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Single-molecule enzyme kinetics in the presence of inhibitors

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Recent studies in single-molecule enzyme kinetics reveal that the turnover statistics of a single enzyme is governed by the waiting time distribution that decays as mono-exponential at low substrate concentration and multi-exponential at high substrate concentration. The multi-exponentiality arises due to protein conformational fluctuations, which act on the time scale longer than or comparable to the catalytic reaction step, thereby inducing temporal fluctuations in the catalytic rate resulting in dynamic disorder. In this work, we study the turnover statistics of a single enzyme in the presence of inhibitors to show that the multi-exponentiality in the waiting time distribution can arise even when protein conformational fluctuations do not influence the catalytic rate. From the Michaelis-Menten mechanism of inhibited enzymes, we derive exact expressions for the waiting time distribution for competitive, uncompetitive, and mixed inhibitions to quantitatively show that the presence of inhibitors can induce dynamic disorder in all three modes of inhibitions resulting in temporal fluctuations in the reaction rate. In the presence of inhibitors, dynamic disorder arises due to transitions between active and inhibited states of enzymes, which occur on time scale longer than or comparable to the catalytic step. In this limit, the randomness parameter (dimensionless variance) is greater than unity indicating the presence of dynamic disorder in all three modes of inhibitions. In the opposite limit, when the time scale of the catalytic step is longer than the time scale of transitions between active and inhibited enzymatic states, the randomness parameter is unity, implying no dynamic disorder in the reaction pathway. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.4737634]

I. INTRODUCTION

The ability to visualize single molecule enzyme kinetics using spectroscopic techniques has revealed that many interesting and non-trivial aspects of enzymatic activity can be inferred from the kinetics of a single enzyme molecule. Single-molecule enzymology shows that several enzymes undergo temporal fluctuations in the catalytic rates[1–5] due to protein conformational fluctuations,[5–8] an effect which is hard to perceive from an ensemble experiment. The conformational fluctuations along with other discrete and random molecular events, referred to as molecular noise,[9] render the course of an enzymatic reaction stochastic. In single-molecule enzymology, therefore, the waiting time for the turnover of an enzyme is a stochastic event, resulting in a distribution of waiting times.

Recent experimental studies have obtained the distribution of waiting times for the turnover of a single enzyme using fluorescence spectroscopy.[5,6] Interestingly, the waiting time distribution for enzymatic turnovers follows a mono-exponential decay at low substrate concentration but multi-exponential decay at high substrate concentration. The first moment of the distribution yields the mean waiting time of enzymatic turnover, the reciprocal of which follows the classical Michaelis-Menten (MM) equation[10] at low substrate concentration. However, at high substrate concentration, there are systematic deviations from the classical behavior, believed to be due to protein conformational fluctuations. The multi-exponential form of the distribution is a signature of multiple competing time-scales in the reaction. This effect, known as dynamic disorder,[11] occurs when the time-scale of product formation is comparable to the time-scale of transitions between different conformational states resulting in temporal fluctuations in the catalytic rates. Single-molecule enzymology, therefore, provides useful information about temporal fluctuations in the reaction rates, which is often obscured in an ensemble average kinetics.

It is well known that enzyme inhibition provides useful insight into the control and mechanism of enzymatic activity.[12–14] Many drugs regulate enzyme action by binding to the specific sites of an enzyme, thereby reducing its activity. At the single molecule level, does the inhibited enzyme kinetics result in enhanced temporal fluctuations in the reaction rate? If so, does the presence of dynamic disorder manifest differently in different modes of inhibitions? To answer this, starting from the MM mechanism for inhibited enzymes, we study the effects of inhibitors on single-molecule enzyme kinetics[15] in reversible inhibitions, namely, competitive, uncompetitive, and mixed inhibitions. Our key result is that the presence of inhibitors can result in dynamic disorder in the reaction pathway in all three modes of inhibition due to transitions between the active and inhibited states of enzymes. We show that dynamic disorder in inhibited Michaelis-Menten kinetics can arise even when protein conformational fluctuations do not influence the reaction rate, that is, when they act on time scales shorter than the catalytic step.

The MM kinetics in the presence of inhibitors is represented by the following reaction mechanism:[12–14]

$$E + S \underset{k^0_{-1}}{\overset{k^0_1}{\rightleftharpoons}} ES \xrightarrow{k^0_2} P + E',\ E' \xrightarrow{\Delta} E,$$

$$E + I \underset{k^0_{-3}}{\overset{k^0_3}{\rightleftharpoons}} EI,$$

$$ES + I \underset{k^0_{-4}}{\overset{k^0_4}{\rightleftharpoons}} ESI. \tag{1}$$

In this reaction mechanism, enzymes (E) can either react reversibly with the substrates to form an enzyme-substrate (ES) complex, or with the inhibitors to form an enzyme-inhibitor (EI) complex. The enzyme-substrate complex can follow two distinct reaction pathways. It can either dissociate to form the product (P) while regenerating the free enzyme (E′), or can react reversibly with the inhibitors to form the enzyme-substrate-inhibitor (ESI) complex. The regenerated enzyme is instantaneously converted into E.[5] The rate of product formation in the presence of inhibitors is given by $v = \frac{d[P]}{dt} = k^0_2[ES]$. The steady state approximation of $\frac{d[ES]}{dt} \approx 0$ yields

$$v = \frac{k^0_2[E]_0}{\beta + \alpha K_M/[S]}, \tag{2}$$

where $[E]_0 = [E] + [ES] + [ESI] + [EI]$ and $K_M = (k^0_{-1} + k^0_2)/k^0_1$. The fast equilibrium approximation between enzymes and enzyme-inhibitor gives $\alpha = 1 + [I]/K_{EI}$ with $K_{EI} = [E][I]/[EI]$. Similarly, $\beta = 1 + [I]/K_{ESI}$ with $K_{ESI} = [E][I]/[ESI]$. In a double reciprocal form, the above expression can be rewritten as

$$\frac{1}{v} = \frac{\beta}{k^0_2[E]_0} + \frac{\alpha K_M}{k^0_2[E]_0[S]}. \tag{3}$$

In the absence of inhibitors, $\alpha = \beta = 1$, Eq. (1) yields the classical MM equation. In the presence of inhibitors, the inhibition mechanism is broadly classified as competitive, uncompetitive, and mixed. In competitive inhibition, $\alpha > 1$ and $\beta = 1$, the inhibitor competes with the substrate to bind to the active site of the enzyme. In uncompetitive inhibition, $\alpha = 1$ and $\beta > 1$, the inhibitor binds with the active site of the enzyme only if the enzyme-substrate complex has already formed. In mixed inhibition, $\alpha > 1$ and $\beta > 1$, the inhibitor binds to a site other than the active site because of which the binding affinity of the substrate for the active site is reduced.[12–14]

In this work, we study single molecule enzyme kinetics for competitive, uncompetitive, and mixed inhibitions using a stochastic approach. At the single-molecule level, stochasticity arises due to molecular discreteness, which requires that the change in the number of active and inhibited enzymatic states be considered discrete. Each discrete state is specified by a joint probability, the time evolution of which follows the chemical master equation (CME). This is in contrast to classical chemical kinetics, where deterministic mass action kinetics governs the time evolution of the concentrations of the various species.

In a previous study, the effects of stochasticity due to protein conformational fluctuations are included in a single-molecule Michaelis-Menten kinetics by considering a multistate model.[7] In this model, a single enzyme at any time t can exist in any one of the n conformer states depicted by $E_1, E_2, \ldots, E_i, \ldots, E_n$. The same is true for the enzyme-substrate ($ES_1, ES_2, \ldots, ES_i, \ldots, ES_n$) and the regenerated enzyme ($E'_1, E'_2, \ldots, E'_i, \ldots, E'_n$) states. Assuming that dynamic disorder manifests only in the catalytic step of the reaction, an analytical distribution for the catalytic rate $w(k_2)$ is chosen from which a closed form expression for the waiting time distribution is derived. The waiting time distribution shows mono-exponential decay at low and intermediate substrate concentrations and multi-exponential decay at high substrate concentrations, which is in qualitative agreement with the experimental findings.[5,6] The multi-exponential behavior at high substrate concentration is due to interconversions between different conformers, which occur on time scales longer than or comparable to the catalytic reaction step resulting in dynamic disorder. The presence of dynamic disorder is indicated by the randomness parameter (dimensionless variance), which becomes larger than unity at high substrate concentrations. Here, we show that the multiexponential waiting time distribution and dynamic disorder can arise in competitive, uncompetitive, and mixed inhibition kinetics of a single enzyme even in the absence of protein conformation fluctuations, solely due to transitions between active and inhibited enzyme states.

In what follows, starting from the chemical master equation for multiple enzymes and inhibitors, we obtain coupled ordinary differential equations for a single enzyme in the presence of inhibitors. The solution of these equations provides exact expressions for the waiting time distribution for the enzymatic turnover. From the first and the second moments of the waiting time distribution, we calculate the mean waiting time and the randomness parameter for three distinct modes of inhibitions to show how the presence of inhibitors can result in temporal fluctuations in the waiting time-scales for the enzymatic turnovers.

II. SINGLE-ENZYME KINETICS IN THE PRESENCE OF INHIBITORS

The chemical master equation (CME) for the Michaelis-Menten kinetics[16,17] in the presence of inhibitors includes the effects of stochasticity by considering the number of enzymes, enzyme-substrate, enzyme-inhibitor, and enzyme-substrate-inhibitor complexes as discrete random variables, which can only take a finite number of "allowed" positive integral states. At any time t, the state is specified by the number of molecules of each type in the system. If N is the total number of enzymes at $t = 0$ and n_E, n_{ES}, n_{EI}, n_{ESI}, n_P, $n_E{}'$ are the number of enzymes, enzyme-substrate, enzyme-inhibitor, enzyme-substrate-inhibitor complexes, products, and regenerated enzyme at time t, then the chemical master equation[18,19] for the time-evolution of the joint probability $P(n_E, n_{ES}, n_{EI}, n_{ESI}, n_P, n_E{}'; t)$ for the Michalis-Menten mechanism in the

presence of inhibitors is given by

$$\begin{aligned}
&\frac{\partial P(n_E, n_{ES}, n_{EI}, n_{ESI}, n_P, n'_E; t)}{\partial t} \\
&= k_1(n_E+1)P(n_E+1, n_{ES}-1, n_{EI}, n_{ESI}, n_P, n'_E; t) \\
&\quad +k^0_{-1}(n_{ES}+1)P(n_E-1, n_{ES}+1, n_{EI}, n_{ESI}, n_P, n'_E; t) \\
&\quad +k^0_2(n_{ES}+1)P(n_E, n_{ES}+1, n_{EI}, n_{ESI}, n_P-1, n'_E-1; t) \\
&\quad +k_3(n_E+1)P(n_E+1, n_{ES}, n_{EI}-1, n_{ESI}, n_P, n'_E; t) \\
&\quad +k^0_{-3}(n_{EI}+1)P(n_E-1, n_{ES}, n_{EI}+1, n_{ESI}, n_P, n'_E; t) \\
&\quad +k_4(n_{ES}+1)P(n_E, n_{ES}+1, n_{EI}, n_{ESI}-1, n_P, n'_E; t) \\
&\quad +k^0_{-4}(n_{ESI}+1)P(n_E, n_{ES}-1, n_{EI}, n_{ESI}+1, n_P, n'_E; t) \\
&\quad -\big[(k_1+k_3)n_E+\big(k^0_{-1}+k^0_2+k_4\big)n_{ES} \\
&\quad +k^0_{-3}n_{EI}+k^0_{-4}n_{ESI}\big]P(n_E, n_{ES}, n_{EI}, n_{ESI}, n_P, n'_E; t),
\end{aligned} \tag{4}$$

where $k_1 = k^0_1[S]$, $k_3 = k^0_3[I]$, and $k_4 = k^0_4[I]$ are the pseudo first-order rate constants.

In a recent work,[20] the CME for the Michaelis-Menten kinetics in the absence of inhibitors has been solved exactly to obtain the waiting time distribution for multiple enzymes. The waiting time distribution of a single-enzyme is a special case of the latter. The CME for multiple enzymes in the presence of inhibitors, Eq. (4), is difficult to solve analytically. For a single-enzyme, however, the joint probabilities in Eq. (4) are mutually exclusive as a single enzyme at any time t can exist in only one of the four possible states implying $P_E(t) = P(1, 0, 0, 0, 0, 0; t)$, $P_{ES}(t) = P(0, 1, 0, 0, 0, 0; t)$, $P_{EI}(t) = P(0, 0, 1, 0, 0, 0; t)$, $P_{ESI}(t) = P(0, 0, 0, 1, 0, 0; t)$, and $P_P(t) = P(0, 0, 0, 0, 1, 1; t)$. The last expression implies that the product formation is always accompanied by enzyme regeneration. Since $E' \rightarrow E$ is instantaneous, $P_{E'}(t) \approx 0$ at all times. For a single enzyme, therefore, the chemical master equation reduces to the following set of coupled ordinary differential equations:

$$\begin{aligned}
\frac{dP_E(t)}{dt} &= -(k_1+k_3)P_E(t) + k^0_{-1}P_{ES}(t) \\
&\quad +k^0_{-3}P_{EI}(t), \\
\frac{dP_{ES}(t)}{dt} &= k_1P_E(t) - \big(k^0_{-1}+k^0_2+k_4\big)P_{ES}(t) \\
&\quad +k^0_{-4}P_{ESI}(t), \\
\frac{dP_{ESI}(t)}{dt} &= k_4P_{ES}(t) - k^0_{-4}P_{ESI}(t), \\
\frac{dP_{EI}(t)}{dt} &= k_3P_E(t) - k^0_{-3}P_{EI}(t), \\
\frac{dP_P(t)}{dt} &= k^0_2P_{ES}(t).
\end{aligned} \tag{5}$$

These probabilities satisfy the initial conditions $P_E(0) = 1$, $P_{ES}(0) = 0$, $P_{EI}(0) = 0$, and $P_{ESI}(0) = 0$ at t = 0 along with the constraint $P_E(t) + P_{ES}(t) + P_{EI}(t) + P_{ESI}(t) = 1$.

The waiting time probability that an enzymatic turnover occurs between t and $t + \Delta t$ is the same as the waiting time for the formation of product P between t and $t + \Delta t$, that is, $f(t)\Delta t = \Delta P_P$ (Ref. 7). Therefore, in the limit of infinitesimal time interval Δt, the expression for the waiting time distribution is given by

$$f(t) = \frac{dP_P(t)}{dt} = k^0_2P_{ES}(t). \tag{6}$$

Equation (5) represents the temporal variation of the probabilities of a single enzyme as it fluctuates between its active and inhibited states. The solution of the above equations to obtain the waiting time distribution for competitive, uncompetitive, and mixed inhibitions is given in the Appendix.

It is to be noted that in the absence of $E \rightarrow E'$ step, the Michaelis-Menten (MM) kinetics is cyclic, and the temporal variation of the concentration of ES, obtained from the deterministic MM equations, shows initial transient rise, which after a time lag results in a steady-state flux. The initial time lag corresponds to the time required for the enzyme-substrate complex to reach its steady state value. In a single turnover event, however, the presence of $E \rightarrow E'$ step is crucial to obtain the distribution of turnover times, the first moment of which yields the mean turnover (waiting) time. The steady-state flux in the former case yields the number of turnovers per unit time, which is related to the reciprocal of the mean turnover time in the latter case.[21,22]

III. RESULTS AND DISCUSSION

The waiting time distribution is a key quantity in discerning the kinetics of a single enzyme. The first moment of the distribution yields the mean waiting time, $\langle t \rangle = \int_0^\infty tf(t)dt$, for the enzymatic turnover, the reciprocal of which, as we show below, is related to the classical enzymatic velocity in the presence of inhibitors. The latter quantity can be obtained from deterministic kinetics.

The higher moments of the distribution contain information about fluctuations that cannot be inferred from deterministic kinetics. The dimensionless variance of the distribution, for instance, yields information about temporal fluctuations around the mean waiting time, which can be characterized by the randomness parameter, $r = \frac{\langle t^2\rangle - \langle t\rangle^2}{\langle t\rangle^2}$, the square root of which is also referred to as the coefficient of variation (CV).[7,23,24] The randomness parameter, r, can be viewed as a kind of noise-to-signal ratio. For a distribution that decays as mono-exponential, the randomness parameter is unity, implying that the reaction pathway is determined by a single time scale—the time scale of the slowest step in the reaction pathway. When the distribution decays as multi-exponential, there are several competing time scales. This effect, known as dynamic disorder, leads to temporal fluctuations in the reaction rate. The randomness parameter in the latter case is greater than unity.[7]

The mean waiting time and the randomness parameter for mixed, competitive, and uncompetitive inhibitions can be calculated analytically from the waiting time distributions given by Eqs. (A5), (A7), and (A10), respectively. However, since the resulting analytical expressions are unwieldy, we directly solve Eqs. (A4), (A8), and (A11) numerically for the parameter values given in Table I to compute the waiting time

TABLE I. Parameter values for the rate constants.

	Various dimensionless rate constants $k_i' = k_i/k_2^0$				
Inhibition type	$k_{-1}^{0'}$	k_3'	$k_{-3}^{0'}$	k_4'	$k_{-4}^{0'}$
Competitive	1.0	3.0	0.5	...	...
Uncompetitive	1.0	...	...	3.0	0.5
Mixed I	1.0	1.0	1.0	3.0	0.5
Mixed II	1.0	3.0	0.5	1.0	1.0

distributions from Eqs. (A5), (A7), and (A10). The results are summarized below.

A. Competitive and uncompetitive inhibitions

Figures 1(a) and 1(b) show the temporal variation of the dimensionless waiting time distribution for competitive and uncompetitive inhibitions, respectively. On log-linear scale, the temporal decay of the dimensionless waiting time distributions show multi-exponential decay characterized by a broad distribution of the waiting time scales. In competitive inhibition, the temporal variation of the dimensionless waiting time distribution shows multi-exponential decay at low and intermediate substrate concentrations, which becomes mono-exponential at high substrate concentration. In contrast, the temporal decay of the dimensionless waiting time distribution in uncompetitive inhibition follows multi-exponential decay at both intermediate and high substrate concentrations. The multi-exponential decay profiles arise due to transitions between the active and inhibited states of an enzyme resulting in multiple competing time scales in the reaction pathway. In competitive inhibition, the dominance of the active state as opposed to the inhibited state leads to mono-exponential decay at high substrate concentration. In uncompetitive inhibition, in comparison, both the active and inhibited states dominate even at high substrate concentration resulting in multi-exponential behavior.

The dimensionless waiting time distribution shows a broad distribution of the waiting times suggesting significant temporal fluctuations about the mean waiting time. The temporal fluctuations about the mean can be characterized by the randomness parameter r. Figures 1(c) and 1(d) show the variation of the randomness parameter as a function of the

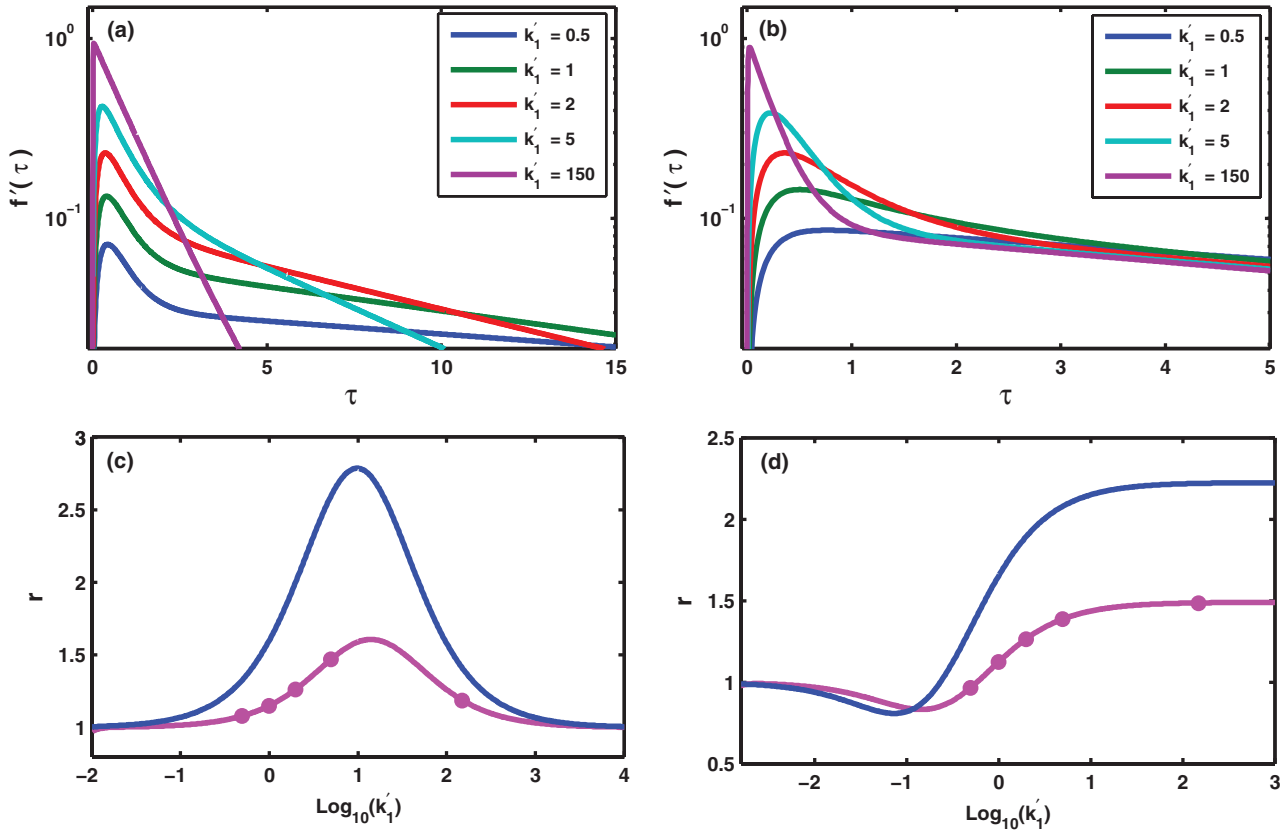

FIG. 1. The dimensionless waiting time distribution $f'(\tau) = f(\tau)/k_2^0$ as a function of the dimensionless time $\tau = k_2^0 t$ for different dimensionless $k_1' = k_1^0[S]/k_2^0$ on log-linear scale for (a) competitive and (b) uncompetitive inhibitions show multi-exponential decay due to transitions between the active and inhibited states of enzymes. In both cases, the waiting time distributions have large dimensionless variance signaling significant temporal fluctuations about the mean waiting time. These fluctuations are quantified by the randomness parameter, $r = (\langle\tau^2\rangle - \langle\tau\rangle^2)/\langle\tau\rangle^2$ for (c) competitive and (d) uncompetitive inhibitions. In competitive inhibition, significant temporal fluctuations ($r > 1$) about the mean occur at intermediate substrate concentrations, which subside at high substrate concentrations. In contrast, uncompetitive inhibition shows significant temporal fluctuations at both intermediate and high substrate concentrations. At very low substrate concentration ($k_1' \lesssim 0.1$), the randomness parameter is unity for both competitive and uncompetitive inhibitions, but for uncompetitive inhibition, there occurs a slight decrease from unity with a small increase in k_1'. The pink and blue curves correspond to the dimensionless rate constants tabulated in Tables I and II, respectively. These parameter values show that increase in k_2^0 leads to enhanced temporal fluctuations. The dots in (c) and (d) correspond to the k_1' values in (a) and (b) for the parameter values tabulated in Table I.

substrate concentration for competitive and uncompetitive inhibitions, respectively.

In competitive inhibition at very low substrate concentrations ($k_1' \lesssim 0.1$), Fig. 1(c), the binding of the substrate to the enzyme is the rate determining step, which occurs at a rate much slower than the rapid transitions between active and inhibited states of the enzyme. Due to slow substrate binding, the effects of the rapid transitions are averaged out resulting in a single dominating time scale—the time scale of the substrate binding. This effect is manifested in the randomness parameter, which is unity implying that the distribution is mono-exponential with the square of the mean equal to its variance. The increase in the substrate concentration suppresses the inhibited state of enzyme resulting in mono-exponential decay at high substrate concentration. As a result, the randomness parameter follows a non-monotonic variation with a pronounced maximum ($r > 1$) at intermediate substrate concentration, which decays to unity at high substrate concentrations.

In uncompetitive inhibition at very low substrate concentrations, the randomness parameter is unity because the substrate binding is the rate determining step. However, a slight increase in the substrate concentration leads to a small decrease in the randomness parameter from unity. This is because at very low substrate concentrations, the probability that an enzyme exists in the enzyme-substrate state $P_{ES}(t)$ is very small, making the probability of transitions between the enzyme-substrate (ES) and enzyme-substrate-inhibitor (ESI) states very small. As a result, the square of the mean waiting time, which is long due to slow rate of dissociation of the enzyme-substrate complex, is larger than the variance resulting in $r < 1$. Alternatively, the decrease in r can be understood by viewing the uninhibited kinetics of a single enzyme as a two-step linear cascade process. For very small k_1, there is a single rate determining step resulting in a mono-exponential waiting time distribution. However, with the increase in k_1, when k_1 is closer to k_2^0, the enzymatic cascade becomes a multi-step process with the waiting time distribution approaching a gamma distribution.[25,26] The dimensionless variance in the latter case is less than unity, $r < 1$. In the presence of inhibitors at very small k_1, the effect of uncompetitive inhibition is small and the waiting time distribution is governed by mono-exponential decay resulting in $r = 1$. In the presence of inhibitors, therefore, the combined effect of the decrease in r due to the multi-step cascade and the increase in r due to dynamic disorder leads to a non-monotonic variation of r as a function of k_1. When k_1 becomes very large, k_2^0 is the rate determining step and decrease in r due to multi-step cascade is absent. In the latter case, the presence of inhibitors results in the increase of r due to dynamic disorder.

The increase in the substrate concentration leads to enhanced temporal fluctuations due to transitions between active and inhibited states of the enzyme-substrate complex resulting in $r > 1$ at intermediate and high substrate concentrations. The value of $r > 1$ indicates dynamic disorder in the reaction pathway, signaling significant temporal fluctuations in the reaction rates.

The increase in the value of k_2^0 leads to an increase in the number of competing pathways since the rate of product formation is no more the rate determining step. This results in an increase in the randomness parameter [Figs. 1(c) and 1(d)]. It is to be noted that in the present study, the low, intermediate, and high substrate concentration ranges roughly correspond to $0.1 \lesssim k_1' \lesssim 1$, $1 \lesssim k_1' \lesssim 10$, and $k_1' > 10$, respectively, and have been defined with respect to the parameters chosen in Table I. It is clear from Figs. 1(c) and 1(d) that for larger k_2^0 [Table II] dynamic disorder can arise even at very low substrate concentrations.

TABLE II. Parameter values for the rate constants.

	Various dimensionless rate constants $k_i' = k_i/k_2^0$				
Inhibition type	$k_{-1}^{0'}$	k_3'	$k_{-3}^{0'}$	k_4'	$k_{-4}^{0'}$
Competitive	0.4	1.2	0.2	...	...
Uncompetitive	0.4	...	...	1.2	0.2
Mixed I	0.4	0.4	0.4	1.2	0.2
Mixed II	0.4	1.2	0.2	0.4	0.4

The first moment of the distribution yields the mean waiting time for the enzymatic turnover. The mean waiting time can be compared with the reciprocal of the enzymatic velocity v, Eq. (2), in the presence and absence of inhibitors. Figure 2 shows the double reciprocal plot for the variation

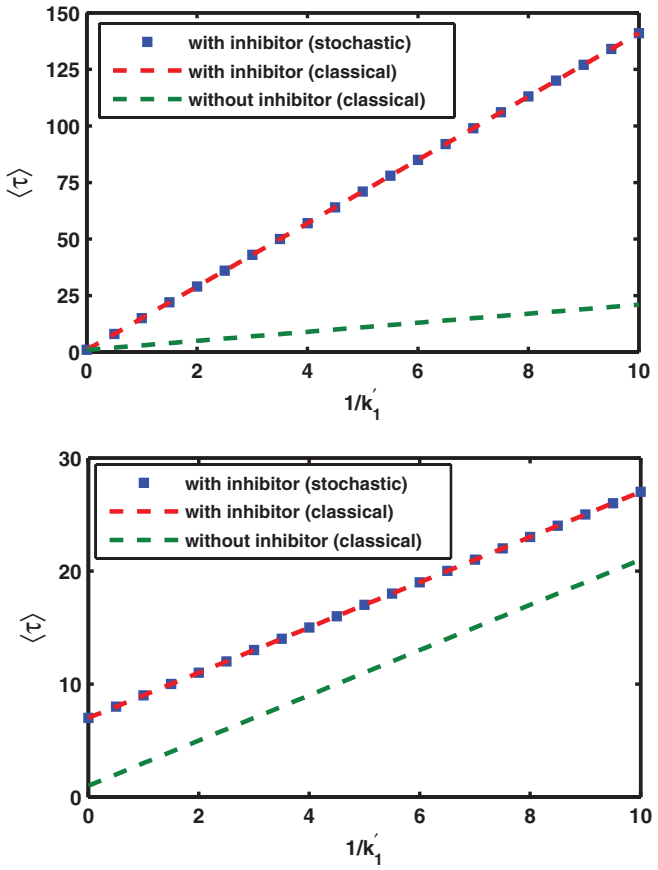

FIG. 2. Double-reciprocal plots for competitive (top) and uncompetitive (bottom) inhibitions show that the reciprocal of the dimensionless mean waiting time $\langle\tau\rangle = k_2^0 \langle t\rangle$ obtained from the first moment of the waiting time distribution is the same as the classical enzymatic velocity v/k_2^0 in the presence and absence of inhibitors. The parameter values are tabulated in Table I.

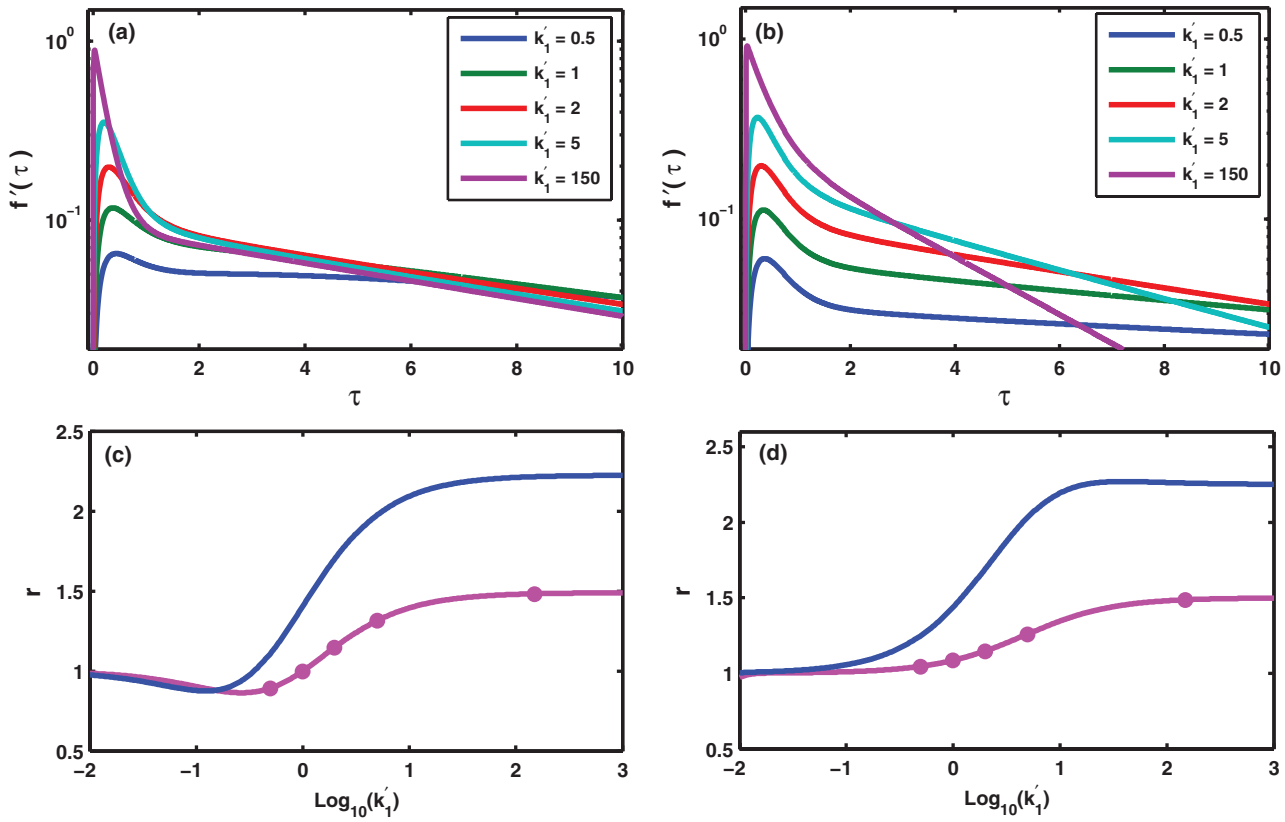

FIG. 3. Dimensionless waiting time distributions $f'(\tau) = f(\tau)/k_2^0$ as a function of the dimensionless time, $\tau = k_2^0 t$, for different dimensionless $k_1' = k_1^0[S]/k_2^0$ on log-linear scale for mixed inhibition show multi-exponential decay for both (a) mixed inhibition I ($k_4' > k_3'$) and (b) mixed inhibition II ($k_3' > k_4'$). Randomness parameter as a function of the substrate concentration for mixed inhibition for (c) mixed inhibition I and (d) mixed inhibition II shows $r > 1$ for intermediate and high substrate concentration signaling dynamic disorder in the reaction pathway. The pink and blue curves correspond to the dimensionless rate constants tabulated in Tables I and II, respectively. These parameter values show that increase in k_2^0 leads to enhanced temporal fluctuations. The dots in (c) and (d) correspond to the k_1' values in (a) and (b), respectively, for the parameter values tabulated in Table I.

of the dimensionless mean waiting time $\langle\tau\rangle = k_2^0 \langle t\rangle$ with the reciprocal of the substrate concentration for competitive and uncompetitive inhibitions. While the red dashed line shows the variation of k_2^0/v as a function of $1/[S]$ (Eq. (2)) in the presence of inhibitors for $\alpha = 7, \beta = 1$ (competitive); $\alpha = 1, \beta = 7$ (uncompetitive), the blue squares represent the first moment of the waiting time distribution for competitive, Eq. (A7), and uncompetitive inhibition, Eq. (A10). The two curves fall exactly on each other implying $v = 1/\langle t\rangle$. The green dashed line in the same figure represents the Lineweaver-Burk plot for k_2^0/v versus $1/[S]$ (Eq. (2)) in the absence of inhibitors, $\alpha = \beta = 1$. The presence of competitive inhibitors only increases the slope of the linear curves without affecting the y-intercept at high substrate concentration. In contrast, uncompetitive inhibitors change the y-intercept without affecting the slope. This is because in uncompetitive inhibition, the increase in the mean waiting time is solely due to the average time delay caused by the transition of the enzyme-substrate complex to its inhibited state (*ESI*), and is independent of the substrate concentration.

B. Mixed inhibition

In mixed inhibition, an enzyme can fluctuate between the enzyme state, the enzyme-substrate state, the enzyme-inhibitor state, or the enzyme-substrate-inhibitor state. Figures 3(a) and 3(b) show the temporal variation of the dimensionless waiting time distribution for mixed-inhibition I ($k_4' > k_3'$) and mixed-inhibition II ($k_3' > k_4'$), respectively, where $k_3' = k_3/k_2^0$ and $k_4' = k_4/k_2^0$. In both cases, the presence of multiple competitive pathways leads to multi-exponential temporal decay. The multi-exponential form of the decay profile makes the randomness parameter greater than unity in both these limits (Figs. 3(c) and 3(d)). However, at high substrate concentrations, the randomness parameter is greater for mixed-inhibition II compared to mixed-inhibition I. This is because in mixed inhibition II, apart from the dominant effect of transitions between *ES* and *ESI* states (uncompetitive inhibition) at high substrate concentration, transitions between *E* and *EI* states (competitive inhibition) act as an additional source of dynamic disorder resulting in greater randomness parameter compared to mixed inhibition I.

At very low substrate concentration, the randomness parameter is unity for mixed inhibitions I and II. However, in mixed inhibition I, a small increase in the substrate concentration leads to a slight decrease in the randomness parameter from unity. This is because of the dominating effect of uncompetitive inhibition in mixed inhibition I. In mixed inhibition II, in contrast, the dominance of competitive inhibition at very low substrate concentration leads to $r = 1$.

The double reciprocal plot for mixed-inhibition I and mixed-inhibition II is presented in Fig. 4. The comparison

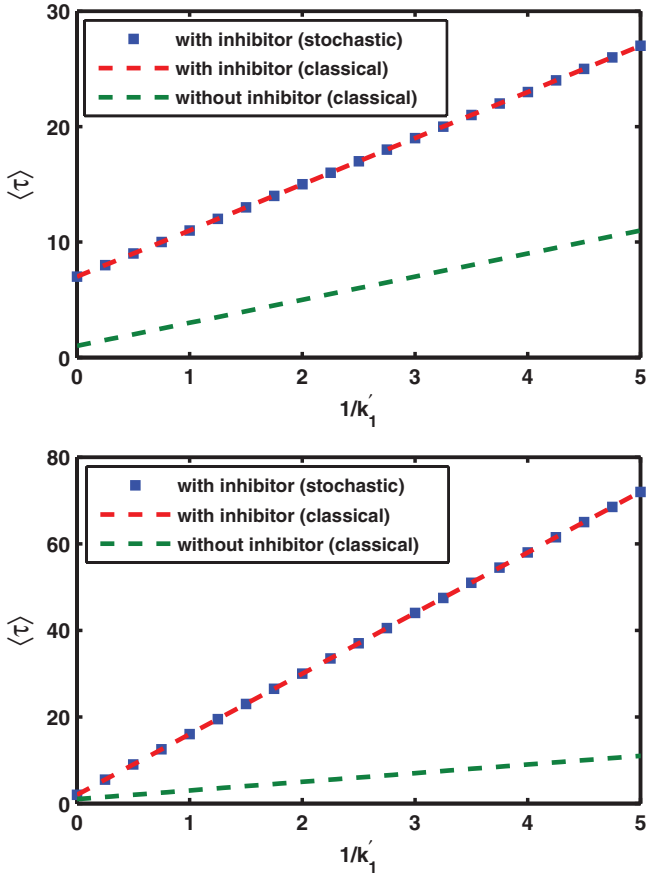

FIG. 4. Double reciprocal plots for mixed inhibition show that the reciprocal of the dimensionless mean waiting time, $\langle\tau\rangle = k_2^0\langle t\rangle$, obtained from the first moment of the waiting time distribution is the same as the classical enzymatic velocity v/k_2^0 in the presence and absence of inhibitors. Points (blue squares) are the first moment of Eq. (6) for mixed inhibition I (top) and mixed inhibition II (bottom), respectively; red dashed lines are the solutions of Eq. (2) for $\alpha = 2$, $\beta = 7$ (top), and $\alpha = 7$, $\beta = 2$ (bottom), respectively; green dashed lines are the solutions of Eq. (2) for $\alpha = 1$, $\beta = 1$. The parameter values are tabulated in Table I.

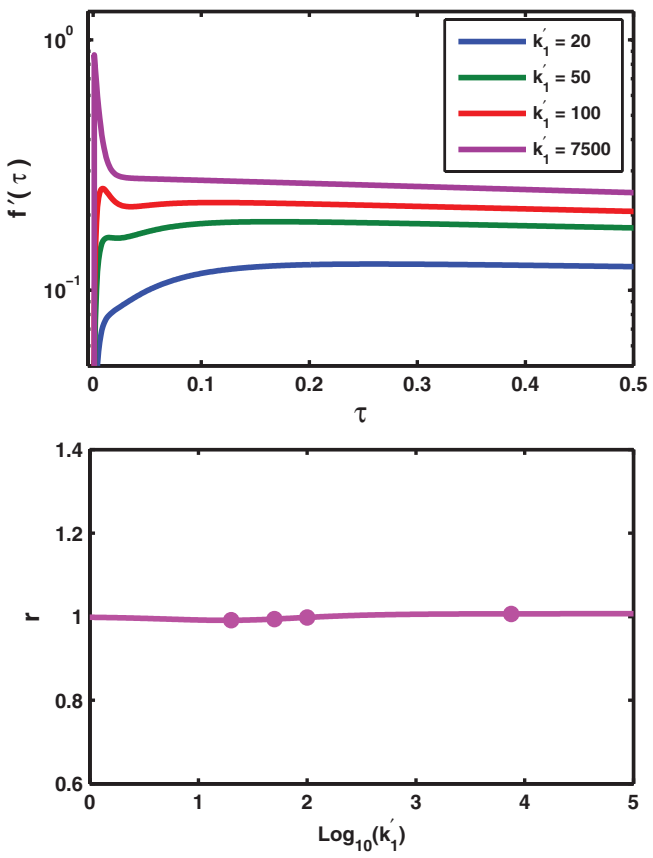

FIG. 5. The variation of the dimensionless waiting time distribution $f'(\tau) = f(\tau)/k_2^0$ as a function of dimensionless time, $\tau = k_2^0 t$, for different dimensionless $k_1' = k_1^0[S]/k_2^0$ for mixed inhibition I for $k_2^0 < 1$. When the time scale of product formation is longer than the time scales of transitions between the active and inhibited states of an enzyme, the waiting time distribution shows distinct transitions between different states before decaying as a single exponential at long times (top). The randomness parameter is (approximately) unity at all values of the substrate concentration signaling the absence of dynamic disorder (bottom). The dots in the bottom figure correspond to $k_1' = 20$, 50, 100, and 7500. All the rate constants have been non-dimensionalized with respect to k_2^0, and are given by $k_3' = 40$, $k_4' = 150$, $k_{-1}^{0'} = 40$, $k_{-3}^{0'} = 40$, $k_{-4}^{0'} = 60$.

shows that the dimensionless mean waiting time, $\langle\tau\rangle = k_2^0\langle t\rangle$ for the former case is less than the latter because the formation of the *EI* complex prolongs the formation of product. In mixed inhibition I, both the slope and the y-intercept for the inhibited case are different from that of the uninhibited one. In mixed inhibition II, the y-intercept is the same as that of the uncompetitive inhibition. However, for the parameter values $k_4' = k_{-4}^{0'} = 1.0$ at $1/k_1' = 10^{-5}$ in Fig. 4, the difference in $\langle\tau\rangle$ for uncompetitive inhibition ($\langle\tau\rangle = 2.0$) and uninhibited kinetics ($\langle\tau\rangle = 1.0$) is very small.

These results show that for a single-enzyme kinetics, the mean waiting time is related to reciprocal of the classical enzymatic velocity in the presence of inhibitors. However, there are significant temporal fluctuations in the waiting time for the turnover of a single enzyme. The magnitude of these fluctuations strongly depends on the type of inhibition mechanism, the concentrations of the substrates and inhibitors, and the rate of the catalytic step.

An interesting behavior emerges when the rate constant for product formation, k_2^0, is very small. Figure 5 shows the temporal variation of the waiting time distribution for mixed inhibition I. At short times, transitions between different enzymatic states are slow and appear as the oscillatory rise and fall of P_{ES}. Since the time scale of product formation is large, transitions between different enzymatic states are well separated such that each curve rises and falls as a single exponential. At long times, the dimensionless waiting time distribution follows a single exponential decay governed by the slowest step of the reaction. Since there is only one time scale governing the course of reaction, the randomness parameter (Fig. 5) is (approximately) unity for all values of substrate concentrations. In this limit, therefore, the reaction pathway shows no dynamic disorder.

In a previous study, the presence of dynamic disorder in single-enzyme Michaelis-Menten kinetics in the absence of inhibitors has been explained by including interconversions between enzyme's conformers, which occur on time scales longer than or comparable to the catalytic step.[7] It has been assumed that dynamic disorder is manifested only in the catalytic step through a distribution for k_2^0. When such

a distribution is considered for the rate constant for substrate release (k_{-1}), the randomness parameter does not exceed unity implying that dynamic disorder due to protein conformational fluctuations manifests only in the catalytic step. In the present study, in contrast, dynamic disorder arises due to transitions between the active and inhibited states of enzymes, which occur on time scales longer than or comparable to the catalytic step. From the exact solution of the waiting time distribution, we find that dynamic disorder is not manifested in any particular step of the reaction, and therefore does not require an *a priori* assumption for the form of distribution for k_2^0 or any other rate constants. The multiexponential decay, for instance, is governed by the effective rate constants (A, B, C, and D) that are non-trivial combinations of the rate constants for inhibitor and substrate binding and release.

In the present work, the first moment of the waiting time distribution yields the mean waiting time for enzyme turnover, the reciprocal of which is the same as the classical enzymatic velocity in the presence of inhibitors. The dimensionless variance (randomness parameter) of the multi-exponential distribution, however, shows significant fluctuations in the waiting time signifying that more than one time-scale govern the turnover statistics of an inhibited enzyme. This behavior is in contrast to turnover statistics of multiple enzymes in the absence of inhibitors, where multi-exponentiality of the waiting time distribution leads to significant deviation of the reciprocal of the mean waiting time from the classical enzymatic velocity.[20]

In a recent experiment, inhibition kinetics of single-β-galactosidase in the presence of the inhibitor D-galactal and the substrate resorufin-β-D-galactopyranoside was studied using fluorescence spectroscopy.[15] β-galactosidase is a tetrameric enzyme molecule with four binding sites and five inhibition states. In this reaction, the rate constant for the catalytic step is much larger than the rate constants for inhibition release and binding. For competitive inhibition mechanism, it was found that the autocorrelation functions for single-enzyme substrate turnovers showed single exponential decay when the substrate concentration (100 μM) was taken to be much larger than the inhibitor concentration (20 μM). Although the present study does not directly calculate the autocorrelation of substrate turnovers at different times, it shows that for competitive inhibition mechanism, the dimensionless variance (randomness parameter) of the waiting time distribution for a repeated turnover of a single enzyme becomes unity at large substrate concentration clearly indicating that the turnover statistics of a single-enzyme is governed by a mono-exponential distribution (single-exponential) with a single time-scale of decay. Since the variance of the waiting time distribution is an equal-time autocorrelation of the deviation of the enzyme turnover time from the mean, $\langle \delta t^2 \rangle$ with $\delta t = t - \langle t \rangle$, the dimensionless variance of the distribution (randomness parameter) is a good indicator of dynamic disorder, as has been pointed out in previous studies.[6,7] Interestingly, the single-exponential decay of autocorrelation function suggests that protein conformational fluctuations do not affect the turnover statistics of a single enzyme even at high substrate concentrations.

IV. CONCLUSION

We conclude that the presence of inhibitors in single-enzyme kinetics can result in significant fluctuations in the waiting time for enzymatic turnover in all three modes of inhibitions. Crucially, the time scale of catalytic reaction step governs the magnitude of temporal fluctuations in the enzymatic turnover time. When the time-scale of catalytic step is comparable to or shorter than the time-scale of transitions between the active and inhibited states of enzymes, the waiting time distributions follow multi-exponential decay with a broad distribution of the waiting times for uncompetitive and mixed inhibitions. In contrast, the waiting time distribution for competitive inhibition is multiexponential at low and intermediate substrate concentrations and becomes single-exponential at large substrate concentrations. When the time-scale of catalytic step is longer than the time-scale of transitions between different enzymatic states, the temporal decay profile is governed by a single time-scale with no dynamic disorder in the reaction pathway. Our results imply that dynamic disorder in the presence of inhibitors can arise even when protein conformational fluctuations do not influence the catalytic rate, that is, when they act on time scales shorter than the catalytic step. A single-exponential decay for autocorrelation function of substrate turnovers in competitive inhibition, observed in a recent experiment, suggests that protein conformational fluctuations do not affect the turnover statistics of a single enzyme even at high substrate concentrations. The waiting time distribution calculated in the present study can, therefore, provide reliable estimates of the effective rate constant for the turnover statistics of a single enzyme at the different concentrations of inhibitors and substrates for competitive, uncompetitive, and mixed inhibitions, especially when the decay profile is governed by more than one time-scale of decay.

The presence of protein conformational fluctuations can be an additional source of stochasticity in turnover statistics of an inhibited enzyme. Recent fluorescence spectroscopy measurements on uninhibited kinetics of a single-enzyme show that dynamic disorder due to interconversions between different conformers occurs at high substrate concentrations ($k_1 \approx 1000\ s^{-1}$) (Ref. 5). However, the present study reveals that in the presence of inhibitors, depending on the time scale of the catalytic step, dynamic disorder can arise even at low and intermediate substrate concentrations due to transitions between active and inhibited states of enzymes. At such low and intermediate substrate concentrations, where the effects of dynamic disorder can arise solely due the inhibition mechanism, the results of the present study can be verified using fluorescence spectroscopy measurements. At high substrate concentrations, where protein conformational fluctuations can act as an additional source of dynamic disorder, the present study can be extended to include the effects of interconversions between different active and inhibited enzyme conformers. Work is underway to explore these possibilities.

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APPENDIX: EXACT SOLUTION OF THE WAITING TIME DISTRIBUTION FROM CME

1. Mixed inhibition

In mixed inhibition, an enzyme can exist in the enzyme state, the enzyme-substrate state, the enzyme-inhibitor state, or the enzyme-substrate-inhibitor state. The underlying mechanism is given by

$$E + S \underset{k^0_{-1}}{\overset{k^0_1}{\rightleftharpoons}} ES \xrightarrow{k^0_2} P + E',\ E' \xrightarrow{\Delta} E,$$

$$E + I \underset{k^0_{-3}}{\overset{k^0_3}{\rightleftharpoons}} EI,$$

$$ES + I \underset{k^0_{-4}}{\overset{k^0_4}{\rightleftharpoons}} ESI. \tag{A1}$$

The coupled differential equations for mixed inhibition, Eq. (5), can be solved exactly for $P_{ES}(t)$ by carrying out the Laplace transform. Taking the Laplace transform of $P_E(t)$, $P_{ES}(t)$, $P_{ESI}(t)$, and $P_{EI}(t)$ in Eq. (5) and applying the initial conditions yield, after simplification, the following expression for P_{ES}:

$$P_{ES}(s) = \frac{k_1\left(s + k^0_{-3}\right)\left(s + k^0_{-4}\right)}{s^4 + \lambda_1 s^3 + \lambda_2 s^2 + \lambda_3 s + \lambda_4}. \tag{A2}$$

The inverse Laplace transform of the above equation yields,

$$\begin{aligned} P_{ES}(t) = k_1\Bigg[& \frac{e^{At}(A + k^0_{-3})(A + k^0_{-4})}{(A - B)(A - C)(A - D)} \\ & - \frac{e^{Bt}(B + k^0_{-3})(B + k^0_{-4})}{(A - B)(B - C)(B - D)} \\ & + \frac{e^{Ct}(C + k^0_{-3})(C + k^0_{-4})}{(A - C)(B - C)(C - D)} \\ & - \frac{e^{Dt}(D + k^0_{-3})(D + k^0_{-4})}{(A - D)(D - C)(D - B)}\Bigg], \end{aligned} \tag{A3}$$

where A, B, C, and D are the effective rate constants, which can be obtained from the solutions of the quartic equation $s^4 + \lambda_1 s^3 + \lambda_2 s^2 + \lambda_3 s + \lambda_4 = 0$ with

$$\begin{aligned} \lambda_1 &= k_1 + k^0_2 + k_4 + k_3 + k^0_{-1} + k^0_{-4} + k^0_{-3}, \\ \lambda_2 &= k^0_{-4}k^0_{-3} + \left(k^0_{-4} + k^0_{-3}\right)\left(k_1 + k^0_2 + k_3 + k_4 + k^0_{-1}\right) \\ &\quad + \left(k_1 + k_3\right)\left(k^0_{-1} + k^0_2 + k_4\right) \\ &\quad - \left(k^0_{-4}k_4 + k^0_{-1}k_1 + k^0_{-3}k_3\right), \\ \lambda_3 &= k^0_{-4}k^0_{-3}\left(k_1 + k^0_2 + k_3 + k_4 + k^0_{-1}\right) + \left(k_1 + k_3\right) \\ &\quad \left(k^0_{-1} + k_4 + k^0_2\right)\left(k^0_{-4} + k^0_{-3}\right) \\ &\quad - \left(k^0_2 + k_4 + k^0_{-1} + k^0_{-4}\right)k^0_{-3}k_3 \\ &\quad - \left(k^0_{-4} + k^0_{-3}\right)k^0_{-1}k_1 - \left(k_3 + k_1 + k^0_{-3}\right)k^0_{-4}k_4, \\ \lambda_4 &= \left(k_1 + k_3\right)\left(k^0_{-1} + k^0_2 + k_4\right)k^0_{-3}k^0_{-4} \\ &\quad - k_3k^0_{-4}k^0_{-3}\left(k^0_{-1} + k^0_2 + k_4\right) + k^0_{-1}k^0_{-4}k^0_{-3}k_1 \\ &\quad + \left(k_1 + k_3\right)k^0_{-3}k_4k^0_{-4} - k^0_{-4}k_4k_3k^0_{-3}. \end{aligned} \tag{A4}$$

Substituting Eq. (A3) into (6) yields the following expression for the waiting time distribution for mixed inhibition:

$$\begin{aligned} f(t) = k^0_2k_1\Bigg[& \frac{e^{At}(A + k^0_{-3})(A + k^0_{-4})}{(A - B)(A - C)(A - D)} \\ & - \frac{e^{Bt}(B + k^0_{-3})(B + k^0_{-4})}{(A - B)(B - C)(B - D)} \\ & + \frac{e^{Ct}(C + k^0_{-3})(C + k^0_{-4})}{(A - C)(B - C)(C - D)} \\ & - \frac{e^{Dt}(D + k^0_{-3})(D + k^0_{-4})}{(A - D)(D - C)(D - B)}\Bigg]. \end{aligned} \tag{A5}$$

The specific cases of competitive and uncompetitive inhibitions are given below.

2. Competitive inhibition

In competitive inhibition, the inhibitor molecule is a structural analogue of the substrate. Its binding site is the same as the substrate molecule. Hence, it binds to the active site of the enzyme, thereby inhibiting the attachment of the substrate. A competitive inhibition is usually reversible if sufficient substrate molecules are available to displace the inhibitor. The underlying mechanism is

$$E + S \underset{k^0_{-1}}{\overset{k^0_1}{\rightleftharpoons}} ES \xrightarrow{k^0_2} P + E,\ E' \xrightarrow{\Delta} E,$$

$$E + I \underset{k^0_{-3}}{\overset{k^0_3}{\rightleftharpoons}} EI. \tag{A6}$$

In the limit of $k_4 = k^0_{-4} = 0$, Eqs. (A3)–(A5) yield the following expression for the waiting time distribution for competitive inhibition:

$$\begin{aligned} f(t) = k_1k^0_2\Bigg[& \frac{e^{At}(A + k^0_{-3})}{(A - B)(A - C)} - \frac{e^{Bt}(B + k^0_{-3})}{(A - B)(B - C)} \\ & + \frac{e^{Ct}(C + k^0_{-3})}{(A - C)(B - C)}\Bigg], \end{aligned} \tag{A7}$$

where A, B, and C are the solutions of the cubic equation $s^3 + \lambda_1 s^2 + \lambda_2 s + \lambda_3 = 0$ with

$$\begin{aligned} \lambda_1 &= k_1 + k^0_2 + k_3 + k^0_{-1} + k^0_{-3}, \\ \lambda_2 &= k^0_{-3}\left(k_1 + k^0_2 + k_3 + k^0_{-1}\right) + \left(k_1 + k_3\right)\left(k^0_{-1} + k^0_2\right) \\ &\quad - \left(k^0_{-1}k_1 + k^0_{-3}k_3\right), \\ \lambda_3 &= \left(k_1 + k_3\right)\left(k^0_{-1} + k^0_2\right)k^0_{-3} \\ &\quad - \left(k^0_2 + k^0_{-1}\right)k^0_{-3}k_3 - k^0_{-3}k^0_{-1}k_1. \end{aligned} \tag{A8}$$

3. Uncompetitive inhibition

In this type of inhibition, the inhibitor has no structural analogy with the substrate. The inhibitor binds to a site other than the active site only if the substrate is already present.

Unlike competitive inhibition, uncompetitive inhibition cannot be reduced by adding more substrate molecules. The underlying mechanism is

$$E + S \underset{k^0_{-1}}{\overset{k^0_1}{\rightleftharpoons}} ES \overset{k^0_2}{\to} P + E,\ E' \overset{\Delta}{\to} E,$$

$$ES + I \underset{k^0_{-4}}{\overset{k^0_4}{\rightleftharpoons}} ESI. \tag{A9}$$

In the limit of $k_3 = k^0_{-3} = 0$, Eqs. (A3)–(A5) yield the following expression for the waiting time distribution for non-competitive inhibition:

$$f(t) = k_1 k^0_2 \left[\frac{e^{At}(A + k^0_{-4})}{(A-B)(A-C)} - \frac{e^{Bt}(B + k^0_{-4})}{(A-B)(B-C)} + \frac{e^{Ct}(C + k^0_{-4})}{(A-C)(B-C)} \right], \tag{A10}$$

where A, B, and C are the solutions of the cubic equation $s^3 + \lambda_1 s^2 + \lambda_2 s + \lambda_3 = 0$ with

$$\begin{aligned} \lambda_1 &= k_1 + k^0_2 + k_4 + k^0_{-1} + k^0_{-4}, \\ \lambda_2 &= k^0_{-4}\left(k_1 + k^0_2 + k_4 + k^0_{-1}\right) + k_1\left(k^0_{-1} + k^0_2 + k_4\right) \\ &\quad -\left(k^0_{-4}k_4 + k^0_{-1}k_1\right), \\ \lambda_3 &= k_1\left(k^0_{-1} + k_4 + k^0_2\right)k^0_{-4} \\ &\quad -k^0_{-4}k^0_{-1}k_1 - k_1 k^0_{-4}k_4. \end{aligned} \tag{A11}$$

Equations (A5), (A7), and (A10) are the waiting time distributions for mixed, competitive, and uncompetitive inhibitions, respectively.

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