Generic Schemes for Single-Molecule Kinetics. 2: Information Content of the Poisson Indicator

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2: Information Content of the Poisson Indicator

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Abstract

Recently, we described a pathway analysis technique (paper 1) for analyzing generic schemes for single-molecule kinetics based upon the first-passage time distribution. Here, we employ this method to derive expressions for the Poisson indicator, a measure of stochastic variation (essentially equivalent to the Fano factor and Mandel’s Q parameter), for various renewal (memoryless) enzymatic reactions. We examine its dependence on substrate concentration, without assuming all steps follow Poissonian kinetics. Based upon fitting to the functional forms of the first two waiting time moments, we show that, to second order, the non-Poissonian kinetics are generally underdetermined but can be specified in certain scenarios. For an enzymatic reaction with an arbitrary intermediate topology, we identify a generic minimum of the Poisson indicator as a function of substrate concentration, which can be used to tune substrate concentration to the stochastic fluctuations and estimate the largest number of underlying consecutive links in a turnover cycle. We identify a local maximum of the Poisson indicator (with respect to substrate concentration) for a renewal process as a signature of competitive binding, either between a substrate and an inhibitor or between multiple substrates. Our analysis explores the rich connections between Poisson indicator measurements and microscopic kinetic mechanisms.

I. Introduction

Single-molecule spectroscopy techniques have allowed the study of single biomolecular complexes at a level of detail previously unattainable. Escaping the averaging of measured quantities inherent in ensemble measurements, single-molecule studies offer insights into the details of the dynamic behavior of biomolecules. In particular, these studies provide information on the underlying kinetic scheme that is unavailable through traditional, bulk measurements of chemical kinetics. At their core, single-molecule studies of enzymes and motor proteins interrogate the waiting time between reaction events, such as the conversion
of substrate to product or the stepping of a motor protein along a filament. The waiting time varies stochastically over the course of the observation of the molecule, and sufficiently long time traces allow the waiting time probability distribution to be described. Given a kinetic mechanism, a mathematical expression for this waiting time distribution in terms of kinetic parameters, such as rate constants and reactant or product concentrations, may be derived. Furthermore, expressions for the moments of the distribution and the correlations between events may be obtained and compared to experimental observations.

From a theoretical standpoint, it is important to first determine the information content available from single-molecule data and then make connections to a generic scheme. Previous work has addressed the relation of single-molecule data to reaction network connectivity and developed a mathematical framework for treating data within a given reaction scheme. In a complementary fashion, we have described a pathway analysis approach to generic reaction schemes for single-molecule kinetics (paper 1). In contrast to other approaches, pathway analysis may be easily adapted to arbitrary reaction scheme topologies. This method provides a straightforward prescription for decomposing a proposed scheme via two basic kinetic motifs, sequential and branching. Secondly, our approach requires no assumption of Poissonian kinetics (i.e., rate processes), allowing each step to be treated with the greatest possible generality. As in paper 1, the current study deals with renewal (memoryless) processes and, as a result, does not capture memory effects in the action of single enzymes, as described previously experimentally and theoretically. A subsequent paper will generalize our method to arbitrary nonrenewal processes (paper 3).

This previous work provided calculation of the first waiting time moment (i.e., mean first-passage time) for generalized enzymatic schemes, which is directly related to the turnover rate for the process. The turnover rate and mean first-passage time can be determined from ensemble-averaging; however, higher-order moments, which contain information on the underlying kinetic scheme of the enzymatic reaction, are unique to single-molecule measurements. In particular, the Poisson indicator, a normalized measure of stochastic fluctua-
captures deviation from Poissonian statistics, taking on a positive value for bunching behavior, a negative value for anti-bunching behavior, and vanishing for a Poisson process. The dependence of the Poisson indicator on substrate concentration can then inform which steps adhere to or violate Poissonian statistics. Moreover, the Poisson indicator is essentially equivalent to other normalized measures of the variance, including Mandel’s Q parameter from photon statistics, the randomness parameter from studies of molecular motors, and the Fano factor.

This paper is organized as follows: in section II, we extend the previously introduced pathway analysis to the calculation of the second moment of the waiting time distribution. We examine a generic model of enzymatic reactions that can generate all possible kinetic models with the same basic topological connectivity and contains no assumptions upon the form of the kinetic scheme. As stated earlier, the only constraint is that the overall reaction be a renewal process. In section III, we employ this approach for the generic enzymatic reaction to evaluate the maximal information content of measurements of the second waiting time moment and, in particular, to examine the dependence of the second moment on substrate concentration. Our results include functional forms for the dependence of both the first (related to the turnover rate) and second (related to the Poisson indicator) reaction waiting time moments on substrate concentration, as well as explicit expressions in terms of the waiting time moments for individual steps. We analyze these functional forms and explore their connections to important experimental limits. In section IV, we extend earlier, similar results to the more complex cases of competitive inhibition and competition between multiple substrates. To our knowledge, these are the first calculations of higher-order waiting time moments for these more complex cases without assuming all steps follow Poissonian kinetics, and the resulting expressions for the Poisson indicator differ qualitatively from earlier results. In section V, we conclude.
II. Self-consistent Pathway Analysis

Let $\phi(t)$ represent the waiting time distribution, which describes the distribution of times between successive reaction events. The moments of the waiting time distribution are given by $\langle t^n \rangle = \int_0^\infty t^n \phi(t) \, dt = (-1)^n \left. \frac{d^n \hat{\phi}(s)}{ds^n} \right|_{s=0}$, where $\hat{\phi}(s)$ denotes the Laplace transform of $\phi(t)$, defined as $\hat{\phi}(s) = \int_0^\infty e^{-st} \phi(t) \, dt$. Our challenge is then to formulate the waiting time distribution for a generic enzymatic reaction. The model we treat is illustrated in Figure 1.

Here, states 1 and 2 are connected by a reversible step, with an arbitrary topology after state 2, before a final, irreversible transition (or set of transitions) to product P. Upon the creation of a product molecule, we assume that the enzyme regenerates quickly and irreversibly to state 1 (the initial free enzyme state), where it begins another turnover. In our model, enzyme turnover is a renewal process because it always begins in the same state. In keeping with the Michaelis-Menten model of enzymatic reactions, the first step corresponds to substrate binding to the enzyme, which we assume to have a single substrate-binding site, making this the only step with dependence on substrate concentration. There may exist many intermediate underlying states between the substrate binding step and final transition(s) to product. We refer to this (possible) aggregate of states as the bound/intermediate state B, which may undergo non-Poissonian decay due to its (possible) internal dynamics, some of which may involve branching out of the chain as well as cyclic loops.

Now, if we let $Q_{ij}(s)$ denote the waiting time distribution for the $i$-to-$j$ transition in the
Laplace domain, we can write the overall waiting time (i.e., first-passage time) distribution in the Laplace domain as

\[ \hat{\phi}(s) = \frac{Q_{1P}(s)}{1 - \hat{Q}_{11}(s)} = Q_{1P}(s) \left[ 1 + \hat{Q}_{11}(s) + \hat{Q}_{11}(s)^2 + \hat{Q}_{11}(s)^3 + \ldots \right] \]  

(1)

where \( Q_{1P}(s) \) is the waiting time distribution for the passage from state 1 to the product, and \( \hat{Q}_{11}(s) \) represents the waiting time distribution for the passage out of and back to state 1. Each term in the infinite summation can be understood as follows: the first term corresponds to the passage from state 1 to product P without returning to state 1, the second term corresponds to the passage from state 1 to product P while returning to state 1 exactly once, the third term corresponds to the passage while returning to state 1 exactly twice, and so on and so forth. Examining only the initial free enzyme state and the bound/intermediate state, we can write

\[ Q_{1P}(s) = Q_{1B}(s)Q_{BP}(s) \]  

(2)

\[ \hat{Q}_{11}(s) = Q_{1B}(s)Q_{B1}(s) \]  

(3)

where \( Q_{1B}(s) \) is the waiting time distribution for substrate binding, \( Q_{B1}(s) \) is the waiting time distribution for substrate unbinding, and \( Q_{BP}(s) \) is the waiting time distribution for product formation (i.e., the conversion of substrate to product after binding). Now, the overall waiting time distribution is given by

\[ \hat{\phi}(s) = \frac{Q_{1B}(s)Q_{BP}(s)}{1 - Q_{1B}(s)Q_{B1}(s)} \]  

(4)

This scheme comprises a generic model for enzyme kinetics. It treats explicitly the substrate binding step with waiting time distribution \( Q_{1B}(s) \), while treating in generality the decay of the bound/intermediate state with the distributions \( Q_{B1}(s) \) and \( Q_{BP}(s) \).

In the Laplace domain, these waiting time distributions can be expanded in terms of
their moments as
\[ Q_{ij}(s) = q_{ij} \left( 1 - s \langle t_{ij} \rangle + \frac{s^2}{2} \langle t_{ij}^2 \rangle - \cdots \right) \tag{5} \]

where the branching probabilities \( q_{ij} \) account for the normalization of probability, with \( \sum_j q_{ij} = 1 \) and \( \sum_j q_{ij} \langle t_{ij} \rangle = \langle \tau_i^k \rangle \), the \( k \)th moment for the decay time of state \( i \). Expanding the overall waiting time distribution in terms of the moments for the individual steps, as in eq 5 yields

\[ \hat{\phi}(s) = \frac{\alpha}{1 - \beta} \tag{6} \]

with

\[ \alpha = q \left[ 1 - s \langle \tau_1 + t_{BP} \rangle + \frac{s^2}{2} \langle (\tau_1 + t_{BP})^2 \rangle - \cdots \right] \tag{7} \]

\[ \beta = (1 - q) \left[ 1 - s \langle \tau_1 + t_{B1} \rangle + \frac{s^2}{2} \langle (\tau_1 + t_{B1})^2 \rangle - \cdots \right] \tag{8} \]

where \( \langle \tau_1 \rangle \) and \( \langle \tau_1^2 \rangle \) are the first and second waiting time moments for the decay of the initial free enzyme state, \( \langle t_{BP} \rangle \) and \( \langle t_{BP}^2 \rangle \) are the first and second waiting time moments for product formation, and \( \langle t_{B1} \rangle \) and \( \langle t_{B1}^2 \rangle \) are the first and second moments for substrate unbinding. The product formation branching probability \( q \) expresses the probability of advancing to product after substrate binding. From an expression for the overall waiting time distribution, the calculation of waiting time moments is straightforward. Given \( \hat{\phi}(s) = \frac{\alpha}{1 - \beta} \), and observing that \( (1 - \beta) \big|_{s=0} = \alpha \big|_{s=0} \), the first moment (i.e., mean first-passage time) is expressed as

\[ \langle t \rangle = - \frac{d\hat{\phi}(s)}{ds} \bigg|_{s=0} = - \left( \frac{\dot{\alpha} + \dot{\beta}}{\alpha} \right) \bigg|_{s=0} \tag{9} \]

where \( \dot{x} \) denotes differentiation of \( x \) with respect to the Laplace variable.

While the mean first-passage time can be determined from bulk measurements, higher-order moments, which contain information on microscopic mechanisms, \[16\,20\,24\] are unique to single-molecule analysis. The Poisson indicator, which measures stochastic fluctuations,
is expressed as \[ Q(t) = \frac{\langle N(t)^2 \rangle - \langle N(t) \rangle^2}{\langle N(t) \rangle} - 1 \], where \( \langle N(t) \rangle \) and \( \langle N(t)^2 \rangle \) are the first and second moments for the number of turnovers \( N \) occurring within the measurement time window \( t \). The first moment \( \langle N(t) \rangle \) is asymptotically related to the mean first-passage time as \[ \langle N(t) \rangle \sim t/\langle t \rangle \]. We are interested in the long-time limit \( P \equiv \lim_{t \to \infty} Q(t) \), which we simply refer to hereafter as the Poisson indicator (essentially equivalent to the Fano factor and Mandel’s Q parameter). Asymptotically, \( N(t) \) is Gaussian distributed for a renewal process, with \[ \langle N(t)^2 \rangle - \langle N(t) \rangle^2 \sim \frac{\langle t^2 \rangle - \langle t \rangle^2}{\langle t \rangle^3} t \], resulting in

\[ P = \frac{\langle t^2 \rangle - 2 \langle t \rangle^2}{\langle t \rangle^2} \] (10)

The Poisson indicator describes the deviation of a statistical process from Poissonian behavior, assuming a positive value for the bunching of events (super-Poissonian statistics), a negative value for the anti-bunching of events (sub-Poissonian statistics), and vanishing for a Poisson process. This quantity and equivalent measures of variation are frequently calculated in experimental studies and can serve to indicate the presence of dynamic disorder in particular reaction steps. Of particular interest, the sign of the Poisson indicator yields information about the topology of the kinetic mechanism: negative values of \( P \) correspond to kinetics dominated by sequential, multi-step reactions, while positive values of \( P \) are associated with kinetics dominated by a competing trapping process. In fact, when no branching occurs out of an enzymatic chain with an irreversible final step, \( P \leq 0 \). Given the above functional form for \( \hat{\phi}(s) \), the numerator of the Poisson indicator can be calculated as

\[ \langle t^2 \rangle - 2 \langle t \rangle^2 = \frac{\dot{\alpha} + \dot{\beta}}{\alpha} - \frac{2 \dot{\alpha}}{\alpha^2} \left( \dot{\alpha} + \dot{\beta} \right) \bigg|_{s=0} \] (11)
III. Generic Enzymatic Reaction

A. Functional Forms and Parameter Specification

Applying eqs 6-9 and 11 to the generic model of enzyme catalysis (Figure 1) yields

\[ \langle t \rangle = \frac{1}{q} \left[ \langle \tau_1 \rangle + \langle \tau_B \rangle \right] \] (12)

\[ \langle t^2 \rangle - 2 \langle t \rangle^2 = \frac{1}{q} \left[ (\langle \tau_1^2 \rangle - 2 \langle \tau_1 \rangle^2) + \langle \tau_B^2 \rangle - 2 \langle t_{BP} \rangle (\langle \tau_1 \rangle + \langle \tau_B \rangle) \right] \] (13)

where \( \langle \tau_B \rangle = q \langle t_{BP} \rangle + (1 - q) \langle t_{B1} \rangle \) and \( \langle \tau_B^2 \rangle = q \langle t_{BP}^2 \rangle + (1 - q) \langle t_{B1}^2 \rangle \) are the first and second waiting time moments, respectively, for bound/intermediate state decay. In order to connect the above expressions to experimental determinations of the Poisson indicator, we must examine their dependence on substrate concentration \([S]\). This dependence can be addressed by treating substrate binding as a pseudo-first-order rate step, which implies that substrate binding is a Poisson process (i.e., \( \langle \tau_1^2 \rangle - 2 \langle \tau_1 \rangle^2 = 0 \)) and \( \langle \tau_1 \rangle = \frac{1}{k_1 B} \), with pseudo-first-order rate \( k_1 B = k_1^0 B [S] \), where \( k_1^0 B \) is the rate constant for substrate binding. Experimental studies of single enzymes have confirmed the validity of this assumption, and its application leads to

\[ \langle t \rangle = \frac{1}{q} \left[ \frac{1}{k_1^0 B [S]} + \langle \tau_B \rangle \right] \] (14)

\[ \langle t^2 \rangle - 2 \langle t \rangle^2 = \frac{1}{q} \left[ -\frac{2 \langle t_{BP} \rangle}{k_1^0 B [S]} + \langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle \right] \] (15)

Finally, the Poisson indicator for the enzymatic reaction is given by

\[ \mathcal{P} ([S]) = q \left[ \frac{-2k_1^0 B \langle t_{BP} \rangle}{[S]} + (k_1^0 B)^2 (\langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle) \right] \left( \frac{1}{[S]} + k_1^0 B \langle \tau_B \rangle \right)^2 \] (16)
This result gives a general functional form for the substrate dependence of the Poisson indicator under the assumption of pseudo-first-order kinetics for substrate binding:

$$P ([S]) = \frac{A}{[S]} + B \left( \frac{1}{[S]} + C \right)^2$$  \hspace{1cm} (17)

for constants $A$, $B$, and $C$ independent of $[S]$, with expressions

$$A = -2qk_{1B}^o \langle t_{BP} \rangle$$  \hspace{1cm} (18)

$$B = q \left( k_{1B}^o \right)^2 \left( \langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle \right)$$  \hspace{1cm} (19)

$$C = k_{1B}^o \langle \tau_B \rangle$$  \hspace{1cm} (20)

This result is analogous to those reported elsewhere.\footnote{18,19,28}

From eqs 14 and 16, we see that, to second order, five parameters are needed to describe the non-Poissonian kinetics of the generic enzymatic reaction (with Poissonian binding): $k_{1B}^o$, $q$, $\langle \tau_B \rangle$, $\langle t_{BP} \rangle$, and $\langle \tau_B^2 \rangle$. However, fitting measured data to these functional forms (with respect to $[S]$) for the first waiting time moment and Poisson indicator together only gives four independent parameters, since $C$ (given in eq 20) is not independent of the two first moment parameters. Thus, to second order, the generic scheme kinetics are underdetermined by one parameter. However, if $k_{1B}^o$ is known or can be estimated, then the kinetics can be specified. Alternatively, if the enzyme is highly efficient (referred to as a “perfectly evolved enzyme”\footnote{29}), such that the turnover rate is limited only by the rate of diffusion of substrate to the active site of the enzyme, we may assume that virtually every substrate binding event leads to product. In our model, this corresponds to $q \approx 1$, which results in $\langle \tau_B \rangle \approx \langle t_{BP} \rangle$ and $\langle \tau_B^2 \rangle \approx \langle t_{BP}^2 \rangle$.

Now, three parameters are needed to describe the kinetics, and three can be obtained from fitting (since $A$ is no longer independent of the two first moment parameters); thus, the kinetics can be specified under this assumption. Additionally, if the bound/intermediate state undergoes Poissonian decay (i.e., the unbinding and product formation transitions are rate
steps), then \( \langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle^2 = 0 \) and \( \langle \tau_B \rangle = \langle t_{B1} \rangle = \langle t_{BP} \rangle \), eliminating two kinetic parameters and causing \( B \) (given in eq 19) to vanish, thereby permitting the kinetics to be specified. It should also be noted that our result for the first waiting time moment (eq 14) follows the Michaelis-Menten functional form; this is consistent with earlier work demonstrating that mechanisms of arbitrary complexity yield a turnover rate with a hyperbolic dependence on \([S]\) for zero conformational current. Representative plots of the Poisson indicator versus \([S]\) appear in Figure 2. Qualitatively, the Poisson indicator approaches finite limits at small and large \([S]\) and may feature a local minimum.

![Figure 2: Plot of the Poisson indicator versus substrate concentration for the generic enzymatic reaction (eq 16). The kinetic parameters chosen are \( k_{1B}^0 = 1 \), \( q = 0.5 \), \( \langle \tau_B \rangle = 0.55 \), and \( \langle t_{BP} \rangle = 1 \), with \( 2 \langle \tau_B^2 \rangle - 0.03 \) (represented by \( \langle t_2^2 \rangle \)) given in the legend.](image)

B. Minimum of Poisson Indicator and Topological Bound

The Poisson indicator can be considered a measure of stochastic noise, with sub-Poissonian statistics essentially corresponding to better signal-to-noise than for a Poisson process. For nonzero \( A \) and \( C \), when \( B > 0 \), \( \mathcal{P} ([S]) < 0 \) for \( 0 < [S] < -A/B \); when \( B \leq 0 \), \( \mathcal{P} ([S]) < 0 \) for \( [S] > 0 \). Thus, sub-Poissonian behavior is achievable for any set of obtainable, nonzero \( A \) and \( C \), as there always exists a (finite or infinite) range of substrate concentrations at which stochastic fluctuations can enhance the statistics of generic enzyme turnover (i.e., \( \mathcal{P} < 0 \)), even when branching occurs within the bound/intermediate state. The Poisson indicator as
a function of \([S]\) has one stationary point at

\[
[S]^* = \left[k_{1B}^0 \left( \frac{\langle \tau_B^2 \rangle}{\langle t_{BP} \rangle} - \langle \tau_B \rangle \right) \right]^{-1}
\]

which is only realizable when \([S]^* \geq 0\). For (i) \(\langle \tau_B^2 \rangle > \langle \tau_B \rangle \langle t_{BP} \rangle\), \(P([S])\) is minimized at \([S]^*\), where \(P([S]) = -q \langle t_{BP} \rangle^2 / \langle \tau_B^2 \rangle\), which can never correspond to a local maximum. For (ii) \(\langle \tau_B^2 \rangle \leq \langle \tau_B \rangle \langle t_{BP} \rangle\), \(P([S])\) is monotonic and achieves a minimum of \(q (\langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle) / \langle \tau_B \rangle^2\) as \([S] \to \infty\) (blue curve in Figure 2). In either case, the minimum of \(P([S])\) essentially corresponds to the point of optimal signal-to-noise (with respect to \([S]\)); thus, \([S]\) can be tuned to the stochastic fluctuations to optimize enzyme turnover statistics.

For the reaction of an enzyme with a single binding site and an irreversible final step (or set of steps), the Poisson indicator is bounded by \(P \geq M_{\text{max}}^{-1} - 1\), where \(M_{\text{max}}\) is the largest value of \(M\), the number of consecutive links in a turnover cycle, with a network possibly containing multiple turnover cycles. In our model, for a unicyclic network (which may still involve branching within the bound/intermediate state), \(M\) (and hence, \(M_{\text{max}}\)) corresponds to the number of underlying sequential (unbranched) rate steps in the scheme; however, since the bound/intermediate state can contain cyclic loops, and since multiple underlying transitions to product can be present, the generic scheme can represent a multicyclic network. The corresponding bound for \(M_{\text{max}}\) is given by \(M_{\text{max}} \geq [P + 1]^{-1}\), which is saturated when all links in the turnover cycle that corresponds to \(M_{\text{max}}\) are irreversible with identical rates and the rates of any branching steps out of this cycle are zero, which corresponds to the longest homogeneous, sequential chain that can be formed in the network. This topological bound can be modified using the minimum of \(P([S])\). For (i) \(\langle \tau_B^2 \rangle > \langle \tau_B \rangle \langle t_{BP} \rangle\), \(M_{\text{max}}\) is bounded by

\[
M_{\text{max}} \geq \left[1 - q \left( \frac{t_{BP}}{\langle \tau_B \rangle} \right)^2 \right]^{-1}
\]
For (ii) \( \langle \tau_B^2 \rangle \leq \langle \tau_B \rangle \langle t_{BP} \rangle \), we have

\[
M_{\text{max}} \geq \left[ 1 + q \frac{\langle \tau_B^2 \rangle}{\langle \tau_B \rangle^2} - 2 \frac{\langle \tau_B \rangle}{\langle \tau_B \rangle} \langle t_{BP} \rangle \right]^{-1} \tag{23}
\]

Thus, eqs \([22]\) or \([23]\) can be used to estimate the largest number of underlying consecutive rate steps in a turnover cycle. Notably, both of these bounds are independent of \( k_1^0 \), as are the inequalities identifying the two cases. We note that even though the generic scheme kinetics are generally underdetermined by one parameter, the expressions in eqs \([21]\)-\([23]\) along with the minimum of \( P([S]) \) (and \( \langle \tau_B^2 \rangle / (\langle \tau_B \rangle \langle t_{BP} \rangle) \)) to identify the case, can be evaluated from measurement of the first two waiting time moments, without the need for any assumptions.

**C. Limiting Behavior of Poisson Indicator**

The pathway analysis described above offers a simple route to the calculation of waiting time moments, without the assumption of a particular rate model. Ultimately, the goal is to connect experimental measurements of waiting time moments to features of the underlying mechanism. From the analytical expressions for the Poisson indicator as a function of substrate concentration, we can now examine the experimentally accessible limits.

As can be seen from eq \([16]\) in the limit of low substrate concentration, the Poisson indicator vanishes. This is a consequence of the assumption that substrate binding is a pseudo-first-order rate process. At very low substrate concentration, substrate binding becomes the rate determining step for the enzymatic process. Since the Poisson indicator reflects the statistical properties of the waiting time for the overall reaction, if the waiting time for the reaction is dominated by a single step, the Poisson indicator will reflect the statistical properties of that step. Hence, at very low substrate concentration, the Poisson indicator vanishes. This is supported by experimental observation of Poissonian kinetics for single enzymes at very low substrate concentrations.\(^{15}\) As is also apparent from eq \([16]\) at
low substrate concentration, we have, to leading order,

\[ P([S]) \approx -2qk_{1B}^{\circ}[S]\langle t_{BP} \rangle \tag{24} \]

indicating that sub-Poissonian behavior, as well as a linear dependence of the Poisson indicator on \([S] \), is always expected at sufficiently low substrate concentration. This corresponds to substrate binding being so much slower than bound/intermediate state decay \((\langle \tau_1 \rangle \gg \langle \tau_B \rangle)\) that the latter process becomes effectively Poissonian \((\langle \tau_B^2 \rangle - 2\langle \tau_B \rangle^2 \approx 0 \) and \(\langle \tau_B \rangle \approx \langle t_{B1} \rangle \approx \langle t_{BP} \rangle\), irrespective of the complexity of the underlying dynamics. That is, the unbinding and product formation transitions behave as rate steps with rates \(k_{B1} = (1 - q)/\langle \tau_B \rangle \) and \(k_{BP} = q/\langle \tau_B \rangle\), respectively, as the generic scheme reduces to the Michaelis-Menten scheme shown in Figure 3(a) (with \(k_{1B}^{\circ}[S] \ll 1/\langle \tau_B \rangle\)), resulting in sub-Poissonian statistics.

![Diagram](a)

\[ \langle \tau_1 \rangle \gg \langle \tau_B \rangle \]

\[ k_{1B}^{\circ} = \frac{1 - q}{\langle \tau_B \rangle} \]

\[ k_{BP} = \frac{q}{\langle \tau_B \rangle} \]

Figure 3: Reduced representations of the generic enzymatic scheme at low [(a)] and high [(b)] substrate concentration. (a) At low substrate concentration, substrate binding is much slower than bound/intermediate state decay \((\langle \tau_1 \rangle \gg \langle \tau_B \rangle)\), resulting in the latter process becoming effectively Poissonian, i.e., the unbinding and product formation transitions behave as rate steps, as the scheme reduces to a Michaelis-Menten model. (b) At high substrate concentration, substrate binding effectively occurs instantaneously \((Q_{1B} \approx 1)\), as turnover begins in state 2 and unbinding proceeds directly back into state 2.
In the limit of high substrate concentration, the Poisson indicator approaches a constant value. Substrate binding becomes arbitrarily fast at high substrate concentrations, so the Poisson indicator will reflect the statistical properties of the steps not dependent upon substrate concentration. For the generic enzymatic reaction, the large-[S] limit is given by

\[ P_{[S] \to \infty} = q \frac{\langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle}{\langle \tau_B \rangle^2} \]  

which is recovered when \( \langle \tau_1 \rangle \ll \langle \tau_B \rangle \). This corresponds to instantaneous substrate binding \((Q_{1B} \approx 1)\), with turnover effectively beginning in state 2 and unbinding proceeding back into state 2, as shown in the reduced scheme in Figure 3(b). We note that \( P_{[S] \to \infty} \) vanishes when the bound/intermediate state is unaggregated (i.e., contains a single underlying state, undergoing Poissonian decay) and can be positive when branching occurs within the bound/intermediate state. The expression for \( P_{[S] \to \infty} \) can be simplified with basic assumptions about the nature of the enzymatic system. Under the aforementioned perfectly evolved enzyme assumption (in which \( q \approx 1 \)), the large-[S] limit of the Poisson indicator simplifies to

\[ P_{[S] \to \infty} \approx \frac{\langle t_{BP}^2 \rangle - 2 \langle t_{BP} \rangle^2}{\langle t_{BP} \rangle^2} = P_{BP} \]  

where we have defined \( P_{BP} \) as the Poisson indicator for product formation. Therefore, for an enzyme of this type, determination of the Poisson indicator at high substrate concentration directly informs upon the statistical properties of the step(s) converting substrate to product after substrate binding. In a similar vein, we can consider the case of an enzyme where product formation is much slower than substrate unbinding, which corresponds to the limit \( q \to 0 \) in our model. The large-[S] limit of the Poisson indicator is then given by

\[ P_{[S] \to \infty} \approx \frac{q}{1-q} \frac{\langle t_{B1}^2 \rangle - 2 \langle t_{B1} \rangle \langle t_{BP} \rangle}{\langle t_{B1} \rangle^2} \]

\[ = \frac{q}{1-q} \left( P_{B1} + \frac{2(\langle t_{B1} \rangle - \langle t_{BP} \rangle)}{\langle t_{B1} \rangle} \right) \]  

(27)
where \( P_{B1} \equiv \frac{(\langle t_{B1}^2 \rangle - 2 \langle t_{B1} \rangle^2)}{\langle t_{B1}^2 \rangle} \). Hence, in this case, the large-[S] limit depends upon the Poisson indicator for the substrate unbinding process and a normalized measure of the difference in average waiting time for substrate unbinding and product formation. These limits offer another means of tying experimental measurements of the Poisson indicator to the underlying statistics, in addition to the possibility of directly fitting experimental data to the general functional form of the Poisson indicator.

We now proceed to extend our approach to more complex reaction schemes.

### IV. Inhibition and Selective Binding

#### A. Competitive Inhibition

As a further example of our approach, we examine a generalized scheme for enzymatic reactions with competitive inhibition (Figure 4). We note that inhibited single-molecule reactions have experimental relevance and have been the subject of theoretical studies involving rate processes.

![Figure 4: Generalized enzymatic reaction incorporating competitively inhibited state I, which can be an aggregate of states with an arbitrary internal topology. \( p \) and \( q \) are the branching probabilities for binding substrate (versus inhibitor) and for advancing to product from state 2, respectively.](image-url)

\[ p \quad 1-q \quad 1-p \]
Now, the free enzyme may bind either substrate or inhibitor, reaching state 2 or 2* with probability $p$ or $(1 - p)$, respectively. Like the bound/intermediate state, the inhibited state I may be an aggregate of states with an arbitrary internal topology; thus, it may undergo non-Poissonian decay. Following the same analysis as before, the overall waiting time distribution takes the form

$$\hat{\phi}(s) = \frac{Q_{1B}(s)Q_{BP}(s)}{1 - Q_{1B}(s)Q_{B1}(s) - Q_{1I}(s)Q_{II}(s)} = \frac{\alpha}{1 - \beta}$$

(28)

where $Q_{1B}(s)$ and $Q_{B1}(s)$ are the waiting time distributions for substrate binding and unbinding, $Q_{1I}(s)$ and $Q_{II}(s)$ are the distributions for inhibitor binding and unbinding, and $Q_{BP}(s)$ is the distribution for product formation. The constants $\alpha$ and $\beta$ are then

$$\alpha = pq \left[ 1 - s \langle t_{1B} + t_{BP} \rangle + \frac{s^2}{2} \langle (t_{1B} + t_{BP})^2 \rangle - \ldots \right]$$

(29)

$$\beta = p(1 - q) \left[ 1 - s \langle t_{1B} + t_{B1} \rangle + \frac{s^2}{2} \langle (t_{1B} + t_{B1})^2 \rangle - \ldots \right] + (1 - p) \left[ 1 - s \langle t_{1I} + \tau_I \rangle + \frac{s^2}{2} \langle (t_{1I} + \tau_I)^2 \rangle - \ldots \right]$$

(30)

where $\langle t_{1B} \rangle$ and $\langle t_{1B}^2 \rangle$ are the first and second moments for the substrate binding waiting time, $\langle t_{1I} \rangle$ and $\langle t_{1I}^2 \rangle$ are the first and second moments for the inhibitor binding waiting time, and $\langle \tau_I \rangle$ and $\langle \tau_I^2 \rangle$ are the first and second moments for the decay time of the inhibited state. Again, we can examine the dependence on the concentrations of substrate and inhibitor by assuming that the binding of each is a rate process. This assumption leads to $\langle t_{1B}^k \rangle = \langle t_{1I}^k \rangle = \langle \tau_I^k \rangle$, where $\langle \tau_I^k \rangle = p \langle t_{1B}^k \rangle + (1 - p) \langle t_{1I}^k \rangle$, with

$$\langle \tau_I \rangle = \frac{1}{k_{1B} + k_{1I}}$$

(31)

$$p = \frac{k_{1B}}{k_{1B} + k_{1I}}$$

(32)

and $\langle \tau_I^2 \rangle - 2 \langle \tau_I \rangle^2 = 0$ for pseudo-first-order rate $k_{1I} = k_{1I}^0 [I]$, where $k_{1I}^0$ is the rate constant for inhibitor binding, and $[I]$ is the inhibitor concentration.

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The first overall waiting time moment for the enzymatic reaction in the presence of a competitive inhibitor is then

\[
\langle t \rangle = \frac{1}{q} \left[ \frac{1 + k_{1I}^o [I] \langle \tau_I \rangle}{k_{1B}^o [S]} + \langle \tau_B \rangle \right]
\] (33)

Now, calculation of the Poisson indicator as before yields

\[
P([S]) = \frac{A}{[S]} + B \left( \frac{1}{[S]} + C \right)^2
\] (34)

where \( A, B, \) and \( C \) now depend upon the inhibitor concentration and are given by

\[
A = \frac{q k_{1B}^o}{(1 + k_{1I}^o [I] \langle \tau_I \rangle)^2} \left[ -2 \langle t_{BP} \rangle + k_{1I}^o [I] \left( \langle \tau_I^2 \rangle - 2 \langle \tau_I \rangle \langle t_{BP} \rangle \right) \right]
\] (35)

\[
B = \frac{q (k_{1B}^o)^2}{(1 + k_{1I}^o [I] \langle \tau_I \rangle)^2} \left[ \langle \tau_B^2 \rangle - 2 \langle \tau_B \rangle \langle t_{BP} \rangle \right]
\] (36)

\[
C = \frac{k_{1B}^o \langle \tau_B \rangle}{1 + k_{1I}^o [I] \langle \tau_I \rangle}
\] (37)

Notably, this is the same basic functional form (with respect to \([S]\)) as that in the uninhibited case (eq 17). To second order, eight parameters are needed to describe the non-Poissonian kinetics (with Poissonian binding): \(k_{1B}^o, k_{1I}^o, q, \langle \tau_B \rangle, \langle \tau_I \rangle, \langle t_{BP} \rangle, \langle \tau_B^2 \rangle\), and \(\langle \tau_I^2 \rangle\). However, eqs 33-37 indicate that fitting (with respect to \([S]\) and \([I]\)) to second order only gives six independent parameters, making the kinetics underdetermined by two parameters. However, the number of underdetermined parameters can be reduced in several situations. (i) If either \(k_{1B}^o\) or \(k_{1I}^o\) is known, then one kinetic parameter can be eliminated (two if both are known). (ii) If inhibitor unbinding is a rate process with rate \(k_{1I}\), then \(\langle \tau_I \rangle = 1/k_{1I}\) and \(\langle \tau_I^2 \rangle - 2 \langle \tau_I \rangle^2 = 0\), which eliminates one kinetic parameter. (iii) If the aforementioned perfectly evolved enzyme assumption holds, then the number of underdetermined parameters is reduced by one (as shown in section III.A). (iv) If the bound/intermediate state undergoes
Poissonian decay, then the number of underdetermined parameters is also reduced by one (as shown in section III.A). Thus, the kinetics can be specified in a variety of ways.

Figure 5(a) illustrates the dependence of the Poisson indicator on substrate concentration across a range of inhibitor concentrations. As was the case for the uninhibited reaction, the Poisson indicator vanishes at very low substrate concentration and adopts the form given in eq 25 at high substrate concentration. The large-\([S]\) limits match for these two cases because, from eqs 31 and 32, when \(k_{1B}[S] \gg k_{1I}[I]\), \(\langle \tau_1 \rangle \approx 1/k_{1B}[S]\) and \(p \approx 1\). Qualitative differences are evident between Figures 2 and 5(a). In particular, a local maximum (with respect to \([S]\)) can be achieved with a competitive inhibitor when \(AC > B\) and \(C > 2B/A\). This unique feature essentially corresponds to the point of poorest signal-to-noise for a given, obtainable \(A\), \(B\), and \(C\) (capable of achieving one). We note that \(P([S])\) may instead achieve a local minimum or no realizable local extremum. In the presence of a competitive inhibitor, eq 24 for low \([S]\) does not generally apply (except in the limit of vanishing \([I]\)). In fact, under certain conditions, the Poisson indicator can be non-negative at all substrate concentrations, precluding sub-Poissonian behavior. Similarly, the Poisson indicator can be non-positive at all substrate concentrations under certain conditions [behavior not shown in Figure 5(a)], even when a competitive inhibitor is present.

The inherent asymmetry between inhibitor and substrate is demonstrated in Figure 5(b), where the Poisson indicator is plotted against inhibitor concentration across a range of substrate concentrations. The Poisson indicator can attain a local maximum (with respect to \([I]\)), which corresponds to the point of optimal inhibition (i.e., poorest signal-to-noise, essentially) for a given set of conditions (under which one can be achieved). This extremum is important in the context of drug design, since many drugs function by acting as inhibitors. In such cases, \([I]\) can be selectively tuned to attain optimal inhibition statistics. We note that \(P([I])\) may instead achieve a local minimum or no realizable local extremum [cases not shown in Figure 5(b)]. In the limit of saturating \([I]\), \(P\) vanishes because inhibitor binding becomes the only feasible transition. As is to be expected, eqs 35–37 above reduce to the
Figure 5: (a) Plot of the Poisson indicator versus substrate concentration at fixed inhibitor concentration. The numerical parameters are $k_{1B}^0 = 1$, $k_{1I}^0 = 1$, $q = 0.5$, $\langle \tau_B \rangle = 0.55$, $\langle \tau_I \rangle = 3$, $\langle t_{BP} \rangle = 1$, $\langle t_B^2 \rangle = 1.765$, and $\langle t_I^2 \rangle = 20$, with [I] given in the legend. (b) Plot of the Poisson indicator versus inhibitor concentration at fixed substrate concentration. The numerical parameters are identical to those in (a), except now [S] is given in the legend.
results for the generic enzymatic reaction in the limit of vanishing $[I]$.

**B. Multiple Substrates**

Our methodology can also be applied to more complex systems. In fact, generalization to a reaction with multiple substrates is straightforward. The scheme for this case is illustrated in Figure 6. The waiting time distribution for the conversion of any one of the $n$ substrates to its corresponding product is given by

$$
\hat{\phi}(s) = \sum_i Q_{EB_i}(s)Q_{B_iP}(s) = \frac{\alpha}{1 - \beta}
$$

(38)

where $Q_{EB_i}(s)$ is the waiting time distribution for the binding of substrate $S_i$, $Q_{B_iE}(s)$ is the distribution for the unbinding of substrate $S_i$, and $Q_{B_iP}(s)$ is the distribution for the conversion of bound/intermediate state $B_i$ to the corresponding product $P_i$. In terms of the

![Diagram](image-url)
waiting time moments for the individual steps,

\[
\alpha = \sum_i p_i q_i \left[ 1 - s \langle t_{EB_i} + t_{B_i,P} \rangle + \frac{s^2}{2} \langle (t_{EB_i} + t_{B_i,P})^2 \rangle \right] \tag{39} \\
\beta = \sum_i p_i (1 - q_i) \left[ 1 - s \langle t_{EB_i} + t_{B_i,E} \rangle + \frac{s^2}{2} \langle (t_{EB_i} + t_{B_i,E})^2 \rangle \right] \tag{40}
\]

where \( q_i \) is the branching probability for the formation of product \( P_i \), \( \langle t_{EB_i} \rangle \) and \( \langle t_{EB_i}^2 \rangle \) are the first and second waiting time moments for the binding of substrate \( S_i \), \( \langle t_{B_i,E} \rangle \) and \( \langle t_{B_i,E}^2 \rangle \) are the first and second waiting time moments for the unbinding of substrate \( S_i \), and \( \langle t_{B_i,P} \rangle \) and \( \langle t_{B_i,P}^2 \rangle \) are the first and second moments for the formation of product \( P_i \). Assuming that the binding of any substrate is a rate process, then \( \langle t_{EB_i} \rangle = \langle \tau_E \rangle \) and \( \langle t_{EB_i}^2 \rangle = \langle \tau_E^2 \rangle \), where \( \langle \tau_E \rangle = \sum_i p_i \langle t_{EB_i} \rangle \) and \( \langle \tau_E^2 \rangle = \sum_i p_i \langle t_{EB_i}^2 \rangle \) are the first and second waiting time moments, respectively, for the decay of the initial free enzyme state, with \( p_i \) representing the probability of binding substrate \( S_i \). We now have \( p_i \) and \( \langle \tau_E \rangle \) given by

\[
p_i = \frac{k_{EB_i}}{\sum_i k_{EB_i}} \tag{41} \\
\langle \tau_E \rangle = \frac{1}{\sum_i k_{EB_i}} \tag{42}
\]

as well as \( \langle \tau_E^2 \rangle - 2 \langle \tau_E \rangle^2 = 0 \), with pseudo-first-order rate \( k_{EB_i} = k_{EB_i}^0 [S_i] \), where \( k_{EB_i}^0 \) is the rate constant for the binding of substrate \( S_i \).

The first moment for the overall waiting time in the presence of multiple substrates is expressed as

\[
\langle t \rangle = \frac{1 + \sum_i k_{EB_i}^0 [S_i] \langle \tau_{B_i} \rangle}{\sum_i q_i k_{EB_i}^0 [S_i]} \tag{43}
\]

where \( \langle \tau_{B_i} \rangle = q_i \langle t_{B_i,P} \rangle + (1 - q_i) \langle t_{B_i,E} \rangle \) is the first waiting time moment for the decay of bound/intermediate state \( B_i \). Now, if we choose to examine the dependence of the Poisson indicator on the concentration of a single substrate \( [S_k] \), it will have the following functional
form:

\[ P ([S_k]) = \frac{A}{[S_k]^2} + \frac{B}{[S_k]} + C \left( \frac{1}{[S_k]} + D \right)^2 \]  

(44)

which notably departs from the functional form presented above for the single-substrate and competitively inhibited cases (eq 17). The constants \( A, B, C, \) and \( D, \) all independent of \([S_k],\) have expressions

\[
A = \aleph^2 \left[ -2 \sum_{i \neq k} q_i k^{o}_{EB_i} [S_i] \langle t_{B_iP} \rangle + \sum_{i \neq k, j \neq k} k^{o}_{EB_i} [S_i] q_j k^{o}_{EB_j} [S_j] \left( \langle \tau_{B_i}^2 \rangle - 2 \langle \tau_{B_i} \rangle \langle t_{B_jP} \rangle \right) \right] 
\]  

(45)

\[
B = \aleph^2 \left[ -2 q_k k^{o}_{EB_k} \langle t_{B_kP} \rangle + \sum_{i \neq k} q_i k^{o}_{EB_k} k^{o}_{EB_i} [S_i] \left( \langle \tau_{B_k}^2 \rangle - 2 \langle \tau_{B_k} \rangle \langle t_{B_kP} \rangle \right) + \right. 
\]

\[
\left. \sum_{i \neq k} q_k k^{o}_{EB_k} k^{o}_{EB_i} [S_i] \left( \langle \tau_{B_k}^2 \rangle - 2 \langle \tau_{B_k} \rangle \langle t_{B_kP} \rangle \right) \right] 
\]  

(46)

\[
C = \aleph^2 q_k \left( k^{o}_{EB_k} \right)^2 \left( \langle \tau_{B_k}^2 \rangle - 2 \langle \tau_{B_k} \rangle \langle t_{B_kP} \rangle \right) 
\]  

(47)

\[
D = \aleph k^{o}_{EB_k} \langle \tau_{B_k} \rangle 
\]  

(48)

where \( \langle \tau_{B_i}^2 \rangle = q_i \langle t_{B_iP}^2 \rangle + (1 - q_i) \langle t_{B_iE}^2 \rangle \) is the second waiting time moment for the decay of bound/intermediate state \( B_i, \) and we have defined

\[
\aleph = \frac{1}{1 + \sum_{i \neq k} k^{o}_{EB_i} [S_i] \langle \tau_{B_i} \rangle} 
\]  

(49)

As a simple example of the behavior of the Poisson indicator in the presence of multiple substrates, the Poisson indicator is calculated for two competing substrates \( S_a \) and \( S_b. \) In Figure 7, the Poisson indicator is plotted against the concentration of \( S_a \) at a fixed concentration of \( S_b. \) We note that in this plot, \( \langle t_{B_iE}^2 \rangle \) and \( \langle t_{B_kP}^2 \rangle \) are held fixed while \( q_b \) is varied, causing \( \langle \tau_{B_k}^2 \rangle \) to also vary, but \( q_b \) could instead be varied while holding \( \langle \tau_{B_k}^2 \rangle \) fixed.

For \([S_a] = 0,\) the single-substrate result at fixed \([S_b] \) is obtained as \( P ([S_a] = 0) = A, \)
Figure 7: Plot of the Poisson indicator versus $[S_a]$ for two competing substrates $S_a$ and $S_b$. The numerical parameters are $k^{0}_{EB_a} = 1$, $k^{0}_{EB_b} = 1$, $q_a = 0.5$, $[S_b] = 1$, $\langle \tau_{B_a} \rangle = 0.55$, $\langle t_{B_aP} \rangle = 1$, $\langle t_{B_bE} \rangle = 0.3$, $\langle t_{B_bP} \rangle = 10$, $\langle t^{2}_{B_aE} \rangle = 1.765$, $\langle t^{2}_{B_bE} \rangle = 0.25$, and $\langle t^{2}_{B_bP} \rangle = 250$, with $q_b$ given in the legend. Note that the case of $q_b = 0$ is equivalent to substrate $S_a$ competing with inhibitor $S_b$.

which can be nonzero, differing from $\mathcal{P} ([S] = 0)$ for the above two cases. In the limit of saturating $[S_a]$, the single-substrate form for $\mathcal{P}_{[S_a] \to \infty}$ (eq 25) is obtained. It should be noted that earlier results are recovered in the appropriate limits: setting $[S_i \neq k] = 0$ recovers the single-substrate expression for $\mathcal{P} ([S_k])$ (eq 16). In addition, for only two competing substrates $S_a$ and $S_b$, as in Figure 7, setting the branching probability $q_b = 0$ recovers the competitive inhibition result for $\mathcal{P} ([S_a])$ (eq 34-37), where $[S_b]$ corresponds to the inhibitor concentration. In fact, $\mathcal{P} ([S_a])$ can achieve a local maximum similar to that shown in Figure 3(a) for competitive inhibition. We identify the presence of such a maximum for a renewal process as a signature of competitive binding, either between a substrate and an inhibitor or between multiple substrates. Finally, if the substrates are taken to be identical, that is $[S] = [S_1] = [S_2] = \ldots = [S_n]$, then eq 44 describes the Poisson indicator for an enzymatic reaction of a single substrate with multiple, parallel pathways, nearly analogous to earlier results for ion channel statistics.
V. Concluding Remarks

A general methodology for calculating second moments for the waiting time between reaction events has been introduced and applied to the analysis of enzymatic reactions. All of the flexibility conferred by the self-consistent pathway analysis method (paper 1) is retained, and the approach can be applied to many diverse cases. Our approach is currently restricted to renewal processes but will be extended to nonrenewal processes in a subsequent paper (paper 3). In the current study, the principal results concern a generic enzymatic reaction as well as the first explicit calculations (to our knowledge) of higher-order waiting time moments for the more complex cases of competitive inhibition and multiple substrates without assuming all states undergo Poissonian decay. The use of a generic model of enzyme catalysis allows the determination of the maximum information content of measurements of the Poisson indicator and first waiting time moment. Furthermore, analytical expressions for the Poisson indicator as a function of substrate concentration allow connections to be made between experimental data and kinetic models.

Our specific findings are summarized as follows: (i) based upon fitting to the functional forms of the first two waiting time moments, the non-Poissonian kinetics are generally underdetermined to second order but can be specified under certain circumstances. (ii) For a generic enzymatic scheme with an arbitrary intermediate topology, sub-Poissonian statistics can always (for non-trivial kinetics) be achieved for a certain range of substrate concentrations, even when branching occurs out of the intermediate state(s). (iii) We have identified a generic minimum of the Poisson indicator (with respect to substrate concentration), and this can be used to tune substrate concentration to the stochastic fluctuations, attaining optimal turnover statistics, and to estimate the largest number of underlying consecutive rate steps in a turnover cycle. (iv) At high and low substrate concentration, the Poisson indicator reflects the effective reduction of the generic enzymatic scheme based upon the rate-determining process. (v) We have identified a local maximum of the Poisson indicator as a function of substrate concentration for a renewal process as a signature of competitive
binding, either between a substrate and an inhibitor or between multiple substrates. Our analysis may be easily extended to other single-molecule experiments, offering the same benefits. In particular, application to the study of motor proteins may be fruitful due to the presence of reaction steps dependent upon substrate concentration as well as the applied mechanical force.\textsuperscript{35}

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