

Quasi-steady-state kinetics at enzyme and substrate concentrations in excess of the Michaelis–Menten constant

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Abstract

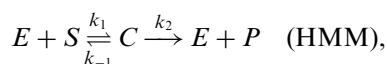
In vitro enzyme reactions are traditionally conducted under conditions of pronounced substrate excess since this guarantees that the bound enzyme is at quasi-steady-state (QSS) with respect to the free substrate, thereby justifying the Briggs–Haldane approximation (BHA). In contrast, intracellular reactions, amplification assays, allergen digestion assays and industrial applications span a range of enzyme-to-substrate ratios for which the BHA is invalid, including the extreme of enzyme excess. The quasi-equilibrium approximation (QEA) is valid for a subset of enzyme excess states. Previously, we showed that the total QSSA (tQSSA) overlaps and extends the validity of the BHA and the QEA, and that it is at least roughly valid for any total substrate and enzyme concentrations. The analysis of the tQSSA is hampered by square root nonlinearity. Previous simplifications of the tQSSA rate law are valid in a parameter domain that overlaps the validity domains of the BHA and the QEA and only slightly extends them. We now integrate the tQSSA rate equation in closed form, without resorting to further approximations. Moreover, we introduce a complimentary simplification of the tQSSA rate law that is valid in states of enzyme excess when the absolute difference between total enzyme and substrate concentrations greatly exceeds the Michaelis–Menten constant. This includes a wide range of enzyme and substrate concentrations where both the BHA and the QEA are invalid and allows us to define precisely the conditions for zero-order and first-order product formation. Remarkably, analytical approximations provided by the tQSSA closely match the expected stochastic kinetics for as few as 15 reactant molecules, suggesting that the conditions for the validity of the tQSSA and for its various simplifications are also of relevance at low molecule numbers.

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1. Introduction

The Henri–Michaelis–Menten (HMM) scheme (Henri, 1902; Michaelis and Menten, 1913) is extensively used in biochemistry to describe enzymatic processes in solution. This scheme reads



where E and C denote the free and bound enzyme, respectively, S denotes the free substrate and P denotes the product, k_1 is the second-order bimolecular rate constant of enzyme–substrate association, k_{-1} the rate constant of dissociation of the enzyme substrate complex

and k_2 is the catalysis rate constant. Typically, *in vitro* enzymatic reactions are studied subject to the following uniform initial conditions

$$(E, S, C, P) = (E_T, S_T, 0, 0), \quad (1)$$

where E_T and S_T denote total enzyme and substrate concentrations. Namely, at the beginning of the experiment the enzyme is entirely free, the substrate is at its maximal concentration and there is no product.

Early researchers sought to understand the HMM scheme and similar classical kinetic schemes using qualitative techniques to derive rate laws. Henri (1902) and later Michaelis and Menten (1913) argued that the enzyme–substrate complex is in thermodynamic equilibrium during the reaction such that $C \approx ES/K_S$, where K_S is its dissociation constant k_{-1}/k_1 . This approximation is equivalent to the

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assumption that $dS/dt \approx 0$ and is variously referred to as the fast equilibrium approximation, the quasi-equilibrium approximation (QEA) or the reverse quasi-steady-state approximation (rQSSA). Briggs and Haldane (1925) argued semi-quantitatively that classical *in vitro* conditions of substrate excess ($S_T \gg E_T$) justify the quasi-steady-state approximation (QSSA) $dC/dt \approx 0$ throughout the course of the reaction. This assumption implies that $C \approx E \cdot S/K_M$, where

$$K_M = (k_{-1} + k_2)/k_1 \quad (2)$$

is the Michaelis–Menten constant. The Briggs–Haldane approximation (BHA) therefore extended the treatment of Henri (1902) and Michaelis and Menten (1913) to settings where the catalysis rate constant is comparable to or larger than the dissociation rate constant. Noting that the HMM scheme conserves enzyme

$$E_T = E + C, \quad (3)$$

Briggs and Haldane obtained $C \approx E_T S/(S + K_M)$ and the Michaelis–Menten rate law (Table 1). Segel (1988) used scaling arguments to estimate two error measures proposed by Schauer and Heinrich (1979) and showed that the validity of the BHA is guaranteed by the more general criterion

$$E_T \ll K_M + S_T. \quad (4)$$

As emphasized by Segel (1988), this criterion does not constrain the enzyme–substrate ratio for substrate concentrations that are small compared to the Michaelis–Menten constant, as is the case for many intracellular glycolytic reactions (Fersht, 1985, Table 12.5). One can find unusual intracellular reactions that satisfy the BHA (inequality 4) even though the total enzyme concentration exceeds the total substrate concentration. For example, the concentration of the enzyme trisphosphate isomerase is 2.5-fold higher than the concentration of its substrate dihydroxyacetone-phosphate in rabbit muscle (Albe et al., 1990), and the Michaelis–Menten constant of this reaction is 17-fold higher than the substrate concentration (Fersht, 1985, Table 12.5), providing for $E_T/(K_M + S_T) = 0.14$. However, the concentration of many glycolytic enzymes is in excess not only of the concentration of their substrates (Srivastava and Bernhard, 1986; Albe et al., 1990) but also of the Michaelis–Menten constant, thereby rendering the BHA (inequality 4) invalid. Moreover, the BHA is invalid for a

range of *in vitro* conditions typical of enzymatic amplification techniques (Lowry et al., 1961; Cha and Cha, 1965; Cha, 1970), industrial catalysts (Carbonell and Kostin, 1972) and a range of protein degradation assays (Lin et al., 1999; Tzafiriri et al., 2002; Karmac et al., 2002; Herman et al., 2005). These examples and others (Sicher, 1984; Hertel et al., 1994) have motivated the ongoing search for alternative approximations of the HMM scheme that are valid when the BHA breaks down.

Guided by the observation that the breakdown of the BHA is associated with significant substrate depletion during the initial fast transient, Goldstein (1944) and others (Reiner, 1959; Cha and Cha, 1965) proposed the total QSSA (tQSSA), wherein the total *intact* substrate concentration

$$\bar{S} \equiv S + C = S_T - P \quad (5)$$

replaces the free substrate as the slow variable, while retaining the quasi-steady-state assumption $dC/dt \approx 0$. Borghans et al. (1996) used the methods of Segel (1988) to delineate the validity of the tQSSA rate equation (Table 1) and derived the first order tQSSA rate law (Table 1) using a two point Pade approximant for the tQSSA complex concentration. They found that the validity domain of the first order tQSSA overlaps the validity domain of the BHA and includes the high enzyme concentration limit $E_T \gg K_M$ (Borghans et al., 1996). Tzafiriri (2003) later showed that the tQSSA rate equation (Table 1) is always a fair approximation, and that the two point Pade approximant employed by Borghans et al. is equivalent to a first order binomial expansion in the parameter $r \equiv 4E_T \bar{S}/(K_M + E_T + \bar{S})^2$. The validity domain of this first order tQSSA rate equation is spanned by the union of inequality (4) and the inequalities (Tzafiriri, 2003)

$$E_T + K_M \gg S_T \quad \text{and} \quad k_{-1} \gg k_2, \quad (6)$$

$$E_T \gg S_T \quad \text{and} \quad E_T \gg K_M \quad \text{and} \quad k_{-1} \ll k_2. \quad (7)$$

An attractive use of analytical approximations is the simplification of parameter estimation and model validation (Schnell and Maini, 2000, 2003; Tzafiriri et al., 2002; Tzafiriri and Edelman, 2004). However, while the validity domain of the first order tQSSA is significantly larger than that of the BHA, the union of inequalities (4), (6) and (7) represent only a restricted range of potential initial

Table 1
Rate laws and their validity

| Name | Validity | Rate equation |
|-------------------|------------------------------|--|
| Michaelis–Menten | Inequality (4) | $dS/dt \approx -k_2 E_T S/(S + K_M)$ |
| First-order tQSSA | Inequalities (4), (7) or (8) | $d\bar{S}/dt \approx -k_2 E_T \bar{S}/(\bar{S} + E_T + K_M)$ |
| tQSSA | Inequality (12) | $d\bar{S}/dt \approx -k_2 C_-(\bar{S})$ with $C_-(\bar{S}) \approx \frac{1}{2} \left((\bar{S} + K_M + E_T) - \sqrt{(\bar{S} + K_M + E_T)^2 - 4E_T \bar{S}} \right)$ |

conditions (Fig. 2, Tzafiriri, 2003). We now integrate the tQSSA rate equation in closed form, without resorting to further approximations. Moreover, we introduce an alternative simplification of the tQSSA rate law that is valid in states of enzyme excess when the absolute difference between total enzyme and substrate concentrations greatly exceeds the Michaelis–Menten constant. This includes a wide range of enzyme and substrate concentrations where the first-order tQSSA is invalid (e.g., $r \approx 1$), thereby considerably enhancing the characterization of HMM kinetics. The new simplification of the tQSSA provides simple yet tight criteria for apparent zero order or first-order product formation. These approximations and their domains of validity are supported by numerical simulations. It is noteworthy that under the appropriate conditions these simple analytical approximations also closely match the expected stochastic kinetics (Rao and Arkin, 2003; Goutsias, 2005).

2. Theoretical background

Applying the law of mass action to the HMM scheme and using the definition of the total intact substrate Eq. (5) to eliminate S yields the following set of differential equations (Tzafiriri, 2003)

$$d\bar{S}/dt = -k_2 C, \quad (8)$$

$$dC/dt = k_1 (C - C_+(\bar{S}))(C - C_-(\bar{S})), \quad (9)$$

where

$$C_{\pm}(\bar{S}) = \frac{(K_M + E_T + \bar{S}) \pm \sqrt{(K_M + E_T + \bar{S})^2 - 4E_T\bar{S}}}{2} \quad (10)$$

are the roots of the quadratic equation

$$C^2 - (K_M + E_T + \bar{S})C + E_T\bar{S} = 0.$$

Similarly, initial condition (1) can be rewritten as

$$(\bar{S}, C) = (S_T, 0), \quad t = 0. \quad (11)$$

Using the methodology developed by Segel (1988) we showed (Tzafiriri, 2003) that the condition

$$\varepsilon \equiv \frac{k_2/k_1}{S_T} \left(\frac{C_-(S_T)}{C_+(S_T) - C_-(S_T)} \right) = \frac{t_C}{t_{\bar{S}}} \ll 1 \quad (12)$$

guarantees a separation of time scales, where

$$t_C \equiv \frac{1}{k_1(C_+(S_T) - C_-(S_T))} = \frac{1}{k_1 \sqrt{(K_M + E_T + S_T)^2 - 4E_T S_T}} \quad (13)$$

is the time scale for an initial binding transient with negligible product formation and

$$t_{\bar{S}} = \frac{S_T}{k_2 C_-(S_T)} \quad (14)$$

is the time scale for observable product formation. Moreover, inequality (12) guarantees that the exact rate equation Eq. (8) is well approximated by the tQSSA rate equation

$$d\bar{S}/dt \approx -k_2 C_-(\bar{S}) \quad (15)$$

throughout the reaction and that the concentration of the enzyme–substrate complex can be approximated as

$$C \approx C_-(\bar{S}) \left(\frac{1 - e^{-t/t_C}}{1 - (C_-(S_T)/C_+(S_T))e^{-t/t_C}} \right), \quad t \in [0, \infty). \quad (16)$$

Analysis of the a priori tQSSA error Eq. (12) as a function of initial concentrations revealed that it has a global maximum such that (Tzafiriri, 2003)

$$\varepsilon(S_T, E_T) \leq \frac{k_2}{4(k_2 + k_{-1})} < \frac{1}{4}. \quad (17)$$

This entails that the tQSSA is always a fair approximation, and that the smaller the ratio k_2/k_{-1} , the better the approximation. This result is noteworthy as $k_2/(k_{-1} + k_2) < 0.4$ for many metabolic enzymes (Table 2), thereby guaranteeing that $\varepsilon < 0.1$.

3. Results

3.1. Analytical solution

In Appendix A, we show that the change of variable

$$\begin{aligned} y &= \bar{S} + \sqrt{(K_M + E_T + \bar{S})^2 - 4E_T\bar{S}} \\ &= S_T - P + \sqrt{(K_M + E_T + S_T - P)^2 - 4E_T(S_T - P)} \end{aligned} \quad (18)$$

allows a direct integration of the tQSSA rate equation Eq. (15) as

$$\begin{aligned} t &= \frac{y_0 - y}{2k_2 E_T} - \left(\frac{K_M + E_T}{k_2 E_T} \right) \ln \frac{y - (K_M + E_T)}{y_0 - (K_M + E_T)} \\ &\quad + k_2^{-1} \ln \frac{y - (E_T - K_M)}{y_0 - (E_T - K_M)}, \end{aligned} \quad (19)$$

where

$$y_0 \equiv y(0) = S_T + \sqrt{(K_M + E_T + S_T)^2 - 4E_T S_T}. \quad (20)$$

Eq. (19) is implicit in the product concentration, as it cannot be rearranged to express P as an explicit function of the other variables in this equation. To verify Eq. (19) we solved the tQSSA rate equation Eq. (15) numerically for given values of S_T , E_T , K_M and k_2 and inserted the calculated product concentrations on the right-hand side of Eq. (19) to obtain an estimate of t . Similarly, the fact that Eq. (19) provides a one-to-one correspondence between product concentrations and the time elapsed after initiation of the catalytic reaction makes it possible to simulate the approximate progress curves. Given the values of S_T , E_T ,

Table 2
Binding off rate and turnover constants for various enzyme–substrate pairs

| Enzyme | Substrate | k_{-1} (s ⁻¹) | k_2 (s ⁻¹) | $k_2/(k_{-1}+k_2)$ |
|--------------------------------|---|--------------------------------|--------------------------|--------------------|
| Alcohol dehydrogenase | NADH | 44 ^a | 85 ^b | 0.66 |
| Chymotrypsin | Acetyl-L-phenylalanine ethyl ester | 90 ^c | 10 ^c | 0.1 |
| | Acetyl-L-tryptophan p-nitrophenyl ester | 6.0×10^4 ^d | 4.4–61.7 ^d | 0.00 |
| | Furylacryloyl-L-tryptophanamide | 2.7×10^4 ^e | 100–1000 ^f | <0.36 |
| | Indole | 5800 ^g | 100–1000 ^f | <0.15 |
| Fumerase | Proflavine | 2150–8300 ^{e,g} | 100–1000 ^f | <0.32 |
| | Fumerate | 4.5×10^4 ^h | 800 ⁱ | 0.02 |
| | L-Malate | 4.0×10^4 ^h | 900 ⁱ | 0.02 |
| Lactate dehydrogenase (rabbit) | NADH | 1.0×10^4 ^e | ≤ 1000 ^j | ≤ 0.09 |
| Lactate dehydrogenase (pig) | NADH | 39 ^e | ≤ 1000 ^j | ≤ 0.96 |
| | Oxmate | 17 ^g | ≤ 1000 ^j | ≤ 0.98 |
| Lysozyme | (NAG) ₂ | 950 ^a | ≤ 0.5 ⁱ | 0.00 |
| Peroxidase | Hydrogen peroxide | 0–0.2 ^k | 4.5 ^k | 0.96–1.0 |
| Ribonuclease | Cytidine 2',3'- cyclic phosphate | 1.5×10^4 ^e | 790 ^l | 0.05 |
| Seryl-tRNA synthetase | tRNA Ser | 550 ^a | 0.83 ^b | 0.00 |
| Tyrosyl-tRNA synthetase | Tyrosine | 24 ^e | 7.6 ^l | 0.24 |
| Trypsin | Benzoyl-L-arginine ethyl ester | 25 ^c | 15 ^c | 0.38 |

^aTable 5.2 Hiromi (1979).

^bTable 1.3 Hiromi (1979).

^cWong (1965).

^dRenard and Fersht (1973).

^eTable 6.1 Hammes (1982).

^fTable 6.2 Hammes (1982).

^gTable 4.3 Fersht (1985).

^hTable 3.5 Bailey and Ollis (1986).

ⁱTable 4.4 Fersht (1985).

^jTable 8–3 Stryer (1995).

^kChance (1943).

^lTable 11.1 Matthews et al. (2000).

K_M and k_2 one then uniformly samples the interval of potential product concentrations $P \in [0, \min(E_T, S_T)]$, and for each concentration solves for time using Eq. (19). In this way, one can generate a time series of product concentrations that can then be plotted in the usual manner against the corresponding time points.

Although useful, Eq. (19) is not illuminating. While it is possible to simplify this analytical solution to a more manageable form (Appendix A) at conditions that validate the first order tQSSA (inequalities 4, 6 and 7), other cases of interest do not seem to be amenable to simplification. To further our understanding of HMM kinetics we shall therefore take the complimentary approach of directly simplifying the tQSSA rate equation at conditions that transcend the first order tQSSA.

3.2. High enzyme and substrate concentrations

The first order tQSSA is invalid when $E_T \approx \bar{S}$ and $|E_T - \bar{S}| \gg K_M$ since these conditions imply that $r \approx 4E_T^2/(K_M + 2E_T)^2 \approx 1$. However, it can be shown that the condition $|E_T - \bar{S}| \gg K_M$ warrants that Eq. (10) can be approximated as (Appendix B)

$$C_-(\bar{S}) \approx \min(E_T, \bar{S}), \quad (21a)$$

$$C_+(\bar{S}) \approx \max(E_T, \bar{S}). \quad (21b)$$

As the Michaelis–Menten constant is at its core an apparent equilibrium dissociation constant of the enzyme–substrate complex, result (21a) states that binding depletes the minority species when the absolute difference between the enzyme and substrate concentrations greatly exceeds the apparent equilibrium dissociation constant, *even* when the ratio of minority concentration to majority concentration is close to unity. Conditions of enzyme excess are discussed in the next subsection. Substrate excess is discussed in Appendix C.

3.2.1. Enzyme excess

In states of excess enzyme ($E_T > S_T$) Eqs. (21a,b) reduce to

$$C_-(\bar{S}) \approx \bar{S}, \quad (22a)$$

$$C_+(\bar{S}) \approx E_T. \quad (22b)$$

Correspondingly, the tQSSA rate equation Eq. (15) reduces to

$$d\bar{S}/dt \approx -k_2\bar{S} \Leftrightarrow S = S_T e^{-k_2 t} \quad (23)$$

and the concentration of the enzyme–substrate complex Eq. (16) to

$$C \approx S_T e^{-k_2 t} \left(\frac{1 - e^{-k_1(E_T - S_T)t}}{1 - (S_T/E_T)e^{-k_1(E_T - S_T)t}} \right), \quad t \in [0, \infty). \tag{24}$$

Since experimental assays typically measure the concentration of product we shall use the expression $P = S_T - \bar{S}$ to rewrite Eq. (23) as

$$P \approx S_T(1 - e^{-k_2 t}). \tag{25}$$

The tQSSA error Eq. (12) for this case is

$$\varepsilon \approx \frac{k_2}{k_1(E_T - S_T)} < \frac{K_M}{E_T - S_T} \tag{26}$$

and implies that

$$E_T - S_T \gg K_M \tag{27}$$

is a *sufficient* condition for the validity of the tQSSA in the form of Eqs. (23)–(25). When $S_T \ll E_T$, the time scale of the initial binding transient becomes $t_C^{-1} \approx k_1 E_T$ and the denominator of Eq. (24) approaches unity, so that we recover the high enzyme limit of the first-order tQSSA, $C \approx S_T e^{-k_2 t} (1 - e^{-k_1 E_T t})$ (Tzafiriri, 2003).

3.2.2. Numerical verification

To highlight conditions that ensure maximal substrate binding Eq. (22a) we numerically evaluated the fraction of bound substrate $C_-(S_T, E_T)/S_T$ provided by Eq. (10) over a range of initial enzyme and substrate concentrations (Fig. 1). The fraction of total substrate that is bound

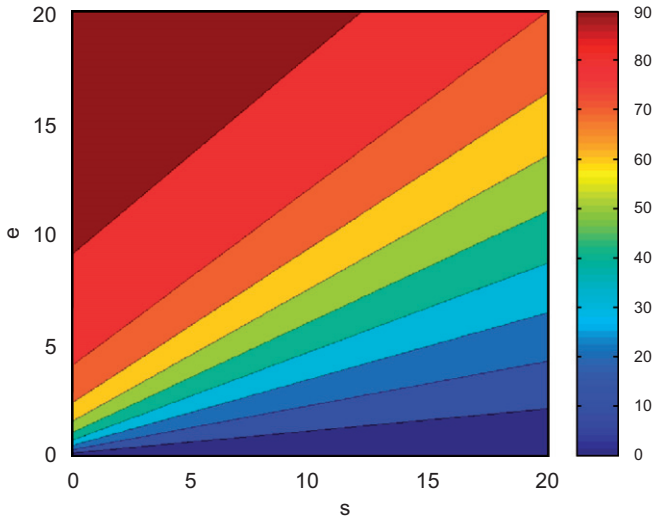


Fig. 1. The steady-state percentage of bound substrate is plotted as a function of specific substrate and enzyme concentrations, $s \equiv S_T/K_M$ and $e \equiv E_T/K_M$. This plot is based on a numerical evaluation of Eq. (10) and illustrates that $e - s \gg 9$ (e.g., $E_T - S_T > 9K_M$) is the necessary and sufficient condition for $C_-(s,e)/s \geq 0.90$.

approaches unity ($C_-(S_T, E_T) \geq 0.90 S_T$) provided that $E_T - S_T > 9K_M$, validating that inequality (27) is a *sufficient* and *necessary* condition for maximal substrate binding Eq. (22a). To span a range of conditions that invalidate the first order tQSSA we considered a range of enzyme to substrate ratios at fixed kinetic parameters and total enzyme concentration ($E_T = 10K_M$, Fig. 2). We found a good fit between the first-order approximation of product formation Eq. (25) and the numerical solution of the exact

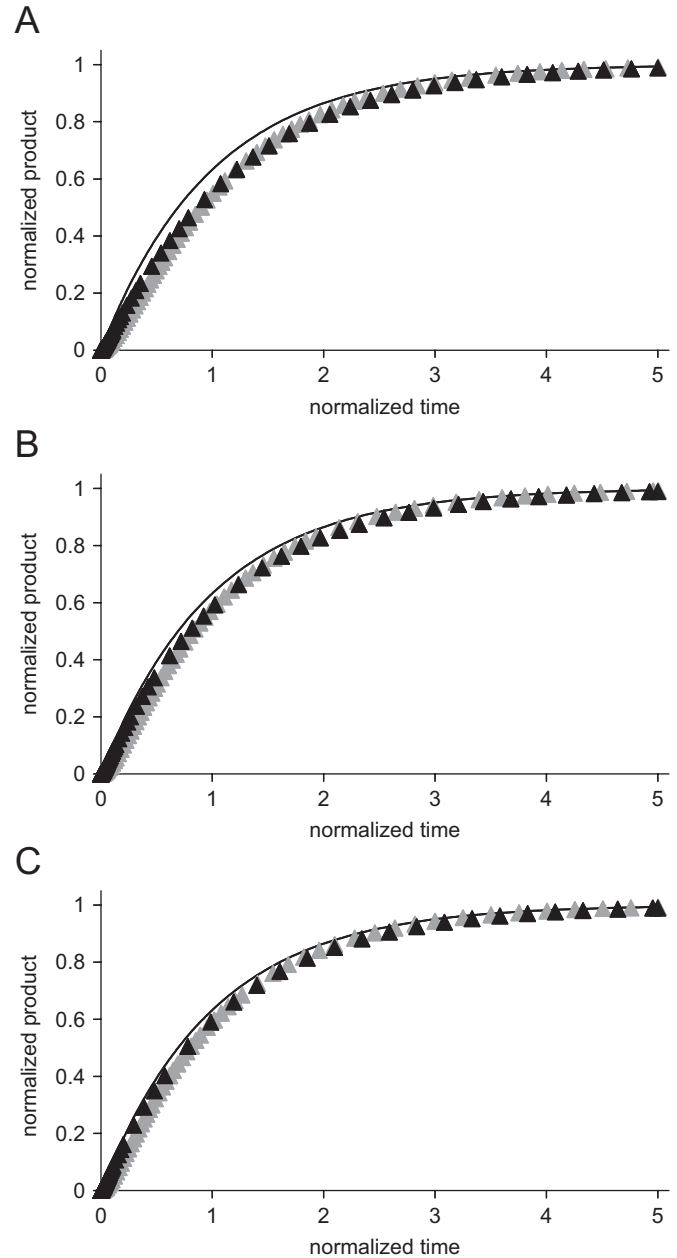


Fig. 2. Normalized product concentration P/S_T as a function of normalized time $k_2 t$ for $E_T = 10K_M$ and $S_T = 10K_M$ (A) $S_T = 5K_M$ (B) and $S_T = K_M$ (C). First-order product formation (Eq. (25), line) is contrasted with the numerical solution (triangles) for $k_2/(k_{-1} + k_2) = 0.1$ (black) and $k_2/(k_{-1} + k_2) = 0.9$ (gray). Inequality (4) is invalid for all 6 examples. Inequality (6) is only valid for $k_2/(k_{-1} + k_2) = 0.1$ in panel C. Inequality (7) is only valid for $k_2/(k_{-1} + k_2) = 0.9$ in panel C.

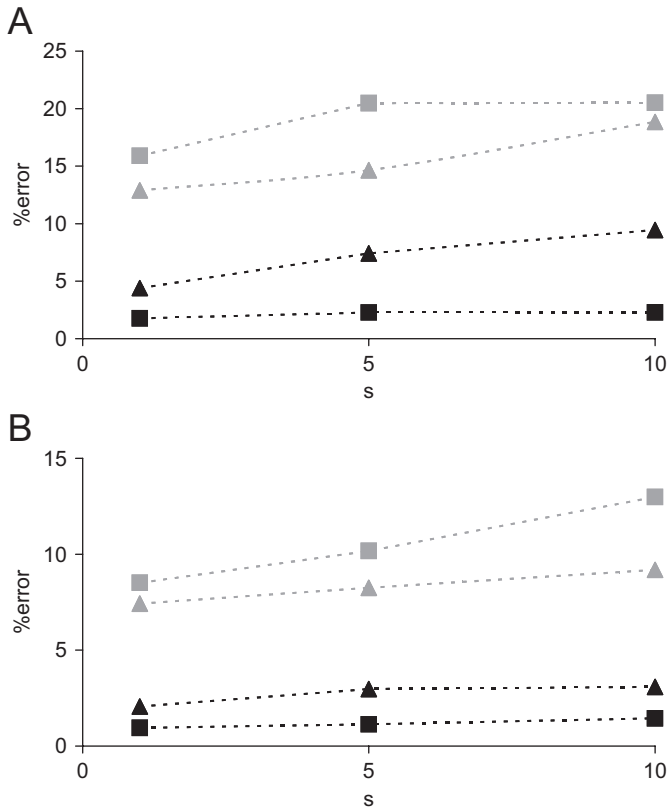


Fig. 3. Percent errors as a function of specific substrate concentration, $s \equiv S_T/K_M$. (A) $E_T = 10K_M$; (B) $E_T = 20K_M$. A posteriori percent errors associated with the first-order approximation of product formation Eq. (25) were estimated as $100\sqrt{1 - R^2}$ (triangles) and contrasted with the corresponding a priori percent errors (squares) 100ε at the extremes of relationships between the catalysis and dissociation rate constants, $k_2/(k_{-1} + k_2) = 0.1$ (black) and $k_2/(k_{-1} + k_2) = 0.9$ (gray). R^2 was evaluated as $1 - \sum (p_i^{num} - p_i^{app})^2 / \sum (p_i^{num} - p_{av}^{num})^2$, where p_i^{num} are the product concentrations provided by the numerical solutions of Eq. (8) at discrete time points, p_{av}^{num} is the average of p_i^{num} and p_i^{app} are the corresponding approximate concentrations Eq. (25).

rate equation Eq. (8) across enzyme–substrate ratios that, improves as the reaction progresses and the ratio $(E_T - \bar{S})/K_M$ approaches its maximum E_T/K_M (Fig. 3(A)). Decreasing the ratio $k_2/(k_{-1} + k_2)$ improves the fit at early times, in accord with the a priori error estimate of the tQSSA Eq. (26). At late times close to equilibrium the first-order approximation holds even for conditions that seemingly invalidate it, where the initial enzyme and substrate concentrations are equal and in marked excess over the apparent equilibrium constant ($E_T = S_T = 10K_M$) and where the catalysis rate constant k_2 is far greater than the dissociation rate constant k_{-1} (Fig. 2(A), $R^2 = 0.964$). The a priori error ε is correlated with the a posteriori nonlinear least squares regression error estimate $\sqrt{1 - R^2}$ (Fig. 3(A)). Doubling the enzyme concentration from $E_T = 10K_M$ to $20K_M$ reduced the a posteriori errors and increased the correlation with the a priori approximation (Fig. 3(B)). When $k_2 \gg k_{-1}$ the a priori error overestimates the a posteriori error since, unlike the latter, the former depends only on the initial substrate concentration. In

contrast, the a priori error underestimates the a posteriori error when $k_2 \ll k_{-1}$, presumably since this is a case where the error associated with the linearization of the QSS expression of enzyme–substrate complex (Eq. (10), Fig. 1) is higher than the error incurred by neglecting product formation during the initial transient and solving the tQSSA rate equation Eq. (15) subject to the true initial conditions Eq. (11).

4. Discussion

The tQSSA rate equation Eq. (15) has long been used to study enzymatic reactions at significant enzyme–substrate ratios (Goldstein, 1944; Reiner, 1959; Cha and Cha, 1965) and the first-order tQSSA rate equation (Table 1) was already derived by Cha (1970). Palsson (1987) obtained results that are equivalent to the first order tQSSA from a local linearization of the mass action equations around initial condition (1). The novelty and importance of the study by Borghans et al. (1996) was their delineation of the validity domain of the first-order tQSSA by means of the same methodology introduced in the study of the BHA (Segel, 1988). Their work illustrated the strength of the scaling approach advocated in Segel (1988) and highlighted that extensions of the classical QSSA formalism of Briggs and Haldane can be derived by improved approximation of the QSS enzyme–substrate complex. This realization guided subsequent refinements of the QEA (Schnell and Maini, 2000) and the tQSSA (Tzafri, 2003; Tzafri and Edelman, 2004), which are nevertheless only valid for a restrictive range of kinetic parameter values and reactant concentrations that imply negligible substrate binding ($C \ll \bar{S}$, inequality 4) or negligible enzyme binding ($C \ll E_T$, inequalities 6 and 7).

It is therefore noteworthy that we have now integrated the tQSSA rate equation Eq. (15) in closed form, as this analytical solution Eq. (19) inherits the wide validity of the tQSSA, as delineated by inequalities (12) and (17). Although implicit, the closed-form solution Eq. (19) provides a one-to-one correspondence between product concentration and time and can therefore be used to approximate the time course of HMM kinetics. Moreover, by inverting the traditional roles of time and concentration, the closed form solution can also be used for parameter estimation. One then solves for time and minimizes the difference between the experimental time points at which specific product concentrations are observed, and the estimates of the tQSSA for those product values Eq. (19) to fit for k_2 and K_M . To test the feasibility of this parameter estimation scheme we used time series generated by numerically simulating the HMM scheme Eqs. (8) and (9) in place of actual experimental data. Data analysis was performed using GraphPad Prism 3.02. The Levenberg–Marquardt method (Marquardt, 1963) was used to minimize the unweighted sum of squares of the difference between simulated data and the data generated by Eq. (19). We considered two sets of initial conditions and parameter

values for which the tQSSA accurately approximates the total substrate and bound substrate concentrations (see Figs. 6 and 7 in Tzafiriri, 2003). In both examples the kinetic parameters were set at $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 90 \text{ s}^{-1}$, $k_2 = 10 \text{ s}^{-1}$ and the substrate concentration was set equal to the Michaelis–Menten constant ($K_M = 100 \mu\text{M}$). At equal total enzyme and substrate concentrations we obtained the estimates $k_2 = (27.3 \pm 1.9) \text{ s}^{-1}$ and $K_M = (433 \pm 40) \mu\text{M}$ (Fig. 4(A)). When the total enzyme concentration is doubled the fitting procedure converged for the parameter estimates $k_2 = (7.4 \pm 0.2) \text{ s}^{-1}$ and $K_M = (20.0 \pm 0.8) \mu\text{M}$ (Fig. 4(B)). Model fits were

excellent with $R^2 = 0.9999$ for both examples and relative standard errors of less than 25%. Yet, the estimated catalysis rate constant is 2.7-fold higher than the true value in the first example (Fig. 4(A)), and the estimated Michaelis–Menten constant is 4.3-fold too high in the first example and 5-fold too low in the second example (Fig. 4(B)). As the tQSSA also provides an accurate approximation of the fitted cases (not shown), the discrepancies between the true and fitted parameter values reflect a relative insensitivity of the product concentration to the values of the Michaelis–Menten constant and the catalysis rate constant. These findings are consistent with the functional dependence of the closed-form solution of the tQSSA rate equation Eq. (19) on K_M , but are also surprising given that k_2 is a scaling factor in that equation. Importantly, while the product concentrations provided by the numerical solution of the HMM scheme Eqs. (8) and (9) are relatively insensitive to K_M and k_2 in both examples, the concentrations of the enzyme–substrate complex are sensitive to these parameters (Figs. 4(A) and (B)). These examples illustrate that reliable estimation of the steady-state kinetic constants k_2 and K_M cannot be solely based on rate laws, whatever their accuracy. Time series of product concentration are not sufficiently sensitive to these parameters, and must be augmented by time series of the enzyme–substrate complex.

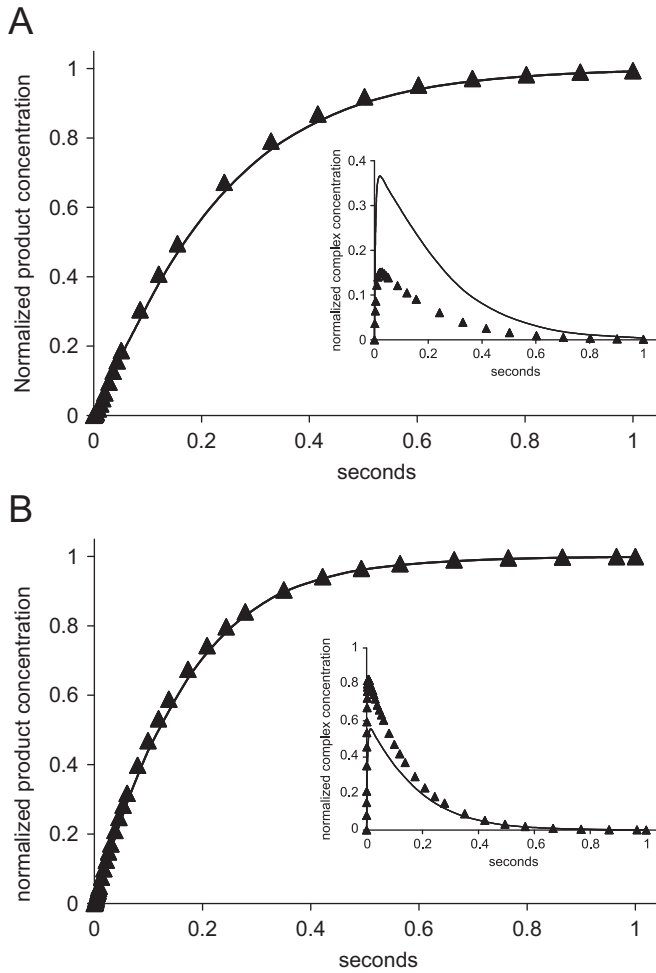


Fig. 4. Parameter estimation is limited by parameter sensitivity. The HMM scheme Eqs. (8) and (9) was simulated for two sets of initial conditions: (A) $E_T = S_T = 100 \mu\text{M}$, or (B) $E_T = 2S_T = 200 \mu\text{M}$. Two sets of parameter values were used: (lines) $k_1 = 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 90 \text{ s}^{-1}$, $k_2 = 10 \text{ s}^{-1}$ or (triangles) $k_1 = K_{M,fit} / (k_{2,fit} + k_{-1})$, $k_{-1} = 90 \text{ s}^{-1}$, $k_{2,fit}$ where $k_{2,fit}$ and $K_{M,fit}$ were provided by the parameter estimation scheme described in the text: (A) $k_{2,fit} = (27.32 \pm 1.92) \text{ s}^{-1}$, $K_{M,fit} = (433 \pm 40) \mu\text{M}$ ($R^2 = 0.9999$) and (B) $k_{2,fit} = (7.4 \pm 0.2) \text{ s}^{-1}$, $K_{M,fit} = (20.0 \pm 4.8) \mu\text{M}$ ($R^2 = 0.9999$). Correspondingly $k_{leff} = 2.71 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (A) or $k_{leff} = 4.87 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (B). An excellent correspondence is observed between the original (solid lines) and fitted (triangles) product concentrations, but not between original and fitted enzyme–substrate complex concentrations (insets). All concentrations are normalized to the initial substrate concentration ($S_T = 100 \mu\text{M}$).

4.1. Redefining states of enzyme excess

Simplification of the QSS enzyme–substrate complex in states of high enzyme and substrate concentrations yielded novel insight on bimolecular binding reactions. In particular, inequality (27) precisely defines the conditions for maximal substrate binding Eq. (22a) and first-order product formation Eq. (25), whereas inequality (C.4) defines the precise conditions for maximal enzyme binding and zero-order product formation Eq. (C.5). These conditions include cases of large enzyme–substrate ratios (inequality (7)) but also a wide range of cases of enzyme to substrate ratios that approach unity (Figs. 1, 2(A) and (B)) and/or such that $k_2 \gg k_{-1}$ (Figs. 2 and 3). This calls for a change in nomenclature; a state of enzyme excess exists when the absolute difference between the enzyme and substrate concentrations is much larger than the corresponding Michaelis–Menten constant, rather than at large enzyme to substrate ratios alone.

4.2. Initial velocity measurements

The HMM scheme is a limiting case for many enzymatic reactions at sufficiently short times such that enzyme stability and product accumulation imperceptibly affect the reaction (Lehninger, 1975). Consequently, the notion of an initial rate of product formation, $v_0 \equiv dP/dt|_{t=0}$, has played a prominent role in the analysis of enzymatic reactions (Lehninger, 1975; Hiromi, 1979). Strictly speaking, initial condition (1) implies that $dP/dt|_{t=0} = k_2 C|_{t=0} = 0$ and

$v_0 = 0$. However, validity of the tQSSA implies that the initial binding transient appears instantaneous compared with the time scale of product formation. In this case, $dP/dt|_T \approx k_2 C_-(S_T, E_T)$ for times T that are sufficiently long to ensure that the enzyme–substrate complex is at steady state, yet sufficiently short to ensure that only a negligible fraction of the initial substrate concentration converts to product, $t_C \leq T \ll t_{\bar{S}}$. Hence, the tQSSA not only supports the approximation (Goldstein, 1944; Reiner, 1959; Cha and Cha, 1965)

$$v_0 = (k_2/2) \times \left((K_M + S_T + E_T) - \sqrt{(K_M + S_T + E_T)^2 - 4E_T S_T} \right), \quad (28)$$

but also provides a self-consistency check for this approximation in that the experimental time points used for evaluating the rate of product formation must be small compared to $t_{\bar{S}} = S_T/v_0$ Eq. (14), yet larger than t_C as defined by Eq. (13). In particular, Eq. (28) inherits the wide validity of the tQSSA and can safely be used at states of enzyme excess (inequalities (6), (7) and (27)), in contrast to recent statements (Schnell and Maini, 2003).

Conditions of pronounced substrate excess such that $S_T \gg K_M + E_T$, guarantee the validity of the Briggs–Haldane rate law Eq. (4) and imply that the enzyme is fully saturated so that $v_0 = k_2 E_T$. We now show that this result

is of much wider validity and holds whenever $S_T - E_T \gg K_M$ (Appendix C). Similarly, $E_T - S_T \gg K_M$ implies that product forms at the maximal rate, $v_0 \approx k_2 S_T$. Drawing upon these results and the first-order tQSSA of the concentration of the enzyme–substrate complex (Tzafiriri, 2003) we mapped the initial rate of product formation as a function of initial substrate and enzyme concentrations (Fig. 5). Remarkably, the initial rate is approximately proportional to the initial substrate or enzyme concentrations for approximately half of the potential initial conditions, regardless of any kinetic considerations. Most other initial conditions do not allow further simplification of (28), while the minority that validate the first-order tQSSA (inequalities (4), (6) or (7)) imply the hyperbolic dependence $v_0 \approx k_2 E_T S_T / (K_M + E_T + S_T)$ (Tzafiriri, 2003). These classifications are approximate and depend on the validity of the tQSSA. When the catalysis rate constant k_2 is smaller than the dissociation rate constant $k_{-1}(k_2/(k_{-1} + k_2) < 0.4)$ the errors associated with this classification are less than 10% (inequality (17)). When the catalysis rate constant k_2 is greater than the dissociation rate constant $k_{-1}(k_2/(k_{-1} + k_2) \geq 0.4)$ the error associated with the tQSSA can reach 25% in the region enclosed by dashes ($0.1 < \varepsilon < 0.25$), and Eq. (28) is only a fair approximation (Tzafiriri, 2003). Importantly, the regimes of maximal rate ($v_0 \approx k_2 S_T$), enzyme saturation ($v_0 \approx k_2 E_T$) and the lower branch of the hyperbolic regime remain valid regardless of the ratio k_2/k_{-1} . The latter regime corresponds to the validity domain of the BHA (inequality (4)).

4.3. Low molecule numbers

One of the motivations for extending the validity of the QSSA, are reports that enzyme–substrate ratios may be high for intracellular reactions, especially due to compartmentalization of reactant molecules. However, compartmentalization also results in low molecule numbers, raising the concern that stochastic effects may invalidate classical kinetics in these cases. In the extreme case of a single enzyme molecule reacting with a single substrate molecule it has been shown that the stochastic expected values can significantly deviate from the deterministic solution for certain values of the rate constants (Aranyi and Toth, 1977). However, the stochastic expected values can already converge to the deterministic solution for as few as several hundred reactant molecules, though individual runs of the stochastic system can still exhibit such deviations (Salwinski and Eisenberg, 2004; Turner et al., 2004). These findings have generated interest in the question whether the QSSA carries through to the stochastic regime. Rao and Arkin (2003) reported a good match between the Michaelis–Menten rate equation (Table 1) and the mean of 50,000 stochastic simulations of the irreversible HMM scheme with 10 enzyme molecules and 100 substrate molecules. The mean number of product molecules is linear in time till about 90% of the substrate has been depleted (Fig. 6, $R^2 = 0.9983$), in agreement with our

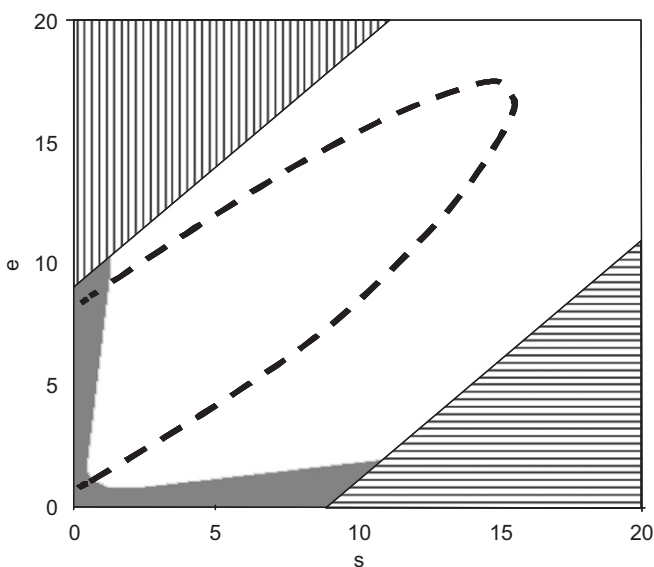


Fig. 5. Initial rate Eq. (28) as a function of initial specific concentrations $s = S_T/K_M$ and $e = E_T/K_M$. When $k_2/(k_{-1} + k_2) < 0.4$ the tQSSA is valid ($\varepsilon \leq 0.1$) for any initial condition and provides the classification: (horizontal lines) maximal rate regime, $v_0 \approx k_2 S_T$; (gray) hyperbolic regime, $v_0 \approx k_2 E_T S_T / (K_M + E_T + S_T)$; (vertical lines) enzyme saturation regime, $v_0 \approx k_2 E_T$; (white) square root regime, $v_0 \approx k_2 C_-(S_T, E_T)$. This classification remains valid when $k_2/(k_{-1} + k_2) \geq 0.4$, except for the region enclosed by the dashed line, for which the tQSSA is only marginally valid ($0.1 < \varepsilon < 0.25$). The regimes of maximal rate and enzyme saturation are valid irrespective of the magnitude of the ratio k_2/k_{-1} .

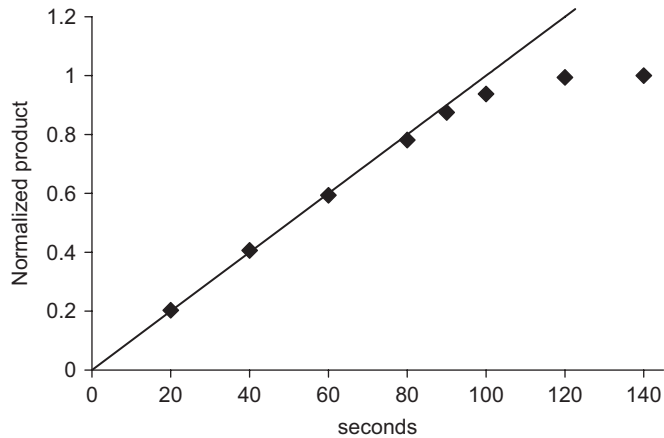


Fig. 6. Normalized product concentration P/S_T as a function of normalized time. Zero-order product formation (Eq. (C.5), line) is contrasted with the mean of 50,000 stochastic runs (diamonds, Rao and Arkin, 2003) for $E_T = 10$ molecules, $S_T = 100$ molecules and $K_M = 1.1$ molecules ($k_2 = 0.1$, $k_2/(k_{-1} + k_2) = 0.09$).

prediction that $\bar{S} - E_T \gg K_M$ guarantees zero order product formation $P \approx k_2 E_T t$ (Appendix C).

For a case of 1000 enzyme molecules and 100 substrate molecules, Rao and Arkin (2003) reported a poor match between the BHA rate law and the mean of 50,000 stochastic simulations. The poor match arises, not because of any stochastic effects, but simply because the condition for the validity of the BHA (inequality (4)) is violated in this example. In contrast, the classical kinetics conditions for first order product formation (inequalities (6) and (27)) are satisfied and an excellent match is obtained between Eq. (25) and the number of product molecules (Fig. 7(A), $R^2 = 0.9998$). Goutsias (2005) recently reported stochastic means for 10 enzyme molecules and 5 substrate molecules, for which the first order tQSSA is invalid ($r = 0.78$) but inequality (26) is valid, ($\varepsilon = 0.02$). As predicted, first-order product formation Eq. (25) closely matches the reported stochastic mean (Fig. 7(B), $R^2 = 0.9894$). Owing to the low number of molecules, the standard deviation of the stochastic runs is significant for this example (Goutsias, 2005). The magnitude of these deviations is captured by the evolution of the standard deviation, which does not follow from the classical analysis.

Taken together, these examples suggest that the expected stochastic kinetics implied by the HMM scheme are adequately captured by classical deterministic kinetics even at relatively low molecule numbers, suggesting that the tQSSA is useful for studying intracellular reactions. A more systematic analysis of this issue is beyond the scope of this paper and will be pursued elsewhere.

4.4. Broader significance

The tQSSA has not only revolutionized our understanding of the irreversible HMM, but has also impacted the modeling and analysis of more complex enzymatic processes (Wu et al., 2001; Tzafirri et al., 2002; Tzafirri and

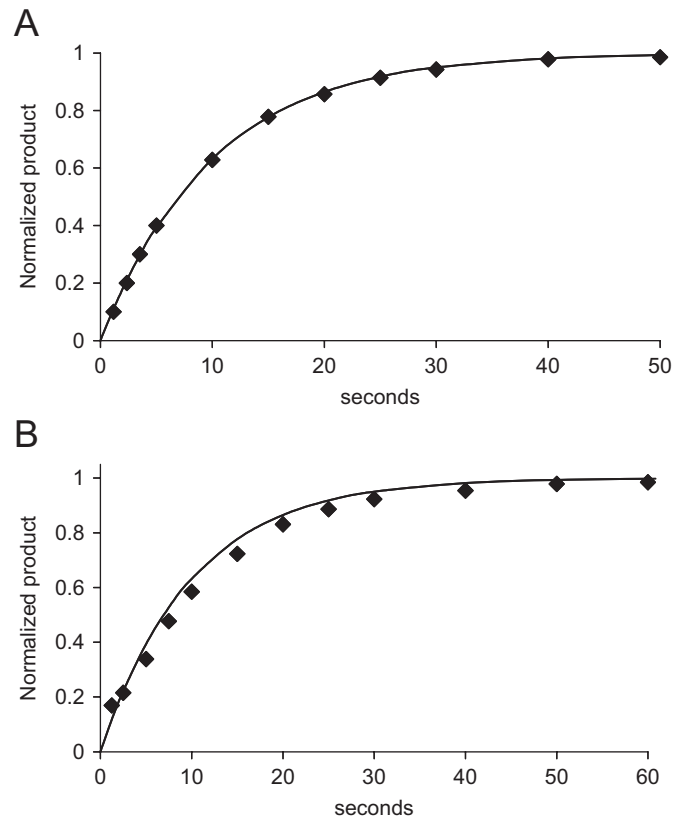


Fig. 7. Normalized product concentration P/S_T as a function of normalized time. First-order product formation (Eq. (25), line) is contrasted with the mean of 5000–50,000 stochastic runs (diamonds) for two examples with the same kinetic parameters ($k_2 = 0.1$, $k_2/(k_{-1} + k_2) = 0.09$ and $K_M = 1.1$ molecules) but different numbers of reactant molecules: (A) $E_T = 1000$ molecules, $S_T = 100$ molecules (Rao and Arkin, 2003) and (B) $E_T = 10$ molecules, $S_T = 5$ molecules (Goutsias, 2005).

Edelman, 2004), predator prey interactions (Borghans et al., 1996), T cell proliferation (De Boer and Perelson, 1995). The current analysis is therefore of potential interest for these related problems. In particular, the finding that for a tight binding pair moderate excess of the majority species can fully saturate the minority species is an important general result for bimolecular binding. As but one example, we recently used the tQSSA to analyze intracellular receptor trafficking (Tzafirri and Edelman, in press). Identifying receptors with the enzyme and ligand molecules with the substrate, we showed that the condition $E_T - S_T \gg K_M$ is the criterion for internalized ligand to remain bound to its receptor following endocytosis Eq. (21a).

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Appendix A. Integration of the tQSSA rate equation

Inspired by Wu et al. (2001) we introduced Eq. (18) and solved for the total substrate concentration to find

$$\bar{S} = \frac{y^2 - (K_M + E_T)^2}{2(K_M + y - E_T)}. \tag{A.1}$$

Differentiation of (A.1) with respect to y yields

$$\frac{d\bar{S}}{dy} = \frac{(y + K_M - E_T)^2 + 4K_M E_T}{2(y + K_M - E_T)^2} > 0. \tag{A.2}$$

Substituting (18) and (A.1) into Eq. (10) we obtain

$$C_-(\bar{S}) = \frac{(K_M + E_T + \bar{S}) - (y - \bar{S})}{2} = \frac{E_T(y - K_M - E_T)}{(y + K_M - E_T)}. \tag{A.3}$$

Applying the chain rule to Eq. (15) we obtain $d\bar{S}/dt = (d\bar{S}/dy)(dy/dt) \approx -k_2 C_-(\bar{S})$, or equivalently

$$\frac{dy}{dt} \approx -\frac{k_2 C_-(\bar{S})}{d\bar{S}/dy} = \frac{-2k_2 E_T(y - K_M - E_T)(y + K_M - E_T)}{(y + K_M - E_T)^2 + 4K_M E_T}. \tag{A.4}$$

Separating variables we obtain

$$\begin{aligned} -k_2 dt &= \left(\frac{1}{2E_T}\right) \left(\frac{y^2 + 2(K_M - E_T)y + (K_M + E_T)^2}{y^2 - 2E_T y + (E_T^2 - K_M^2)}\right) dy \\ &= \left(\frac{1}{2E_T} + \left(\frac{K_M + E_T}{E_T}\right) \frac{1}{y - (K_M + E_T)} - \frac{1}{y - (E_T - K_M)}\right) dy. \end{aligned} \tag{A.5}$$

Integrating both sides of (A.5) subject to the initial condition

$$y(0) \equiv y_0 = S_T + \sqrt{(K_M + E_T + S_T)^2 - 4E_T S_T} \tag{A.6}$$

and rearranging we arrive at Eq. (19). We verified this analytical solution Eq. (19) by solving the tQSSA rate equation Eq. (15) numerically for several combinations of S_T, E_T, K_M and k_2 and inserting these results on the right-hand side of Eq. (19) to obtain an estimate of the time it takes to form the product.

A.1. The first-order tQSSA

Inequalities (4), (6) and (7) are all sufficient conditions for the first-order tQSSA and warrant that $r \equiv 4E_T \bar{S} / (K_M + E_T + \bar{S})^2 \ll 1$ (Tzafriri, 2003). Rewriting Eq. (18) as $y = \bar{S} + (K_M + E_T + \bar{S})\sqrt{1 - r}$ facilitates the binomial expansion

$$\begin{aligned} y &\approx \bar{S} + (K_M + E_T + \bar{S})(1 - r/2) \\ &= 2\bar{S} + K_M + E_T - 2E_T \bar{S} / (\bar{S} + K_M + E_T), \quad r \ll 1. \end{aligned} \tag{A.7}$$

Inequalities (6) and (7) both imply that $S_T \ll E_T + K_M$ and therefore reduce Eqs. (A.7) and (19) to, respectively,

$y \approx K_M + E_T + 2K_M \bar{S} / (K_M + E_T)$ and

$$\begin{aligned} t &= \frac{K_M(S_T - \bar{S})}{k_2 E_T (K_M + E_T)} - \left(\frac{K_M + E_T}{k_2 E_T}\right) \ln \frac{\bar{S}}{S_T} \\ &\quad + k_2^{-1} \ln \frac{K_M + E_T + \bar{S}}{K_M + E_T + S_T} \\ &\approx -\left(\frac{K_M + E_T}{k_2 E_T}\right) \ln \frac{\bar{S}}{S_T}. \end{aligned} \tag{A.8}$$

Solving for the total intact substrate we reproduce the result from Tzafriri (2003),

$$S = S_T \exp\left(-\frac{k_2 E_T}{K_M + E_T} t\right), \quad E_T + K_M \gg S_T. \tag{A.9}$$

On the contrary, the criterion for the validity of the BHA, $E_T \ll S_T + K_M$, reduces Eqs. (A.7) and (19) to, respectively, $y \approx 2\bar{S} + K_M$ and

$$t = \frac{S_T - \bar{S}}{k_2 E_T} - \left(\frac{K_M + E_T}{k_2 E_T}\right) \ln \frac{\bar{S}}{S_T} + k_2^{-1} \ln \frac{K_M + \bar{S}}{K_M + S_T}. \tag{A.10}$$

Up to the third term on the right-hand side, Eq. (A.10) corresponds to the direct integration of the first-order tQSSA rate equation (Tzafriri, 2003, Table 1). The new term is negligible when $\bar{S} \gg K_M$ or $S_T \ll K_M$, but is significant when $\bar{S} \approx K_M$.

Appendix B. Derivation of Eqs. (21a,b)

Here, we consider the consequences of the assumption that

$$K_M \ll |E_T - \bar{S}|. \tag{B.1}$$

At disparate enzyme and substrate concentrations inequality (B.1) provides for the validity of the first-order tQSSA

$$r \equiv 4E_T \bar{S} / (K_M + E_T + \bar{S})^2 \approx 4 \min(E_T, \bar{S}) / \max(E_T, \bar{S}) \ll 1,$$

so that (Tzafriri, 2003)

$$\begin{aligned} C_-(E_T, \bar{S}) &\approx \frac{E_T \bar{S}}{E_T + K_M + \bar{S}} \approx \frac{\min(E_T, \bar{S}) \max(E_T, \bar{S})}{\max(E_T, \bar{S})} \\ &= \min(E_T, \bar{S}) \end{aligned} \tag{B.2}$$

and

$$\begin{aligned} C_+(E_T, \bar{S}) &\approx E_T + K_M + \bar{S} \approx \min(E_T, \bar{S}) \\ &\quad + \max(E_T, \bar{S}) = \max(E_T, \bar{S}). \end{aligned} \tag{B.3}$$

We now show that inequality (B.1) provides for (B.2) and (B.3), even when the total enzyme and total substrate concentrations are comparable, $\bar{S} = O(E_T)$. Introducing the notation

$$a \equiv K_M / E_T, \quad \bar{s} \equiv \bar{S} / E_T \tag{B.4}$$

we can rewrite Eq. (10) as

$$C_{\pm}(\bar{s}, a)/E_T = \frac{(a + 1 + \bar{s}) \pm \sqrt{(a + 1 + \bar{s})^2 - 4\bar{s}}}{2}. \quad (\text{B.5})$$

This is informative, since $\bar{S} = O(E_T)$ implies that $\bar{s} = O(1)$, whereas inequality (B.1) guarantees that a is small and

$$C_{\pm}(\bar{s}, a) \approx C_{\pm}(\bar{s}, 0) + \left. \frac{\partial C_{\pm}(\bar{s}, a)}{\partial a} \right|_{a=0} a, \quad a \ll 1. \quad (\text{B.6})$$

Substituting

$$C_{\pm}(\bar{s}, 0)/E_T = \frac{(1 + \bar{s}) \pm \sqrt{(1 + \bar{s})^2 - 4\bar{s}}}{2} = \frac{(1 + \bar{s}) \pm |1 - \bar{s}|}{2} \quad (\text{B.7})$$

and

$$\begin{aligned} \left. \frac{\partial C_{\pm}(\bar{s}, a)}{\partial a} \right|_{a=0} &= \frac{E_T}{2} \left(1 \pm \frac{(1 + \bar{s})}{\sqrt{(1 + \bar{s})^2 - 4\bar{s}}} \right) \\ &= \frac{E_T}{2} \left(1 \pm \frac{(1 + \bar{s})}{|1 - \bar{s}|} \right) = \frac{C_{\pm}(\bar{s}, 0)}{|1 - \bar{s}|} \end{aligned} \quad (\text{B.8})$$

into Eq. (B.6) we find

$$\begin{aligned} C_{\pm}(\bar{s}, a) &\approx C_{\pm}(\bar{s}, 0) \left(1 + \frac{a}{|1 - \bar{s}|} \right) \\ &= C_{\pm}(\bar{s}, 0) \left(1 + \frac{K_M}{|E_T - \bar{S}|} \right), \quad a \ll 1. \end{aligned} \quad (\text{B.9})$$

Correspondingly, inequality (B.1) warrants the neglect of the linear term in the binomial expansion Eq. (B.9), yielding Eqs. (B.2) and (B.3). Interestingly, the latter results can be shown to hold even when $E_T = \bar{S} \gg K_M$.

Appendix C. The limit of substrate excess

At excess substrate ($E_T < \bar{S}$) Eqs. (21a,b) reduce to

$$C_-(\bar{S}) \approx E_T, \quad C_+(\bar{S}) \approx \bar{S} \quad (\text{C.1})$$

in contradistinction to Eqs. (22a,b). Substituting these approximations into Eqs. (12) and (15) we find, respectively:

$$\varepsilon \approx \frac{k_2 E_T}{k_1 (S_T - E_T) S_T} \leq \frac{k_2/k_1}{S_T - E_T} \quad (\text{C.2})$$

and

$$d\bar{S}/dt \approx -k_2 E_T \Leftrightarrow \bar{S} \approx S_T - k_2 E_T t. \quad (\text{C.3})$$

Thus, the condition

$$\bar{S} - E_T \gg K_M \quad (\text{C.4})$$

guarantees the validity of the tQSSA ($\varepsilon \ll 1$) and implies that the early kinetics of product formation are well approximated as zero order

$$P = S_T - \bar{S} \approx E_T (k_2 t). \quad (\text{C.5})$$

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