Relaxation to statistical equilibrium in stochastic Michaelis-Menten kinetics

Subham Pal,1 Manmath Panigrahy,1 R. Adhikari,2 and Arti Dua1

1Department of Chemistry, Indian Institute of Technology, Madras, Chennai-600036, India
2DAMTP, Centre for Mathematical Sciences, University of Cambridge, Wilberforce Road, Cambridge CB3 0WA, UK

The equilibration of enzyme and complex concentrations in deterministic Michaelis-Menten reaction networks underlies the hyperbolic dependence between the input (substrates) and output (products). This relationship was first obtained by Michaelis and Menten and then Briggs and Haldane in two asymptotic limits: “fast equilibrium” and “steady state”. In stochastic Michaelis-Menten networks, relevant to catalysis at single-molecule and mesoscopic concentrations, the classical analysis cannot be directly applied due to molecular discreteness and fluctuations. Instead, as we show here, such networks require a more subtle asymptotic analysis based on the decomposition of the network into reversible and irreversible sub-networks and the exact solution of the chemical master equation (CME). The reversible and irreversible sub-networks reach detailed balance and stationarity, respectively, through a relaxation phase that we characterise in detail through several new statistical measures. Since stochastic enzyme kinetics encompasses the single-molecule, mesoscopic and thermodynamic limits, our work provides a broader molecular viewpoint of the classical results, in much the same manner that statistical mechanics provides a broader understanding of thermodynamics.

I. INTRODUCTION

Michaelis and Menten, in their pioneering study of 1913, proposed the initial rate method to estimate the kinetic parameters of enzymatic reactions [1]. They rationalized the experimentally observed hyperbolic relationship between substrate concentration (the input) and the enzyme velocity (the output) in terms of a simple mechanism where enzyme $E$ and substrate $S$ instantaneously attained equilibrium with the complex $ES$, characterized by an equilibrium constant $K_M$. The input-output characteristic was parametrized by $K_M$, now known as the Michaelis constant, and $V_{\text{max}}$, the maximum attainable enzymatic velocity to give the celebrated hyperbolic relationship [2–5],

$$V_0 = \frac{V_{\text{max}}[S]}{[S] + K_M}. \quad (1)$$

The assumption that enzymes only weakly bound to the substrate, together with the law of mass conservation, implied that a hyperbolic characteristic curve would be attained when $[E]_0$, the initial enzyme concentration, was much smaller than the substrate concentration. For over a century, this has delineated the limit in which the initial rate method can be reliably used in experimental assays to estimate kinetic parameters [2–5].

Given the importance of the method established by Michaelis and Menten, it is not surprising that it has been subject to refinement and scrutiny since then [6–20]. In 1925, Briggs and Haldane introduced a more detailed mechanism

$$E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow{k_2} P + E \quad (2)$$

in which the dissociation of the complex into product and the regeneration of the enzyme was explicitly included and the assumption of instantaneous equilibrium was relaxed [7]. This allowed, for the first time, the possibility of studying the initial transient in which equilibrium between enzyme and substrate was yet to be established. In a subtle conceptual shift, Briggs and Haldane derived an expression for the steady-state velocity, beyond this initial transient, which, surprisingly, had exactly the same analytic form as the Michaelis-Menten equation, Eq. (1), but now with an explicit expression for the Michaelis constant $K_M = \frac{k_2}{k_{-1}}$, in terms of the rates of the elementary steps of the mechanism. Their analysis established that equilibrium would be rapidly established if the dissociation rate constant was much larger than the product formation rate constant $k_2 \ll k_{-1}$. Under this “steady-state” assumption, the initial velocity $V_0$ in the Michaelis-Menten analysis could be identified with the steady-state velocity $V_{ss}$ in the Briggs-Haldane analysis if both association and dissociation rates were much larger than the product formation rate.

The conditions under which this identification can be made have been refined further by Laidler and remain an active area of research [20–28], see Ref. [16] for a review. The characteristic curve remains a central theoretical tool in both inferring reaction mechanisms from experimental assays and in estimating the rate parameters of the inferred mechanisms.

Over the last two decades, the increasing sophistication of experimental methods has enabled the study of enzymatic catalysis at the single-molecule level [29–40]. The thermodynamic limit implicit in the classical work described above no longer applies to these experiments. Instead, the appropriate formalism is that of continuous-time Markov processes with discrete state spaces, the chemical master equation, reflecting the discrete nature of the transition between catalytic states. The relaxation to statistical equilibrium, consequently, is different from that in the thermodynamic limit and presents, as we have discovered, several subtleties.
Bartholomay was the first to develop a chemical master equation (CME) formalism for enzyme kinetics [41]. This formalism incorporated the randomness of discrete molecular collisions and their uncertainty, termed molecular fluctuations, in each elementary step of the Michaelis-Menten mechanism. Bartholomay used the CME to demonstrate the broader applicability of the stochastic treatment and how number fluctuations influence the mean catalytic response in the thermodynamic limit. Later works used the quasi-steady-state assumption on the CME to reduce the two-dimensional chemical master equation for the Michaelis-Menten (kinetic) mechanism, Eq. (2), to a one-dimensional master equation for a Michaelis-Menten (equilibrium) mechanism [42–44], see Ref. [45] for a review. However, this a priori assumption of stationarity cannot fully characterise the molecular fluctuations arising from individual reaction steps in the kinetic mechanism in the non-stationary transient state, which is crucial for the asymptotic analysis of the classical results.

We have developed a stochastic time-based approach (point process description), which seamlessly combines with the number-based approach (count process description), namely the chemical master equation, and provides a comprehensive statistical analysis of molecular fluctuations in enzymatic mechanisms [46–50]. This treatment encompasses the non-stationary transients and stationary steady states in stochastic enzymatic networks, in time, and single-molecule, mesoscopic and classical limits, in terms of the enzyme numbers.

In this work, we introduce new statistical measures for the count and point process description and apply them to the exact solution of the CME for the Michaelis-Menten mechanism to comprehensively analyse the nature of molecular fluctuations in the transient and stationary state kinetics. This provides a wide-ranging generalisation of the asymptotic analysis of classical enzyme kinetics to stochastic enzyme kinetics, with the subtle role played by molecular fluctuations being clearly delineated. The novelty of our work lies in decomposing the network of stochastic transitions into reversible and irreversible subnetworks, where the reactions $E \to ES$ and $ES \to E$ correspond to the reversible subnetwork, and the reaction $ES \to E + P$ corresponds to the irreversible subnetwork. As we show, the reversible network attains chemical detailed balance while the irreversible network attains a stationary state through a non-trivial transient that we carefully characterise.

Sections II and III describe the salient features of the deterministic and stochastic single-enzyme networks. After introducing the notations and terminology of the count and point process descriptions comprising joint probabilities of discrete species, generating function, waiting time distributions, we develop the detailed statistical formalism in Sections IV-VII. A summary of each section is provided at the end of Section III.

## II. DETERMINISTIC MICHAELIS-MENTEN NETWORK

The deterministic Michaelis-Menten network is a kinetic reaction scheme, Eq. (2), which involves a cyclical process of enzyme turnovers through the catalytic rate constant $k_2$, resulting in product formation over multiple cycles. Over time, the rate of product formation reaches a stationary state, and the enzyme turnover cycles reach an equilibrium state, where enzymes and their complexes satisfy the chemical detailed balance condition, $k_a[E]c_q = k_b[ES]c_q$. Here, $k_a = k_1[S]$ is the pseudo-first-order rate constant and $k_b = k_{-1} + k_2$. This stationary (equilibrium) state underlies the fundamental hyperbolic relation between the steady-state velocity ($V_{ss}$) and substrate concentration, quantified by the Michaelis-Menten equation (MME), $V_{ss} = \frac{k_2k_0}{k_a+k_0} = \frac{k_2[E]_0[S]}{[S]+K_M} = \frac{V_{max}[S]}{[S]+K_M}$, where $[E]_0 = [E] + [ES]$ is the total enzyme concentration, $V_{max} = k_2[E]_0$ is the maximum velocity at saturating substrate concentration, and $K_M = (k_{-1} + k_2)/k_1$.

The classical studies derive the MME using either a fast-equilibrium assumption (FEA) or a quasi-steady-state assumption (QSSA). The FEA assumes a timescale separation between the fast equilibration of enzymes and complexes and slow product formation. This assumption implies a quasi-equilibrium between enzymes and substrates $E \rightleftharpoons ES$, with complexes

$$E \rightleftharpoons ES \rightleftharpoons ES + P,$$

and complexes $ES \rightleftharpoons ES + P$, at the onset of the reaction. The QSSA does not assume an equilibrium between enzymes and complexes but rather a short initial transient. The QSSA bypass this by assuming a quasi-equilibrium or quasi-stationary state immediately after the reaction begins.

While the duration of the initial transient involves non-stationary turnover kinetics, the FEA and SSA bypass this by assuming a quasi-equilibrium or quasi-stationary state immediately after the reaction begins. A simple estimate of the duration of the initial transient follows from the solution of the rate equation for complex in time, $V_c(t) = \frac{k_a[E]_0}{k_a+k_0} e^{-(k_a+k_0)t}$. For $t \gg T^*$, $V_c(t)$ relaxes to its stationary value, $[ES]_{ss} = \frac{k_a[E]_0}{k_a+k_0}$. The QSSA, thus, translates into obtaining the duration of the initial transient as

$$T^* = \frac{|\lambda|}{(k_a + k_b)}$$

where $\lambda$ is a positive constant, which remains undetermined in the mass action kinetics. For times beyond the initial transient regime, the MME is exactly recovered.

The deterministic approach assumes enzymes and substrate concentrations to be thermodynamically large.
However, in vitro and vivo, enzyme concentrations are significantly lower than the substrates. This concentration ratio, $[E]_0 \ll [S]$, is the basis of the initial rate method and ensures that the initial rate of product formation recovers the hyperbolic MME [1–5]. At such low enzyme concentrations, however, the turnover kinetics is inherently stochastic, governed by molecular fluctuations. Whereas the MM mechanism accounts for this concentration ratio through pseudo-first-order rate constant $k_a = k_1 [S]$, which implicitly assumes $[E]_0 \ll [S]$, the underlying mechanism and the rate equations only have a deterministic context. They exclude the possibility of molecular fluctuations, which, we show here, play a crucial role in describing the discrete turnover kinetics in the non-stationary transient regime and determining its duration, Eq. (3).

In the next section, we describe the single-enzyme stochastic MM network, in which the catalytic conversion of substrates to products is carried out by a single enzyme, one substrate at a time, in $p$ discrete turnover cycles. We examine the non-stationary and stationary turnover kinetics of $N$ replicas of this stochastic network from a probabilistic point of view. The stationarity and (statistical) equilibrium conditions for the single or replica network follow from the joint probability of the number of chemical species in the $p$-th turnover cycle. These results provide a stochastic generalization of the FEA, QSSA and their relation to the hyperbolic MME in the $p$-th turnover cycle.

III. STOCHASTIC MICHAELIS-MENTEN NETWORK

The stochastic description of the Michaelis-Menten network begins by defining the state vector $\mathbf{n} = \{n_E, n_{ES}, n\}$, and its joint probability $P(n_E, n_{ES}, n, t)$ at time $t$, where $n_E, n_{ES}, n$ are the number of enzymes, complexes, and products. The time evolution of the joint probability obeys the Markovian chemical master equation (CME) [41, 51–53]:

$$
\frac{\partial P(\mathbf{n}, t)}{\partial t} = k_a (n_E + 1) P(n_E + 1, n_{ES} - 1, n; t) + k_{-1} (n_{ES} + 1) P(n_E - 1, n_{ES} + 1, n; t) + k_2 (n_{ES} + 1) P(n_E - 1, n_{ES} + 1, n - 1; t) - [k_a n_E + (k_2 + k_{-1}) n_{ES}] P(\mathbf{n}, t). \tag{4}
$$

The CME accounts for the molecular discreteness and stochasticity in each elementary step of the MM network. Since $N$ initial enzymes either exist in free or bound state, the stochastic trajectories generated from the CME obey the enzyme conservation law $n_E + n_{ES} = N$ at all times, implying $P(N - n_{ES}, n_{ES}, n, t) = P(n_{ES}, n, t|N)$.

Fig. (1) shows the salient features of a single-enzyme stochastic Michaelis-Menten network with discrete turnover cycles $p = 1, 2, \cdots$, which include equilibrium between $E$ and ES (blue dotted lines), and discrete product turnover events. Each turnover cycle includes $n = p - 1$ number of products. The turnover kinetics is characterized by the $p$-th waiting time between two consecutive product turnovers, $\tau_p = T_p - T_{p-1}$, where $T_p$ is the turnover time for the $p$-th product formation starting from $T_0 = 0$ such that $\tau_1 = T_1$. The product turnovers are described in two complimentary ways: The count process describes the number of products $n = p - 1$ formed in continuous time $t$; The point process description specifies the turnover time for the $p$-th product formation.

Figure 1. A single-enzyme stochastic Michaelis-Menten (MM) network with discrete turnover cycles $p = 1, 2, \cdots$, which include equilibrium between $E$ and ES (blue dotted lines), and discrete product turnover events. Each turnover cycle includes $n = p - 1$ number of products. The turnover kinetics is characterized by the $p$-th waiting time between two consecutive product turnovers, $\tau_p = T_p - T_{p-1}$, where $T_p$ is the turnover time for the $p$-th product formation starting from $T_0 = 0$ such that $\tau_1 = T_1$. The product turnovers are described in two complimentary ways: The count process describes the number of products $n = p - 1$ formed in continuous time $t$; The point process description specifies the turnover time for the $p$-th product formation.
The specification of the number of products \( n = p - 1 \) in time \( t \) is the count process description, described by the joint probability \( P(n_{ES}, n, t|N) \), which is the solution of Eq. (4). The specification of the turnover time \( T_p \) for the \( p \)-th product is the point process description, described in terms of the distributions of turnover times, \( w(T_p|N) \). [54].

The CME, Eq. (4), describes the time evolution of discrete states \( n \) in continuous time \( t \). It can be transformed into a partial differential equation by using the following relation between the generating function \( G(s_1, s_2, t|N) \) and the joint probability \( P(n_{ES}, n, t|N) \) [46]:

\[
G(s_1, s_2, t|N) = \sum_{n_{ES}} \sum_{n} s_{1}^{n_{ES}} s_{2}^{p-1} P(n_{ES}, p-1, t|N) \quad (5)
\]

where \( n_{ES} \) and \( n = p - 1 \) are independent variables. The transformation yields an equation of motion for \( G(s_1, s_2, t|N) \), which is continuous in the variables \( s_1 \) and \( s_2 \):

\[
\frac{\partial G(s_1, s_2, t|N)}{\partial t} = k_3 N (s_1-1) G(s_1, s_2, t|N) + \left[ (1-s_1)(k_b + k_a s_1) - k_2(1-s_2) \right] \frac{\partial G(s_1, s_2, t|N)}{\partial s_1} \quad (6)
\]

The exact solution of the partial differential equation follows from the method of characteristic. The solution, presented in our earlier work [46], is given by

\[
G(s_1, s_2, t|N) = e^{-BNt} \left[ \cosh(A't) + \frac{k_3 - k_a(1-2s_1)}{2A'} \sinh(A't) \right]^N \quad (7)
\]

where \( B = \frac{(k_a+k_b)}{2}, A' = B\sqrt{1-\delta(1-s_2)} \) and \( \delta = \frac{4k_b k_2}{(k_a+k_b)^2} \).

The generating function \( G(s_1, s_2, t|N) \) and its moments describe the turnover kinetics in terms of the joint probability of the species numbers at time \( t \). The point process description complements the latter by incorporating molecular details of stochasticity in the substrate binding and unbinding times in the \( p \)-th cycle through the distributions of turnover times, \( w(T_p|N) \). The fundamental relation between the two, derived in our earlier works [46–48], is given by

\[
w(T_p|N) = e^{-BN_{ES}} \left[ \frac{\partial P(n_{ES}, n, t|N)}{\partial t} \right]_{t=T_p} \quad (8)
\]

Eq. (8) holds for any \( N \) and \( p \) and leads to the following exact expression for the turnover time distributions in terms of the generating function:

\[
w(T_p|N) = \frac{k_2}{(p-1)!} [0_{s_1} \partial_{s_1} G(s_1, s_2, T_p|N)]_{s_1=1, s_2=0} \quad (9)
\]

In this work, we use the count and point process description of stochastic Michaelis-Menten network, Eq. (5)-(9), to address two fundamental questions in enzyme kinetics: Firstly, what is the relationship between the experimental condition, \([E]_0 \ll [S]\) widely used in the initial rate method, and the theoretical condition \( k_2 \ll k_{-1} \) commonly used to analyze stationary (equilibrium) states in kinetic mechanisms? Secondly, what is the duration of the initial transient regime beyond which the enzymatic velocity attains its stationary value and shows hyperbolic substrate dependence?

The answers to these lie in the assumption made in the Michaelis-Menten (MM) work, \([E]_0 \ll [S]\), which is at odds with the deterministic framework used to study their kinetics. At low enzyme concentrations, the deterministic mechanistic is replaced by a single-enzyme Michaelis-Menten network, Fig. (1), and its replicas. This stochastic network admits a more subtle stationary and equilibrium states described by the joint probabilities, \( P(n_{ES}, p-1, T_p) \), of discrete chemical states \( n = (n_{ES}, p-1) \), evaluated at times \( t = T_p \) with \( n_{ES} = N - n_{ES} \).

In Section IV, we derive explicit expressions for the turnover number dependent joint probabilities. We then obtain a static condition on the rate parameters of the Michaelis-Menten network that ensures stationarity of the joint probabilities and leads to a statistical equilibrium between the average number of enzymes and complexes in the first turnover cycle. This condition is a stochastic analogue of the fast-equilibrium assumption that describe the concentration-based quasi-equilibrium in the Michaelis-Menten work.

In Section V, we obtain a dynamic condition on the rate parameters, which yields the duration of the transient regime and provides a stochastic generalization of the quasi-steady-state assumption in deterministic kinetics.

Section VI relates the static and dynamic conditions on the rate parameters with the steady-state velocity in the first and \( p \)-th turnovers. The static and dynamic rate parameter conditions are stochastic generalizations of the fast-equilibrium and steady-state assumption in deterministic kinetics that lead to hyperbolic initial and steady-state velocities, respectively.

In Section VII, we compare our theory with stochastic simulations and summarize the key findings in Section VIII.

**IV. STATIONARITY, STATISTICAL EQUILIBRIUM AND GENERALIZED FAST EQUILIBRIUM CONDITIONS**

We begin with the following axiom on the stationarity of the joint probabilities, \( P(n_{ES}, p, t = T_{p+1}) \), for successive enzyme turnovers:
where \( p = 1, 2, \ldots \).

For stationarity to be realized in the first turnover cycle \( p = 1 \), the joint probabilities of the first \( P(n_{ES}, 0, T_1) \) and second \( P(n_{ES}, 1, T_2) \) turnover cycles in Eq. (10) must be equal. The equality between the two naturally implies that joint probabilities of all successive turnovers are equal. In this notation, \( P(n_{ES}, p, T_{p+1}) \) is the joint probability of the stationary state, which is realized in the asymptotic limit of a large number of products \( p \to \infty \).

In this section, we deduce a condition on the rate parameters of the MM network that permits stationarity in the first turnover cycle. Since this condition only involves rate parameters and not time, we term it a static rate parameter condition (SRPC). This condition, if obeyed, guarantees stationarity and statistical equilibrium between enzymes and complexes in the first turnover cycle. While stationarity condition compares joint probabilities of successive turnover cycles \( p = 1, 2, \ldots \), Eq. (10), the condition of statistical equilibrium follows from the joint distribution of the \( p \)-th cycle. To highlight the difference, we introduce a new kinetic measure, \( R(T_p) \), which relates the SRPC with the condition of statistical equilibrium for the first turnover cycle \( p = 1 \). Non-compliance with the SRPC implies stationarity and statistical equilibrium are not attained in the first turnover. In such cases, a dynamic rate parameter condition is required, which we derive in the next section. We conclude this section with a brief survey of how the SRPC provides a stochastic generalization of the fast equilibrium assumption.

The joint probabilities in Eq. (10) can be derived from the moment-generating function, Eq. (5). The joint probabilities for \( p = 2, 3, \ldots \) turnovers yield unwieldy expressions as they involve partial derivatives with respect to \( s_2 \), similar to Eq. (9). We bypass their evaluation and focus on the joint probabilities of two cases of interest: the joint probability of the first turnover at \( t = T_1 \),

\[
P(m, 0, T_1) = \frac{1}{m!} \frac{\partial^m G(s_1, s_2, t)}{\partial s_1^m} \bigg|_{s_1 = s_2 = 0}
\]

and stationary state,

\[
\lim_{p \to \infty} P(m, p, T_{p+1}) = \frac{1}{m!} \frac{\partial^m G(s_1, s_2, t)}{\partial s_1^m} \bigg|_{s_1 = 0, s_2 = 1}
\]

where \( m \equiv n_{ES} \). Substituting the explicit expression for the generating function, Eq. (7), into Eqs. (11) and (12) yields the joint probability of the first turnover.

\[
P(m, 0, T_1) = \left[ \frac{N}{C_m(k_a)} \frac{k_a}{k_b} \right]^m \sinh^m (At) \left( \cosh(At) + \frac{k_a - k_b}{2k_a} \sinh(At) \right) ^{N-m} e^{-BNt} \bigg|_{t = T_1}
\]

and stationary state,

\[
\lim_{p \to \infty} P(m, p, T_{p+1}) = \frac{[N_C_m(k_a)]^m}{[k_b]} e^{-BNt} \bigg|_{t = T_{p+1}}
\]

where \( A = B\sqrt{1 - \delta} \), \( B = \frac{(k_a + k_b)}{k_a} \) and \( \gamma = \frac{k_a}{k_a + k_b} \).

Comparison of Eqs. (13) and (14) shows that \( P(m, 0, T_1) \) is equivalent to \( \lim_{p \to \infty} P(m, p, T_{p+1}) \) in the asymptotic limit of \( A^n \to B^n \) or \((1 - \delta)^{N/2} \to 1 \). To first order in \( \delta \), it implies \( N \delta \to 0 \) or \( N \delta \ll 1 \). From Eq. (10) and aforementioned analysis, it follows that the condition of stationarity for the first turnover \((p = 1)\) is given by

\[
\lim_{N \to 0} P(n_{ES}, 0, T_1) \equiv \lim_{p \to \infty} P(n_{ES}, p, T_{p+1}).
\]

It is to note that the asymptotic limit of \( N \delta \to 0 \) is the SRPC condition, pertaining to \( N \delta \ll 1 \). It provides a stochastic generalization of the fast equilibrium assumption in deterministic \((N \to \infty)\) kinetics. Below we analyze it for \( N \geq 1 \).

Single enzyme turnovers \((N = 1)\), always satisfy the rate parameter condition \( \delta \ll 1 \) required for stationarity, Eq. (15), \( \lim_{N \to 0} P(1, 0, T_1) = P(1, p, T_{p+1}) \) with \( p = 1, 2, \ldots \). To demonstrate this, we rewrite \( \delta \ll 1 \) as \((k_a + k_b)^2 - 4k_a k_b \gg 0 \). Substituting \( k_a = k_{-1} + k_{2} \) into the latter, we obtain \((k_a - k_{-2})^2 + k_{2}^2 + 2k_{-1}(k_a + k_{2}) \gg 0 \). The latter reveals that \( \delta \ll 1 \) is a positive definite, implying that single-enzyme turnovers are stationary for all \( p \). Another interesting limit emerges by replacing \( k_a \) with \( k_1[S] \) and \( k_a \) with \( K_M \). This permits us to rewrite the inequality as \((S) + K_M \gg 4N[S]k_2/k_{1} \), where \( N = 1 \). At the inflection point, when \( K_M = [S] \), the SRPC for \( N = 1 \) simplifies to \( k_2 \ll k_{1} \). This is a remarkable result as it reveals that for \( N = 1 \), the values of \( k_2 \) are almost unbound. It underpins the renewal characteristics of single-enzyme turnovers. We return to this point in the next section.

For \( N > 1 \), the SRPC corresponds to \( N \delta \ll 1 \) or \((k_a + k_b)^2 \gg 4Nk_a k_{2} \), which can be re-expressed as

\[
\left( 1 + \frac{K_M}{[S]} \right)^2 \gg \frac{N}{[S]} K_c
\]

where \( K_C = \frac{k_1}{k_1 + k_{-2}} \) is the Van Slyke-Cullen constant [6, 15, 16].

Does the compliance with the rate parameter condition for stationarity in the first turnover, Eq. (16), lead to statistical equilibrium between enzymes and complexes at \( t = T_1 \)? To verify this, we define the ratio of the average number of enzymes as complexes in the \( p \)-th turnover

\[
\frac{R(T_p)}{\langle n_{ES}(t) \rangle} \bigg|_{t = T_p}
\]
as the kinetic measure of statistical equilibrium. This ratio asymptotes to its equilibrium value $\overline{R}_p$ at long times,

$$\overline{R}_p \equiv \left[ \frac{\langle n_{E} \rangle_{eq}}{\langle n_{ES} \rangle_{eq}} \right]_p = \frac{k_b}{k_a}$$  \quad (18)

yielding the statistical equilibrium condition, $[k_a \langle n_{E} \rangle_{eq} = k_b \langle n_{ES} \rangle_{eq}]_p$, in the p-th turnover cycle.

Let us now analyze how Eqs. (10)-(18) are related to each other. Eq. (10) is the most general representation of the stationarity condition for p successive turnovers. Eq. (13) is the (non-stationary) joint probability of enzymes and complexes in the first turnover time $T_1$; Eq. (14) represents the (stationary) joint probability at $T_{p+1}$, attained asymptotically in the limit $p \to \infty$. The left-hand side of Eq. (15) signifies that stationarity can be realized in the first turnover provided the rate parameters of the MM network obey the SRPC, Eq. (16), corresponding to $N\delta \ll 1$. In the latter limit, $A \equiv B$ and the expression for the joint probability in Eq. (13) reduces to

$$P(m,0,T_1) = \left[ NC_m \left( \frac{p}{K} \right)^m \left( \frac{1}{2e} \right) \right]^m \left( 1 - e^{-2Bt} \right)^m \times \left( 1 + e^{-2Bt} + \frac{k_b - k_a}{k_a + k_b} \left( 1 - e^{-2Bt} \right) \right)^{N-m} \right]_{t=T_1}$$

Upon further simplification, it leads to the following expression,

$$P(n_{ES},0,T_1) = \left[ NC_{n_{ES}}(\gamma)^N \left( 1 - e^{-2Bt} \right)^{n_{ES}} \times \left( \frac{k_b}{k_a + e^{-2Bt}} \right)^{N-n_{ES}} \right]_{t=T_1}$$  \quad (19)

which is equivalent to Eq. (14), i.e., the right-hand side of Eq. (15) with $m \equiv n_{ES}$. Crucially, thus, the attainment of stationarity in the first turnover time depends on compliance with the SRPC, Eq. (16).

For $N > 1$, the first moments of Eq. (19) are given by

$$\langle n_{E}(t) \rangle_{t=T_1} = N\gamma \left( \frac{k_b}{k_a + e^{-2Bt}} \right)$$

and

$$\langle n_{ES}(t) \rangle_{t=T_1} = N\gamma \left( 1 - e^{-2Bt} \right).$$

Substituting them into Eq. (17) yields $R(T_1) = \left( \frac{k_a/\gamma_{E} + e^{-2Bt_1}}{1 - e^{-2Bt_1}} \right)$, which asymptotes to

$$\overline{R}_1 \equiv \left[ \frac{\langle n_{E} \rangle_{eq}}{\langle n_{ES} \rangle_{eq}} \right]_{p=1} = \frac{k_b}{k_a} \text{ at } T_1 \gg (k_a + k_b)^{-1},$$

recovering the statistical equilibrium condition $[k_a \langle n_{E} \rangle_{eq} = k_b \langle n_{ES} \rangle_{eq}]_{p=1}$ in the first turnover. For $N = 1$, the probability of enzyme and complex states are equal to their average values: $P_E(T_1) = P(0,0,T_1) = \langle n_{E}(t) \rangle_{t=T_1}$ and $P_{ES}(T_1) = P(1,0,T_1) = \langle n_{ES}(t) \rangle_{t=T_1}$. The statistical equilibrium condition for $N = 1$ is thus $[k_a \overline{P}_E = k_b \overline{P}_{ES}]$, which is valid for all turnovers. Here, $\overline{P}_E$ and $\overline{P}_{ES}$ are equilibrium probabilities.

Eq. (16) provides a lower bound on the magnitude of $K_e$ for a specific ratio of $N$ to $[S]$. At the inflection point, $[S] = K_M$, the above inequality can be analyzed in two alternative ways: Firstly, when $[S]$ replaces $K_M$, it reduces to $k_2 \ll \frac{k_b}{k_a}$ or $\frac{N}{S} \ll \frac{N}{S}$. While the former is the condition on the rate parameters of the kinetic mechanism, the latter is the experimental condition that assigns a lower limit to the ratio $N/[S]$. Secondly, when $K_M$ replaces $[S]$ then $k_2 \ll k_1$.

Eq. (16), in essence, is a stochastic generalization of the fast-equilibrium assumption in deterministic kinetics and applies to any $N$. In classical deterministic limit, $N \to \infty$, the condition $k_2 \ll \frac{k_a}{k_a}$ pertains to $k_2 \to 0$. It implies quasi-equilibrium between enzymes and complexes at the onset of the reaction. This limit was implicitly assumed in the work of Michaelis and Menten and forms the basis of the initial rate method. The condition $k_2 \ll k_1$, first appeared in the theoretical work of Briggs and Haldane [7], explains how the kinetic MM network can yield quasi-equilibrium between enzymes and complexes at initial times.

It is worthwhile to highlight the link between the conditions of stationarity, Eq. (15), and statistical equilibrium, Eq. (18), in the first turnover with the SRPC, Eq. (16). If the rate parameters of the MM network satisfy the SRPC, then the stationary state is realized in the first turnover and the corresponding statistical equilibrium equation for $p = 1$ is obtained. If the SRPC is violated, then stationary equilibrium state is not attained in the first turnover cycle. The next section describes how this state is attained dynamically.

V. RELAXATION TO STATISTICAL EQUILIBRIUM AND GENERALIZED STEADY-STATE CONDITION

Eq. (14) evaluates the joint probability of the stationary state in the asymptotic limit of $p \to \infty$. An enzymatic network, however, attains this limit in finite time, i.e., for times beyond an initial transient regime, $T_p \gg p^*$. This notation combines the count and point process description in the following manner: $T^* \equiv T_{p^*}$ is the critical turnover time that defines the duration of the transient regime, and $p^*$ is the corresponding critical turnover number beyond which the product numbers attain their asymptotic value. For times beyond $T^*$, the turnover kinetics relaxes from a non-stationary $t \ll T^*$ to a stationary equilibrium $t \gg T^*$ state. This is a count process description, which occurs in continuous time. Similarly, for turnovers beyond $p^*$, the turnover statistics changes from non-renewal $p \ll p^*$ to renewal $p \gg p^*$. This is the point process description in discrete turnover numbers [46–49]. The combination of the two, $T_p \gg p^*$, specifies the time beyond which the p-th turnover cycle attains stationarity. The stationarity condition, thus, reads as

$$P(n_{ES},p-1,T_p) = P(n_{ES},p,T_{p+1}). \quad (p \gg p^*). \quad (20)$$

Below, we derive an approximate expression for $p^*$ to show that the condition $p \gg p^*$ is the dynamic equiva-
lent of Eq. (16). For this, we obtain $T^*$ from the count process description and $\langle T_p^* \rangle$ from the point process description. The equality between the two yields $p^*$ for short transients. We discuss its relevance to the dynamic rate parameter condition (DRPC) and Eq. (20). We conclude this section with a brief description of how $p^*$ can be evaluated from stochastic simulations.

Eq. (3) quantifies the duration of the classical transient, $T^* = \frac{\lambda}{(k_a + k_b)}$, where $\lambda$ is a positive constant that remains undetermined in the mass action kinetics. To estimate $T^*$ and $\lambda$ in the classical limit, let us assume that the stationarity condition, Eq. (20), is satisfied beyond the first turnover cycle, $p \gg 1$. This assumption implies that the turnover kinetics in the first turnover cycle is non-stationary, governed by Eq. (13). The first moment of Eq. (13) yields the average number of complexes in the non-stationary state, $\langle \rho_{ES}(t) \rangle_{t=T_1} = \frac{N\lambda}{2\lambda}[e^{-(B-A)t} - e^{-(B+A)t}]_{t=T_1}$. The QSSA on this average number, $\frac{d \rho_{ES}(t)}{dt}|_{t=T^*} = 0$ yields the duration of the initial transient,

$$T^* = \frac{(1 - \delta)^{-1/2}}{(k_a + k_b)} \ln \left[ \frac{1 + (1 - \delta)^{1/2}}{1 - (1 - \delta)^{1/2}} \right].$$

Comparing Eqs. (3) and (21) yields an explicit expression for $\lambda$

$$\lambda = (1 - \delta)^{-1/2} \ln \left[ \frac{1 + (1 - \delta)^{1/2}}{1 - (1 - \delta)^{1/2}} \right],$$

where $\delta$ lies between zero and one, $0 < \delta < 1$. It, thus, violates the stationarity, Eqs. (15), and statistical equilibrium, (18), conditions in the first turnover.

The point process description determines the critical turnover number and the duration of the initial transient, statistically, in terms of the mean $p$-th turnover $\langle T_p^*(N) \rangle$ and waiting $\langle \tau_p(N) \rangle$ times [46–48]. The mean $p$-th turnover time is the sum of the waiting times between two consecutive turnovers $\langle T_p^*(N) \rangle = \sum_{k=1}^{p} \langle \tau_k^N \rangle$, where $\langle \tau_p^N \rangle = \langle T_p^*(N) \rangle - \langle T_p^*(N) \rangle$. While $\langle T_p^*(N) \rangle$ is evaluated from the first moment of the turnover time distributions $w(T_p|N)$, Eqs. (8) and (9), $\langle T_p^*(N) \rangle = \int_0^\infty dT_p T_p w(T_p|N)$, the waiting time distribution $w(\tau_p)$ and its mean $\langle \tau_p^N \rangle$ follow from stochastic simulations [55–58]. These temporal distributions show remarkably different statistics for $N = 1$ and $N > 1$.

For $N = 1$, the waiting time distributions are independent and identical $w(\tau_p^N) = w(\tau)$ for all $p$ [46–48]. This implies that the first turnover time distribution $w(\tau_1^N)$ is identical to $w(\tau)$. From Eqs. (8) and (9), the exact expression for $w(\tau)$ is obtained as $w(\tau) = \frac{k_b k_a}{2T_a}[e^{-(B-A)\tau} - e^{-(B+A)\tau}]$. The mean of $w(\tau)$ yields $\langle \tau \rangle = \frac{k_b k_a}{k_a + k_b}$, which is identical for $p = 1, 2, \cdots$. This feature characterizes the renewal turnover statistics for $N = 1$. It emerges from the absence of the waiting time correlations between consecutive turnovers for $N = 1$, $C_{1,q}^{(N)} = \delta \tau_1^N \delta \tau_1^{q(N)} = 0$, where $\delta \tau_1^N = \tau_{1}^{q(N)} - \tau_0^{q(N)}$ and $q = 1, 2, \cdots$. This leads to two interesting results: first, the mean $p$-th turnover time follows the renewal theorem $\langle T_p^{(N)} \rangle = \sum_{k=1}^{p} \langle \tau_k \rangle = p\langle \tau \rangle$; second, the inverse of $\langle \tau \rangle$ yields the single-enzyme velocity in the first turnover $v_1 = (\tau)^{-1} = \frac{k_b k_a}{k_a + k_b}$. The renewal turnover statistics implies that the interval between two consecutive turnovers is the same ($\tau$) for $p = 1, 2, \cdots$. It is worth noting that two important results of the previous section for $N = 1$, $\delta = 0$ and $k_2 \ll \frac{k_a}{N-1}$, are linked to the renewal nature of the single enzyme turnovers. All these results imply that single enzyme turnovers satisfy the stationarity condition, Eq. (15), at $p = 1$ and do not have a transient regime.

For $N > 1$, the waiting time distributions are non-identical $w(\tau_p^N) \neq w(\tau_p^{(N)})$ for turnovers below $p^*$, but become identical $w(\tau_p^{(N)}) = w(\tau_p^N)$ in the asymptotic limit of $p \gg p^*$ [46–48]. This effect emerges from the presence of non-stationary waiting time correlations $C_{1,q}^{(N)} \neq 0$ which persist for $q = 1, 2, \cdots p^*$ and vanish $C_{1,q}^{(N)} = 0$ beyond $p^*$. The critical turnover number, thus, demarcates the non-renewal regime ($p \ll p^*$) where the turnover statistics is non-stationary from the renewal regime ($p \gg p^*$) where the stationarity is obtained asymptotically. In the stationary state, the mean waiting times between successive products turnover $\langle \tau_{p \gg p^*} \rangle$ are identical $\langle \tau_{p \gg p^*} \rangle = \langle \tau \rangle/N$. In the non-renewal regime, these times are non-identical and depend on the turnover number.

In the point process description, the duration of the transient is defined by the mean critical turnover time $\langle T_p^*(N) \rangle = \sum_{k=1}^{p} \langle \tau_k^N \rangle$. In the classical limit, where the kinetics of all but the first turnover is non-stationary $\langle T_p^*(N) \rangle = \sum_{k=1}^{p} \langle \tau_k^N \rangle = (\tau_1^N) + (p^* - 1)\langle \tau \rangle / N$. For short transients, it is reasonable to assume $\langle \tau_1^N \rangle \approx \langle \tau \rangle / N$. This assumption of renewal statistics allows us to write $\langle T_p^*(N) \rangle \approx p^* \langle \tau \rangle / N$. The count process description of this duration is given by Eq. (21). Combining the results of count and point processes, $T^* \approx \langle T_p^*(N) \rangle \approx p^* \langle \tau \rangle / N$, yields an approximate analytical expression for $p^* \approx T^* N / (\langle \tau \rangle) \approx |\lambda| N \delta$, which simplifies to

$$p^* \approx |\lambda| \frac{N[S]K_c}{(S + K_M)^2}. \quad (23)$$

As stated above, $p \gg p^*$ is the dynamic rate parameter condition (DRPC) that marks the critical turnover beyond which the stationarity condition, Eq. (20), is obeyed. From Eq. (23), it follows that the DRPC is given by

$$p \gg |\lambda| \frac{N[S]K_c}{(S + K_M)^2} \quad (24)$$
The DRPC subsumes the SRPC, Eq. (16), and provides a stochastic generalization of the QSSA. The rate parameters that satisfy Eq. (16) yield \( p^* \approx 0 \), implying that the stationary, Eq. (15), and statistical equilibrium, Eq. (18), conditions are obeyed in the first turnover cycle. The rate parameters that disobey Eq. (16) yield \( p \gg p^* \), thus, the stationarity and statistical equilibrium conditions, Eq. (20) and Eq. (18), are realized, dynamically, in the \( p \)-th turnover cycle.

The theoretical estimate for critical turnover number, Eq. (23), assumes renewal turnover statistics and thus provides a lower bound on \( p^* \). The latter is valid for short transients, \( i.e.\), for rate parameters that weakly violate the SRPC. For long transients, the product turnovers follow non-renewal statistics characterized by non-stationary waiting time correlations, \( C_{1q}^{(N)} = \langle \delta t_{1}^{(N)} \delta t_{i+1}^{(N)} \rangle \). Thus, the critical turnover number beyond which \( C_{1p^*} = 0 \) provides a numerical estimate for \( p_{\text{num}}^* \). We obtain the latter from stochastic simulations and present the results in Section VII.

### VI. KINETIC MEASURE OF HYPERBOLICITY

Eqs. (16) and (24) provide the static and dynamic rate parameter conditions for stationarity and statistical equilibrium in the first and \( p \)-th turnover cycles, abbreviated as the SRPC and DRPC, respectively. To link these conditions with the hyperbolic MME, we introduce a dimensionless kinetic measure - the turnover number dependent fractional enzymatic velocity (FEV),

\[
\Theta_{p}^{(N)} = \frac{V_{p}^{(N)}}{V_{ss}^{(N)}}
\]

where \( V_{p}^{(N)} \) is the point process description of the enzymatic velocity in the \( p \)-th turnover cycle and \( V_{ss}^{(N)} = \frac{k_{2}N[S]}{[S] + K_{M}} \) is the classical steady-state velocity. The FEV, Eq. (25), as a ratio of the two velocities, provides a single parameter to quantify the deviation of \( V_{p} \) from hyperbolic velocity \( V_{ss} \) for \( p = 1, 2, \ldots \). In this form, it seamlessly combines two crucial features of the discrete turnover kinetics: first, that the \( V_{p}^{(N)} \) is non-hyperbolic in the non-renewal (or non-stationary) transient regime with \( V_{p \gg p^*} \neq V_{ss}^{(N)} \), implying \( \Theta_{p \gg p^*} \neq 1 \); second, that \( V_{p} \) attains stationarity in the renewal steady-state, \( i.e.\), \( V_{p \gg p^*} = V_{ss}^{(N)} \), implying \( \Theta_{p \gg p^*} = 1 \).

Both these features are included in the \( p \)-th enzymatic velocity, whose functional form \( V_{p}^{(N)} = p(T_{p}^{(N)})^{-1} \) incorporates the renewal turnover statistics, \( \langle T_{p}^{(N)} \rangle = p(\tau)/N \), in the steady state [46–49]. The renewal statistics defines the single-enzyme velocity \( v_{1} \) as a function of \( N \), implying \( \Theta_{1 \gg p^*} = 1 \) for all \( p \). As stated earlier, for \( N = 1 \), the renewal turnover statistics, \( \langle T_{p}^{(1)} \rangle = p(\tau) \), yields the single-enzyme velocity \( v_{1} = \langle \tau \rangle^{-1} = \frac{1}{k_{2} \frac{[S]}{[S] + K_{M}}} \). This is the single-enzyme analogue of the classical MME, \( v_{1} = \frac{V_{ss}^{(N)}}{N} = \frac{k_{2}N[S]}{[S] + K_{M}} \), and represents the hyperbolic relation between \( v_{1} \) and \( [S] \) in the first turnover. For \( N > 1 \), the turnover statistics is non-renewal \( \langle T_{p}^{(n \gg p^*)} \rangle \neq p(\tau)/N \) in the transient state and renewal \( \langle T_{p}^{(N)} \rangle \neq p(\tau)/N \) in the steady-state. This leads to deviation from the MME in the transient state, \( V_{p \gg p^*} \neq V_{ss}^{(N)} \), and its asymptotic recovery in the steady-state, \( V_{p \gg p^*} = p(T_{p}^{(N)})^{-1} = N(\tau)^{-1} = V_{ss}^{(N)} \).

In essence, the \( p \)-dependent FEV, Eq. (25), includes waiting time correlations between successive turnovers, and quantifies the degree of non-hyperbolicity, \( i.e.\), deviation from the MME, in the transient regime. The increase in \( p \) brings about a crossover from \( \Theta_{p}^{(N)} \neq 1 \) in the non-stationary state to \( \Theta_{p}^{(N, \gg p^*)} = 1 \) in the stationary state. The asymptotic recovery of the MME, \( \Theta_{p \gg p^*} = 1 \), in the stationary state is linked to the DRPC. Below, we obtain an exact expression for the substrate dependence of \( \Theta_{1}^{(2)} \). We deduce rate parameter conditions that yield \( \Theta_{1}^{(2)} \neq 1 \) and show how the limit \( \Theta_{1}^{(2)} \rightarrow 1 \) is linked to the SRPC. We generalize these results in the next section.

For \( N = 2 \) and \( p = 1 \), the FEV is given by \( \Theta_{1}^{(2)} = \frac{V_{1}^{(2)}}{V_{ss}} \), where \( V_{1} = (T_{1}^{(2)})^{-1} \). The first moment of \( \mu_{1} = (T_{1}^{(2)})^{-1} \) and the FEV as

\[
\Theta_{1}^{(2)} = \frac{\langle [S] + K_{M} \rangle}{K_{C}[S] + ([S] + K_{M})^{2}}.
\]

To understand the link between Eq. (26) and the SRPC, we consider the variation of \( \Theta_{1}^{(2)} \) as a function of \( [S] \) for three limiting cases, summarized in Table I.

At low \( K_{C} \), the rate parameter conditions for low \( [S] \) case
distributions yield the statistical averages of ES and E as:

\[ P(n_{ES}, p^{-1}, t = T_p) \]

Stationarity, Eq. (15), leading to the equivalence between distributions at \( p = 1 \) and \( p = 100 \). The first moments of these distributions yield the statistical averages of ES and E as \( \langle n_{ES} \rangle_{eq} = 25 \) and \( \langle n_E \rangle_{eq} = N - \langle n_{ES} \rangle_{eq} = 15 \).

\[ P(n_i, p^{-1}, t = T_p) \]

Figure 2. Joint probabilities of the number of enzymes (\( n_E \)), complexes (\( n_{ES} \)) and products (\( n = p - 1 \)) at time \( t = T_p \), \( P(n_i, p - 1, T_p) \), with \( i = \{ E, ES \} \). These distributions are obtained from stochastic simulations for the rate parameter values, \([S] = 2.5\), \( K_M = 1.5\), \( N = 40\), \( K_c = 0.01\). These parameters obey the SRPC, Eq. (16), and hence satisfy the condition of stationarity, Eq. (15), leading to the equivalence between distributions at \( p = 1 \) and \( p = 100 \). The first moments of these distributions yield the statistical averages of ES and E as \( \langle n_{ES} \rangle_{eq} = 23.3 \) and \( \langle n_E \rangle_{p=1} = 16.3 \). These estimates are close to their equilibrium values \( \langle n_{ES} \rangle_{eq} = \frac{NS}{M + K_M} = 25 \) and \( \langle n_E \rangle_{eq} = N - \langle n_{ES} \rangle_{eq} = 15 \).

Figure 3. Joint probability distributions, \( P(n_i, p - 1, t = T_p) \), for parameter values \([S] = 2.5\), \( K_M = 1.5\), \( N = 40\), \( K_c = 1\). These rate parameter values violate the SRPC, Eq. (16), and hence do not satisfy the stationarity condition, Eq. (15), at \( p = 1 \). For these rate parameters, the equilibrium averages are \( \langle n_E \rangle_{eq} = 15 \) and \( \langle n_{ES} \rangle_{eq} = 25 \); and the critical turnover number, obtained from Eq. (23), is \( p_{\text{thres}} = 15 \). The first moment of the distributions at \( t = T_1 \) yields the average number of E and ES \( \langle n_E \rangle_{p=1} = 30 \neq \langle n_E \rangle_{eq} \) and \( \langle n_{ES} \rangle_{p=1} = 10 \neq \langle n_{ES} \rangle_{eq} \), which deviate from the stationary (equilibrium) values. For \( p = 100 \) \( (p \gg p^*) \), the first moments of the joint distributions yield number averages \( \langle n_E \rangle_{p=100} = 15.6 \) and \( \langle n_{ES} \rangle_{p=100} = 24.4 \), which are close to their equilibrium estimates.

\[ P(n_i, p - 1, t = T_p) \]

Ia], intermediate [case IIa], and high [case IIIa] substrate concentrations suggest \( \Theta_1^{(2)} \approx 1 \) for all \([S]\). The latter implies that, at low \( K_c \), the hyperbolic substrate dependence of the MME is the result of statistical equilibrium, between \( N \) independent and identical E and ES states, in the first catalytic turnover cycle. At high \( K_c \), in contrast, the rate parameter conditions for low [case Ib], intermediate [case IIIb], and high [case IIIb] substrate concentrations yield \( \Theta_1^{(2)} < 1 \). For \([S] \ll K_M\), \( \Theta_1^{(2)} \) decreases with increasing \([S]\); reaches a minimum at \([S] \approx K_M\); for \([S] \gg K_M\), \( \Theta_1^{(2)} \) increases with increasing \([S]\) and asymptotically attains \( \Theta_1^{(2)} \approx 1 \) when \( K_c \ll [S] \) [case (3a)].

The above analysis shows the SRPC, if obeyed, yields \( \Theta_1^{(2)} = 1 \). If violated yields \( 0 < \Theta_1^{(2)} < 1 \). In the next section, we generalize these results to show that the presence of the waiting time correlations between first and successive turnovers, \( C_{ij}^{(N)} \neq 0 \), underlies the non-hyperbolic substrate response, \( \Theta_1^{(N)} \neq 1 \), in the transient regime. In the stationary state, vanishing of correlations, \( C_{ij}^{(N)} = 0 \) leads to hyperbolic substrate dependence of the MME, \( \Theta_1^{(N)} = 1 \).

VII. COMPARISON OF THEORY WITH COMPUTER EXPERIMENT

Equations (10), (16), (18), (24), and (25) are the key findings of our theory and provide stochastic generalizations of the stationarity condition, the fast-equilibrium
Figure 4. Variation of \( \Theta_1 \) as a function of substrate concentration \([S]\) (a) for a range of \( N \) at \( K_M = 2.5, K_c = 1 \); (b) for a range of \( K_c \) at \( K_M = 20 \) and \( N = 12 \). The relation between the substrate variation of the waiting time correlations between first and second turnovers, \( C_{12} \), and scaled FEV, \( (\Theta_1 - 1) \), for the rate parameters of (b) is captured in (c). The U-shaped curves represent non-hyperbolic substrate dependence of the enzymatic velocity, \( \Theta_1 < 1 \), in the non-classical transient regime. The recovery of the hyperbolic MME in the steady state is signaled by \( \Theta = 1 \) for (a) and (b) and \( C_{12} = (\Theta_1 - 1) = 0 \) for (c).

Figure 5. Contour plots of \( \Theta_1 \), with \( K_M \) and \([S]\) as independent parameters, for a range of \( N \) at \( K_c = 10 \) (top panel) and a range of \( K_c \) at \( N = 10 \) (bottom panel). From left to right, the non-classical transient region, represented by the iso-values \( \Theta_1 < 1 \), reduces in size with the decrease in \( N \) and \( K_c \). On the right, the approach to the steady state is indicated by the iso-values close to one, \( \Theta_1 \approx 1 \).

assumption, the quasi-equilibrium condition, the quasi-steady-state assumption, and the enzyme velocity, respectively. Below, we combine the results of Sections IV-VI to establish that compliance with the SRPC, Eq. (16), yields stationarity, statistical equilibrium and the MME in the first turnover. Non-compliance implies that these conditions and the MME are recovered dynamically for \( p \gg p^* \), i.e., the DRPC, Eq. (24).

We begin our analysis by establishing the link between the SRPC, and the DRPC with the stationarity Eq. (10), and statistical equilibrium, Eq. (18), conditions. For this, we first obtain the joint probability distributions \( P(n_E, p, t = T_p) \) and \( P(n_{ES}, p, t = T_p) \) from the Doob-Gillespie stochastic simulation algorithm [55–57]. We generate \( 10^6 \) stochastic trajectories and extract the joint distributions of the number of enzymes \( n_E \) and complexes \( n_{ES} \) for \( n = p - 1 \) products at time \( t = T_p \) [58]. The simulation results are shown in Figs. (2) and (3), which we discuss below.

Fig. (2) considers the rate parameter values (lower \( K_C \)) that obey the SRPC in the first turnover cycle \( (p = 1) \). The compliance with the SRPC leads to the equivalence between the joint distributions for \( p = 1 \) and \( p = 100 \) turnovers, \( P(n_i, 0, T_1) \equiv P(n_i, p, T_{p+1}), \) for \( p \gg p^* \), where \( i = \{E, ES\} \). The number averages of \( P(n_E, 0, T_1) \) and \( P(n_{ES}, 0, T_1) \) yield \( \langle n_E \rangle_{p=1} \) and \( \langle n_{ES} \rangle_{p=1} \). These av-
erages satisfy the statistical equilibrium condition in the first turnover cycle, \( [k_a \langle n_E \rangle_{eq} = k_b \langle n_{ES} \rangle_{eq}]_{p=1} \).

Fig. (3), in contrast, considers the rate parameter values (higher \( K_c \)) that do not obey the SRPC. As a result, the conditions of stationarity \( P(n_i, 0, T_1) \neq P(n_i, p, T_{p+1}) \) and statistical equilibrium \( [k_a \langle n_E \rangle_{seq} \neq k_b \langle n_{ES} \rangle_{seq}]_{p=1} \) are not obeyed in the first turnover cycle. The DRPC yields \( p_{\text{theor}} = 15 \). For \( p = 100 \), thus, the number averages of the stationary distribution \( (p \gg p^*) \), \( P(n_i, p, T_{p+1}) \), yield \( \langle n_E \rangle_{p \gg p^*} \), and \( \langle n_{ES} \rangle_{p \gg p^*} \). These averages satisfy the statistical equilibrium condition, \( [k_a \langle n_E \rangle_{eq} = k_b \langle n_{ES} \rangle_{eq}]_{p \gg p^*} \) for \( p \gg p^* \).

A quantitative link between the SRPC and the substrate variation of the first-FEV, \( \Theta_1^{(2)} \), is established in Table I for three limiting cases: \( [S] \ll K_M ; [S] \approx K_M ; [S] \gg K_M \). Together they suggested that at high \( K_c \), the violation of the SRPC indicates non-hyperbolic enzymatic velocity in the transient regime. For the latter, the substrate variation of \( \Theta_1^{(N)} \) yields a U-shaped curve, confined between 0 and 1, with a minimum given by case IIb.

Figs. (4a) and (4b) show the substrate dependence of \( \Theta_1^{(N)} \), computed from Eq. (25), for a range of \( N \) and \( K_c \), in Mathematica. For convenience, all the rate parameters, including \( K_M \), \( K_c \), and \( [S] \) are dimensionless. For large \( N \) and \( K_c \), the SRPC is violated, leading to U-shaped curves for the substrate variation of \( \Theta_1^{(N)} \) (Fig (4a)). With the decrease in the value of \( K_c \), when the SRPC is obeyed, \( \Theta_1^{(N)} = 1 \) for all \( [S] \) (the red curve in Fig. (4b)). This limit recovers the hyperbolic enzymatic velocity \( V_1 \), the MME, in the first turnover. The results captured in Figs. (4a) and (4b) are consistent with the analytical analysis for \( N = 2 \), summarized in Table I.

Fig. (5) represents the contour plots of \( \Theta_1^{(N)} \), with \( K_M \) and \( [S] \) as independent variables, for a range of \( N \) at fixed \( K_c \) (top panel), and a range of \( K_c \) at fixed \( N \) (bottom panel). The iso-values lower than one, \( \Theta_1 < 1 \), correspond to the non-stationary transient regime (blue and green colors), and \( \Theta_1 \approx 1 \) (red color) correspond to the steady-state regime. The top panel shows that the first turnover is in the transient regime for larger \( N \) and close to the steady-state for smaller \( N \). The bottom panel shows that the first catalytic turnover is in the transient regime for higher \( K_c \) and close to the steady-state at lower \( K_c \). Both these are in agreement with the predictions of the SRPC, Eq. (16).

To trace the molecular origins of the U-shaped response curve, we plot the substrate variation of the waiting time correlations between first and second turnovers, \( C_{12}^{(N)} \), and scaled first-FEV, \( (\Theta_1^{(N)} - 1) \), Fig. (4c). The substrate dependence of \( C_{12}^{(N)} \) is negative and shows a non-monotonic response to increase in \( [S] \) (blue stars). This is similar to the U-shaped substrate variation of \( (\Theta_1^{(N)} - 1) \) at high \( K_c \) (blue curve). This implies that at high \( K_c \), when the SRPC is violated, the first turnover is in the (non-renewal) transient regime governed by the waiting time correlations, \( i.e., C_{12}^{(N)} < 0 \implies \Theta_1^{(N)} < 1 \). At low \( K_c \), both \( C_{12}^{(N)} \) and \( (\Theta_1^{(N)} - 1) \) are zero (red stars and curve). This implies that at low \( K_c \), when the SRPC is obeyed, the first turnover is in the (renewal) steady-state regime, \( i.e., C_{12}^{(N)} = 0 \implies \Theta_1^{(N)} = 1 \). The inflection point for both \( C_{12}^{(N)} \) and \( \Theta_1^{(N)} \) is attained at \( [S] \approx K_M \).

The above analysis quantifies the link between the SRPC and the FEV for \( p = 1 \). In particular, it illustrates that the SRPC, if violated, yields \( \Theta_1^{(N)} < 1 \), and if obeyed, leads to \( \Theta_1^{(N)} = 1 \). In the former case, the DRPC estimates the critical turnover number \( p^* \) beyond which the statistical equilibrium between E and ES is established and hyperbolicity is recovered. Eq. (23) provides...
stationarity condition

\[ \Theta \]

\[ \Theta \]

\[ P(n_i, p-1, T_p) = P(n_i, T_{p+1}), p = 1, 2, \ldots \]

\[ k_\alpha (n_E)_{eq} = k_b (n_{ES})_{eq} |_{p=1} \]

\[ \Theta_1^{(N)} = 1 \]

\[ P(n_i, p-1, T_p) \neq P(n_i, T_{p+1}), p = 1, 2, \ldots \]

\[ k_\alpha (n_E)_{eq} \neq k_b (n_{ES})_{eq} |_{p=1} \]

\[ \Theta_1^{(N)} < 1 \]

\[ P(n_i, p-1, T_p) = P(n_i, T_{p+1}), p \gg p^* \]

\[ k_\alpha (n_E)_{eq} = k_b (n_{ES})_{eq} |_{p \gg p^*} \]

\[ \Theta_p^{(N)} = 1 \]

Table II. Quantitative link between the static and dynamic rate parameter conditions, Eqs. (16) and (24), with the stationarity condition, Eq. (10), the statistical equilibrium condition, Eq. (18), and the \( p \)-th dependent fractional enzyme velocity (FEV), \( \Theta_p^{(N)} \), Eq. (25).

Table: Quantitative link between the static and dynamic rate parameter conditions, Eqs. (16) and (24), with the stationarity condition, Eq. (10), the statistical equilibrium condition, Eq. (18), and the \( p \)-th dependent fractional enzyme velocity (FEV), \( \Theta_p^{(N)} \), Eq. (25).

| Rate parameter condition | Stationarity condition | Statistical Equilibrium Condition | FEV |
|--------------------------|------------------------|-----------------------------------|-----|
| SRPC obeyed              | \( P(n_i, p-1, T_p) = P(n_i, T_{p+1}), p = 1, 2, \ldots \) | \( k_\alpha (n_E)_{eq} = k_b (n_{ES})_{eq} |_{p=1} \) | \( \Theta_1^{(N)} = 1 \) |
| SRPC violated            | \( P(n_i, p-1, T_p) \neq P(n_i, T_{p+1}), p = 1, 2, \ldots \) | \( k_\alpha (n_E)_{eq} \neq k_b (n_{ES})_{eq} |_{p=1} \) | \( \Theta_1^{(N)} < 1 \) |
| DRPC obeyed              | \( P(n_i, p-1, T_p) = P(n_i, T_{p+1}), p \gg p^* \) | \( k_\alpha (n_E)_{eq} = k_b (n_{ES})_{eq} |_{p \gg p^*} \) | \( \Theta_p^{(N)} = 1 \) |

an analytical expression for the critical turnover number \( \rho_{\text{theor}}^* \), which is only valid for short transients. To check the validity of the assumption made in deriving Eq. (23), we compare the theoretical and numerical estimates of \( p^* \). Fig. (6a) shows the variation of waiting time correlations \( C_{1p}^{(N)} \) with turnover lag \( p = 1, 2, \ldots \) obtained from stochastic simulations. The critical turnover number beyond which \( C_{1p}^{(N)} = 0 \) provides a numerical estimate for \( p_{\text{num}}^* \). For short transients (low \( K_c \)), the numerical estimate is very close to the theoretical estimate.

To quantify the link between the DRPC and the \( p \)-th dependent FEV, we plot in Fig. (6b) the variation of \( \Theta_p^{(N)} \) as a function of \( p \) for the same parameter as Fig. (6a). The variation of \( C_{1p}^{(N)} \) versus \( p \) is qualitatively similar to \( \Theta_p^{(N)} \) versus \( p \). In particular, for \( p \ll p^* \), the turnover kinetics in transient regime, where negative waiting time correlations \( C_{1p}^{(N)} < 0 \) yield non-hyperbolic response curves \( \Theta_p^{(N)} < 1 \), i.e., \( C_{1p}^{(N)} < 0 \Rightarrow \Theta_p^{(N)} < 1 \). For \( p \gg p^* \), \( C_{1p}^{(N)} = 0 \) yields the hyperbolic MME, \( \Theta_p^{(N)} = 1 \). Table (II) summarizes the link between the conditions of statistical equilibrium and stationarity with the SRPC, DRPC and the fractional enzymatic velocity. These results for mesoscopic enzyme kinetics are valid for any number of enzymes \( (N) \) and turnover number \( (p) \).

VIII. SUMMARY AND CONCLUSION

In this work, we present a novel statistical route to quantify, at the molecular level, the number and temporal fluctuations in the stochastic Michaelis-Menten network of \( N \) enzymes as they form products in discrete turnover times \( T_p \). Our work begins by introducing a condition of stationarity, which demands equality between the joint probabilities of discrete species numbers in the \( p \)-th and \((p+1)\)-th cycles with \( p = 1, 2, \ldots \). We examine the statistical properties of the joint probability through the count and point process descriptions to show that the stationarity condition can be satisfied, statically or dynamically, in the first or the \( p \)-th turnover cycle.

We use the count process description to deduce the static condition on the rate parameters of the network that yield statistical equilibrium in the first turnover. We use the combination of the count and point process descriptions to show that non-compliance with the static rate parameter condition is a transient effect originating from the non-stationary, or equivalently, the non-renewal turnover kinetics. We derive the critical turnover number and time above which the turnover statistics have a renewal character. This feature yields the dynamic rate parameter condition that ensures the simultaneous realization of the stationarity and statistical equilibrium conditions for \( p \gg p^* \) turnovers. It also provides an analytical estimate of the duration of the short transients.

We use the count process description to prove that single enzyme turnovers in the stochastic Michaelis-Menten network, irrespective of the magnitude of \( k_2 \), always obey the static and dynamic rate parameter conditions. This feature corresponds to the renewal turnover statistics in the point process description and implies that single enzyme turnovers do not admit a transient regime [48]. For a mesoscopic number of enzymes, the static and dynamic rate parameter conditions provide the stochastic generalization of the fast-equilibrium and steady-state assumptions.

In conclusion, our work traces the molecular origins of the widely used fast-equilibrium and steady-state assumptions in enzyme kinetics through the count and point process descriptions of turnover probability. It combines the conditions of stationarity and statistical equilibrium with hyperbolic reaction rate, providing stochastic generalizations of the initial and steady-state velocity. The generalized theoretical framework proposed in this study encompasses the classical (deterministic) and single-enzyme (stochastic) Michaelis-Menten kinetics. The dynamic rate parameter conditions, derived here, offer a novel way to quantify the duration of the transient regime in mesoscopic Michaelis-Menten kinetics through the statistical analysis of number and temporal fluctuations.

ACKNOWLEDGMENTS

MP acknowledges the financial support from the Council of Scientific and Industrial Research (CSIR), Government of India.
