaction is very fast for which \( s_2 \) is given by

\[
s_2 = K_{M_2} W \left[ \sigma_2 e^{\sigma_2 - \nu t^2/2K_{M_2}} \right]. \tag{83}
\]

If \( \sigma_2 \ll 1 \), then (83) is approximately

\[
s_2 \approx s_0^0 e^{\sigma_2 - \nu t^2/2K_{M_2}}. \tag{84}
\]

Next, notice that under the QSSA we have

\[
c_2 \approx -\frac{1}{k_4} \frac{ds_2}{dt}, \quad t \geq 0. \tag{85}
\]

If we differentiate both sides of (85), then we see that \( \dot{c}_2 \) vanishes when \( \ddot{s}_2 \) vanishes:

\[
\frac{dc_2}{dt} \approx -frac{1}{k_4} \frac{d^2 s_2}{dt^2}. \tag{86}
\]

Inserting (84) into the right hand side of (86) and setting the left hand side to zero yields an expression for \( t \):

\[
t = \sqrt{\frac{K_{M_2}}{V_2}} \cdot t_{s_1} \equiv t_{c_2}. \tag{87}
\]

The timescale \( t_{c_2} \) is identically the lag time when the indicating reaction is fast and \( \sigma_2 \ll 1 \) and the indicator reaction is fast (see Figure 6).

6.2. Estimation of the lag time for slow indicator reactions

For slow indicator reactions will can employ the RSA

\[
\max_{t \leq t_{s_1}} |\dot{s}_2| \cdot t_{s_1} \ll s_0^0, \tag{88}
\]

and thus we obtain

\[
\dot{c}_2 = k_3(c_2^A - c_2)s_2^0 - (k_{-3} + k_4)c_2, \quad t \leq t_{s_1}. \tag{89}
\]
Figure 6: Validity of the timescale $t^*_c$. The numerical solution to the mass action equations \cite{11} (thick black line in both panels) with $s_1^0 = 100, e_1^0 = 1, k_1 = 1, k_2 = 1, k_{-1} = 1, s_2^0 = 1, k_3 = 1, k_{-3} = 1$ and $k_4 = 100$. The dashed line corresponds to $t^*_c$ and is the time it takes for $c_2$ to reach its maximum value. The total concentration $c_2$ has been scaled by $c_2/c_{2,\text{max}}$.

Furthermore, we will assume that $\max(c_2)$ is given by

$$\max c_2 \approx \frac{s_1^0 s_2^0}{K_{M_2} + s_2^0} \quad (90)$$

when the indicator reaction is slow. The timescale $t_{s_1}$ will serve as a good approximation to the lag time when $\sigma_1$ very large. However, when $\sigma_1$ is small, the asymptotic solution to the MM equation reduces to

$$s_1 = K_{M_1} W [\sigma_1 e^{\sigma_1 - \eta t}] \simeq s_1^0 e^{\sigma_1 - \eta t}, \quad (91)$$

and consequently the timescale $t_{s_1}$ is characteristic, which means roughly $1/3$ of $s_1^0$ still needs to be converted to product when $t = t_{s_1}$. Thus, we need an estimate for the time it takes for the non-observable reaction to complete when $\sigma_1$ is small. To do this we set

$$s_1^0 e^{\sigma_1 - \eta t} = \epsilon, \quad \epsilon \equiv t_{c_1}/t_{s_1} \quad (92)$$

and solve for $t$. This yields

$$t = -t_{s_1} \ln \epsilon \equiv t^*_1 \quad (93)$$
that is a much better estimate of the lag time when \( \sigma_1 \) is small. A similar analysis can be carried out when \( \sigma_1 \) is of order unity, but we will not dive into the detail of this calculation here. Numerical results confirm the lag time estimates \( t_s \) and \( t_s^* \) when the indicator reaction is slow (see Figures 7a–7b).

7. Discussion

The primary contribution of this paper is to introduce methods for the appropriate scaling and timescale estimates of coupled enzyme catalyzed reactions. As a case study, we present the analysis of a zymogen activation coupled to an enzyme catalyzed reaction. The identification of specific parameters through scaling has yielded necessary and sufficient conditions for the QSSA, whereas previous nonlinear studies of the coagulation cascade with zymogen activation coupled to an enzyme catalyzed reaction have employed the QSSA without justification [31]. Moreover, previous analyses [32] do not provide insight as to how to properly estimate kinetic timescales via nonlinear methods, even though the zymogen activation coupled to an enzyme catalyzed reaction are inherently nonlinear. This work outlines a clear procedure for estimating depletion timescales, and serves as a template for the analysis of more complicated reactions. We give a brief summary of the results of the analysis in what follows.

Scaling analysis of the mass action equations that model the kinetics of a zymogen activation coupled to an enzyme catalyzed reaction [1]–[2] has revealed two small parameters, \( \varepsilon \) and \( \lambda_{\text{max}} \),

\[
\lambda_{\text{max}} = \frac{s_1^0}{K_{M_2} + s_2^0} \ll 1 \\
\varepsilon = \frac{c_1^0}{K_{M_1} + s_1^0} \ll 1.
\]

The parameters \( \varepsilon \) and \( \lambda \) regulate the partition of the fast timescales \( t_{c_1}, t_{c_2} \) and
Figure 7: The validity of $t_{s1}$ and $t_{s1}^*$. The former is the approximate lag time when $\sigma_1$ is large (dashed line in panel (a)) and the latter is the lag time when $\sigma_1$ is small (dashed line of panel (b)). The solid black curves are the numerical solutions to the mass action equations of the complete reaction \((\text{8})\). (a) The constants (without units) used in the numerical simulation are: $e_1^0 = 1, s_1^0 = 100, k_1 = 100, k_2 = 10$ and $k_{-1} = 1$. $s_2^0 = 5000, k_3 = 1, k_4 = 1$ and $k_{-3} = 10$. (b) The constants (without units) used in the numerical simulation are: $e_1^0 = 1, s_1^0 = 1, k_1 = 1, k_2 = 100$ and $k_{-1} = 1$. $s_2^0 = 1000, k_3 = 1, k_4 = 1$ and $k_{-3} = 10$. Time has been mapped to the $t_\infty$ scale: $t_\infty(t) = 1 - 1/\ln(t + 1)$.

the slow timescales $t_{s1}$ and $T_{s2}$:

\[
\frac{t_{c1}}{t_{s1}} < \varepsilon, \quad \frac{t_{c2}}{T_{s2}} < \lambda \leq \lambda^{\text{max}}.
\]  

(95)

When these parameters are small, and the timescales $t_{c2}$ and $t_{s1}$ are adequately
separated, the indicator reaction can be assumed to be in a QSS for the duration of the reaction (i.e., for \( t \geq 0 \)). There is a twofold reasoning to this assumption. First, if \( \lambda^{\text{max}} \ll 1 \), then

\[
\lambda = \frac{\max(e_2^A)}{K_{M_2} + s_2^0} \ll 1,
\]

and model reduction from slow manifold projection is valid regardless of which reaction finishes first (non-observable or indicator). Since it is not generally possible to determine which reaction is faster in the typical experiment \textit{a priori}, the condition that \( \lambda^{\text{max}} \ll 1 \) serves as a sufficient qualifier to ensure the validity of the reduced model for the reaction rate of depletion of the indicator substrate:

\[
\dot{s}_2 = -\left(\frac{(K_{M_1} + s_1)\Delta s_1 - e_1^0 s_1}{K_{M_1} + s_1}\right)\left(\frac{k_4}{K_{M_2} + s_2}\right)s_2.
\]

Second, as long as \( t_{c_2} \ll t_{s_1} \), a QSSA will effectively hold for all time since experimental initial conditions lie on the \( c_2 \)-nullcline. From the theory of singular perturbations, the slow manifold, \( \mathcal{M}_\lambda \), is well-approximated by the \( c_2 \)-nullcline when \( \lambda^{\text{max}} \ll 1 \). Because experimental initial conditions lie on the \( c_2 \)-nullcline, the phase space trajectory is already extremely close to the slow manifold, and therefore there is no need for an initial fast transient in order for the trajectory to reach the slow manifold. As we have pointed out, the slow manifold is a geometrical representation of the steady-state rate equation for the reaction. Note that this is very different from the non-observable reaction, since a fast transient (the duration of the fast transient is approximated by the timescale \( t_{c_1} \)) must elapse before the QSSA is justifiable.

In addition, simple asymptotic solutions to the mass action equations were derived that are valid when the indicator reaction is very fast or very slow in comparison to the non-observable reaction. If the indicator reaction is fast, then the time course of the indicator substrate \( s_2 \) is accurately model by

\[
s_2 = K_{M_2}W\left[\sigma_2 \exp\left(\sigma_2 - \frac{k_4 \omega t^2}{2K_{M_2}}\right)\right],
\]

where \( W \) denotes the Lambert-W function. In contrast, if the indicator reaction
is very slow, then the time course of \( s_2 \) can be modeled by

\[
s_2 = K_{M_2} W \left( \sigma_2 \exp \left( \frac{\sigma_2 - V_2 t}{K_{M_2}} \right) \right).
\]

Note that the above two expressions are analogous to the Schnell–Mendoza equation [21].

It should be pointed out that the condition \( \lambda_{\text{max}} \ll 1 \), which can be ensured by requiring an excess of the initial amount of substrate \( s_2 \) (i.e., requiring that \( s_0^1 \) be large enough so that \( s_0^1 \ll s_0^2 \)), is sufficient but not necessary for the validity of the reduced model presented in (63). In general, it is desirable that \( s_0^2 \) be much larger than the maximum amount of available enzyme, \( \text{max}(e_2^A) \), over the timescale of the indicator reaction. If the indicator reaction is fast, then the maximum amount of available enzyme, \( \text{max}(e_2^A) \), will be small, and thus the requirement that \( s_0^1 \ll s_0^2 \) is unnecessary as if \( \text{max}(e_2^A) \ll K_{M_2} \) (see Figure 7). Of course, the integrity of the reduced model does not diminish if \( s_0^1 \ll s_0^2 \).

\[
\text{Time has been mapped to the } t_{\infty} \text{ scale: } t_{\infty}(t) = 1 - 1/\ln(t + e).
\]

Finally, three reduced models have been derived that can be utilized in
the analysis of the inverse problem. Our analysis seems to suggest that a fast indicator reaction is the most beneficial case for parameter estimation. Under this circumstance, two expressions

\[ s_2 = K_{M_2} W \left[ \sigma_2 \exp \left( \sigma_2 - \frac{V_2 V_1 t^2}{2 K_{M_2} (s_1^0 + K_{M_1})} \right) \right], \]

and

\[ \dot{s}_2 = -\left( \frac{(K_{M_1} + s_1) \Delta s_1 - c_1^0 s_1}{K_{M_1} + s_1} \right) \left( \frac{k_4}{K_{M_2} + s_2 s_2} \right) \]

can be simultaneously utilized to estimate the four unknown parameters \( V_1, V_2, K_{M_1}, \) and \( K_{M_2}. \) Additionally, previous studies [6, 1, 7, 4, 2] that have analyzed this reaction mechanism in terms of PFO kinetics can be bridged together with the work here. Since we have treated the first reaction as independent of the second, an additional model could be obtained by imposing PFO kinetics on the non-observable reaction. However, while one can construct many models as a result of our nonlinear analysis, the full understanding of the inverse problem is beyond the scope of this paper. We hope to theoretically investigate the scope of parameter estimation in coupled assays in subsequent future work.

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References

[1] B. Havsteen, M. Garcia-Moreno, E. Valero, M. Manjabacas, R. Varn, The kinetics of enzyme systems involving activation of zymogens, Bull. Math. Biol. 55 (1993) 561–583.

[2] R. Varn, B. Havsteen, M. Garca, A. Vsquez, J. Tudela, F. Cnovas, Kinetics of the trypsinogen activation by enterokinase and/ or trypsin: Coupling of a reaction in which the trypsin acts on one of its substrates, J. Mol. Catal. 66 (1991) 409–419.

[3] D. L. Purich, Enzyme kinetics: Catalysis & control, Academic Press, London, UK, 2010.

[4] R. Varón, A. Román, F. García, F. G. Carmona, Transient phase kinetics of activation of human plasminogen, Bull. Math. Biol. 48 (1986) 149–166.

[5] L. Michaelis, M. L. Menten, Die Kinetik der Invertinwirkung, Biochem. Z. 49 (1913) 333–369.

[6] M. E. Fuentes, E. Valero, M. García-Moreno, E. Vique, R. Varón, Kinetic analysis of the mechanism of plasminogen activation by streptokinase, J. Math. Chem. 42 (2007) 753–774.

[7] R. Varn, B. Havsteen, Kinetics of the transient-phase and steady-state of the monocyclic enzyme cascades, J. theor. Biol. 144 (1990) 397–413.

[8] F. B. Rudolph, B. W. Baugher, R. S. Beissner, Techniques in coupled enzyme assays, Methods Enzymol. 63 (1979) 22–42.

[9] B. A. C. Storer, A. Cornish-bowden, P. O. Box, B. Birmingham, The kinetics of coupled enzyme reactions, Biochem. J. 141 (1974) 205–209.

[10] W. W. Cleland, Optimizing coupled enzyme assays, Anal. Biochem. 99 (1979) 142–145.
[11] W. R. McClure, Kinetic analysis of coupled enzyme assays, Biochemistry 8 (1969) 2782–2786.

[12] J. S. Easterby, Coupled enzyme assays: A general expression for the transient, Biochim Biophys Acta. 293 (1973) 552–558.

[13] O. D. Dang, A. Vindigni, E. Di Cera, An allosteric switch controls the procoagulant and anticoagulant activities of thrombin, Proc. Natl. Acad. Sci. USA 92 (1995) 5977–5981.

[14] S. Schnell, C. Mendoza, The condition for pseudo-first-order kinetics in enzymatic reactions is independent of the initial enzyme concentration, Biophys. Chem. 107 (2004) 165–174.

[15] W. Stroberg, S. Schnell, On the estimation errors of $K_M$ and $V$ from time-course experiments using the Michaelis-Menten equation, Biophys. Chem. 219 (2016) 17–27.

[16] W. Stroberg, S. Schnell, On the validity and errors of the pseudo-first-order kinetics in ligandreceptor binding, Math. Biosci. 287 (2017) 3–11.

[17] W. Klonowski, Simplifying principles for chemical and enzyme kinetics, Biophys. Chem. 18 (1983) 73–87.

[18] J. F. Griffiths, Reduced kinetic models and their application to practical combustion systems, Prog. Energy Combust. Sci. 21 (1995) 25–107.

[19] M. S. Okino, M. L. Mavrovouniotis, Simplification of mathematical models in chemical reaction systems, Chem. Rev. 98 (1998) 391–408.

[20] R. Bertram, J. E. Rubin, Multi-timescale systems and fast-slow analysis, Math. Biosci. 287 (2017) 105–121.

[21] S. Schnell, C. Mendoza, Closed form solution for time-dependent enzyme kinetics, J. Theor. Biol. 187 (1997) 207–212.
[22] A. H. Nguyen, S. J. Fraser, Geometrical picture of reaction in enzyme kinetics, J. Chem. Phys. 91 (1989) 186–193.

[23] M. R. Roussel, S. J. Fraser, Geometry of the steady-state approximation: Perturbation and accelerated convergence methods, J. Chem. Phys. 93 (1990) 1072–1081.

[24] S. M. Hanson, S. Schnell, Reactant stationary approximation in enzyme kinetics, J. Phys. Chem. A 112 (2008) 8654–8658.

[25] L. A. Segel, On the validity of the steady state assumption of enzyme kinetics, Bull. Math. Biol. 50 (1988) 579–593.

[26] S. K. Shoffner, S. Schnell, Approaches for the estimation of timescales in nonlinear dynamical systems: Timescale separation in enzyme kinetics as a case study, Math. Biosci. 287 (2017) 122–129.

[27] L. A. Segel, M. Slemrod, The quasi-steady-state assumption: a case study in perturbation, SIAM Rev. 31 (1989) 446–477.

[28] S. Schnell, Validity of the Michaelis-Menten equation – Steady-state, or reactant stationary assumption: that is the question, FEBS J. 281 (2014) 464–472.

[29] S. Schnell, C. Mendoza, Time-dependent closed form solutions for fully competitive enzyme reactions, Bull. Math. Biol. 62 (2000) 321–336.

[30] S. Schnell, C. Mendoza, Enzyme kinetics of multiple alternative substrates, J. Math. Chem. 27 (2000) 155–170.

[31] M. Khanin, V. Semenov, A mathematical model of the kinetics of blood coagulation, J. theor. Biol. 136 (1989) 127–134.

[32] F. Martorana, A. Moro, On the kinetics of enzyme amplifier systems with negative feedback, Math. Biosci. 21 (1974) 77–84.