Single-enzyme approach predicts natural emergence of inhibitor-activator duality

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The classical theory of enzymatic inhibition aims to quantitatively describe the effect of certain molecules—called inhibitors—on the progression of enzymatic reactions, but "non-classical effects" and "anomalies" which seem to fall beyond its scope have forced practitioners and others to repeatedly patch and mend it ad-hoc. For example, depending on concentrations, some molecules can either inhibit, or facilitate, the progression of an enzymatic reaction. This duality gives rise to non-monotonic dose response curves which seriously complicate high throughput inhibitor screens and drug development, but it is widely believed that the three canonical modes of inhibition—competitive, uncompetitive, and mixed—cannot account for it. To critically test this view, we take the single enzyme perspective and rebuild the theory of enzymatic inhibition from the bottom up. We find that accounting for multi-conformational enzyme structure and intrinsic randomness cannot undermine the validity of classical results in the case of competitive inhibition; but that it should strongly change our view on the uncompetitive and mixed modes of inhibition. In particular, we show that inhibitor-activator duality is inherent to these modes of "inhibition", and state—in terms of experimentally measurable quantities—a condition assuring its emergence. Fundamental and practical implications of our findings are discussed.

Enzymes spin the wheel of life by catalyzing a myriad of chemical reactions central to the growth, development, and metabolism of all living organisms[1,2]. Without enzymes, essential processes would progress so slowly that life would virtually grind to a halt; and some enzymatic reactions are so critical that inhibiting them may even result in immediate death. Enzymatic inhibitors could thus be potent poisons[3,4] but could also be used as antibiotics[5,6] and as drugs to treat other forms of disease[7,8]. Inhibitors have additional commercial uses[9,10], but the fundamental principles which govern their interaction with enzymes are not always understood in full, and have yet ceased to fascinate those interested in the basic aspects of enzyme science. The canonical description of enzymatic inhibition received much exposure and can be found in various texts[1,2,11]. Its limitations are, however, much less appreciated, and while serious attempts to draw attention to this fact and tackle some of the inherent difficulties have been made[12], they have so far been limited by the same bulk based approach that crippled the classical theory from its very inception.

Single molecule approaches have revolutionized our understanding of enzymatic catalysis[13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33], but similar studies of enzymatic inhibition trail behind and are just starting to emerge[34,35,36,37,38]. Given the revolutionary potential of these studies it is somewhat surprising that our understanding of enzymatic inhibition is still based, by and large, on experiments made in the bulk and on a theory, that is now many decades old. Rebuilding the theory of enzymatic inhibition from the bottom up thus looks appealing but is easier said than done. Stochastic, single-molecule, descriptions of inhibited enzymatic catalysis exist, but these are oftentimes based on simple kinetic schemes that fail to capture the multi-conformational nature of enzymes, or properly account for

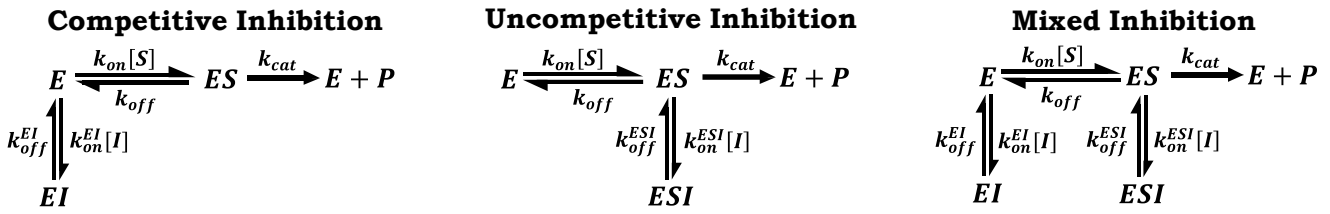

Figure 1. The three canonical modes of enzymatic inhibition (from left to right): competitive, uncompetitive and mixed. Rates govern transitions between the different states: free enzyme (E), enzyme-substrate complex (ES), enzyme-inhibitor complex (EI), enzyme-substrate-inhibitor complex (ESI), and the $(E+P)$ state which represents the end of a turnover cycle.

intrinsic randomness at the microscopic level. From a mathematical perspective, these kinetic schemes are usually built as Markov chains, and while expanding those to account for additional complexity is—in principle—always possible, this then necessitates the introduction of a large number of parameters. These, not only complicate the analysis, but also make it extremely difficult to identify universal results and principles which are key ingredients of any successful theory. Here, we circumvent these problems by adopting a generic, not necessarily Markovian, description of enzymatic catalysis. This approach has recently allowed us to dispense many of the restrictive assumptions that are usually made to shed new light on the role of unbinding in uninhibited enzymatic reactions[39]; and have furthermore opened the door for fundamental advancements to be made in the theory of first passage time processes[40,41,42]. Below, we extend this approach to treat inhibited enzymatic reactions and show that this has far reaching implications on conventional wisdom in this field.

The classical theory of enzymatic inhibition considers the effect of molecular inhibitors on enzymatic reactions in the bulk, and focuses on three canonical modes of inhibition (Fig. 1). In this theory, the concentrations of enzyme, substrate, inhibitor, and the various complexes formed are taken to be continuous quantities and differential equations are written to describe their evolution in time. Assuming that inhibitor molecules can bind either to the free enzyme, E, or the enzyme substrate complex, ES, as in the case of mixed inhibition (Fig. 1), and that all complexes reach fast equilibrium (the quasi-steady-state approximation), it can be shown that the per enzyme turnover rate, k_{turn}, of an inhibited enzymatic reaction obeys[11]

$$\frac{1}{k_{turn}} = \frac{K_m\left(1+\frac{[I]}{K_{EI}}\right)}{v_{max}}\frac{1}{[S]} + \frac{\left(1+\frac{[I]}{K_{ESI}}\right)}{v_{max}} \quad . \quad (1)$$

Here, $[S]$ and $[I]$ respectively denote the concentrations of substrate and inhibitor, v_{max} is the maximal, per enzyme, turnover rate attained at an excess of substrate and no inhibition, K_m is the so-called Michaelis constant—the substrate concentration required for the rate of the uninhibited reaction to reach half its maximal value, and K_{EI} and K_{ESI} denote the equilibrium constants related with reversible association of the inhibitor to form the molecular complexes EI and ESI, respectively. The constants, K_m, K_{EI} and K_{ESI} could then be expressed through the rates of the elementary processes in Fig. 1 as $K_m = (k_{off} + k_{cat})/k_{on}$, $K_{EI} = k_{off}^{EI}/k_{on}^{EI}$, and $K_{\mathrm{ESI}} = k_{off}^{ESI}/k_{on}^{ESI}$, where k_{on} and k_{off} are the rates at which the *substrate* binds and unbinds the enzyme, and k_{on}^{EI} (k_{on}^{ESI}) and k_{off}^{EI} (k_{off}^{ESI}) are the rates at which the *inhibitor* binds and unbinds the enzyme (enzyme-substrate complex). Finally, note that turnover rates for the special cases of competitive and uncompetitive inhibition can be deduced from Eq. (1) by taking the $K_{\mathrm{ESI}} \to \infty$ and $K_{\mathrm{EI}} \to \infty$ limits respectively.

The kinetic schemes described in Fig. 1 also serve as a starting point for a single-molecule theory of enzymatic inhibition. This theory is fundamentally different from the bulk one as it should describe the *stochastic* act of a single enzyme embedded in a "sea" of substrate, and inhibitor, molecules. However, the main observable here is once again the turnover rate, k_{turn}, which is defined as the mean number of product molecules generated by a single enzyme per unit time. Equivalently, this rate can also be defined as the inverse of the average turnover time, $\langle T_{turn} \rangle$, defined as the mean time between successive product formation events. Adopting $\langle T_{turn} \rangle \equiv 1/k_{turn}$ as a convention, the rate based description in Fig.1 can be interpreted as a Markovian scheme which governs the state-to-state transitions of a single enzyme, and it can once again be shown that Eq. (1) holds (SI).

Beyond the Classical Theory. The kinetic schemes presented in Fig. (1) do not account for additional enzymatic states that are often part of the reaction, and it is a priori unclear how these could affect the validity of the result in Eq. (1). For example, it is often necessary to discriminate between distinct enzyme-substrate complexes, but this could be done in a multitude of ways (Fig. 2 left), and the effect of inhibition should then be worked out on a case-by-case basis. This could work well when relevant states and transition rates can be determined experimentally, but doing so is often not possible technically or simply too laborious. Indeed, in the overwhelming majority of cases the number of intermediates and the manner in which they interconvert is simply unknown, resulting in a dire need for a description that will allow these to be effectively taken into account when information is missing or unspecified. Such description would also be useful when trying to generalize lessons learned from the analysis of simple case studies of enzymatic inhibition.

Generic reaction schemes could be built by retaining the same state space as in the classical approach (Fig. 1) while replacing the all so familiar transition rates with generally distributed transition times. This is done in order to account for the coarse grained nature of states, and allows for an equivalent, but much more concise, description of complex reaction schemes. The time for the completion of a transition between two states is then characterized by a generic probability density function (PDF), e.g., $f_{T_{cat}}(t)$ in the case of the catalysis time, T_{cat}, which governs the transition between the *ES* and *E+P* states above (Fig. 2 right). Applied to all other transitions, an infinitely large collection of reactions schemes could then be analyzed collectively, potentially revealing striking universalities that have so far managed to remain hidden.

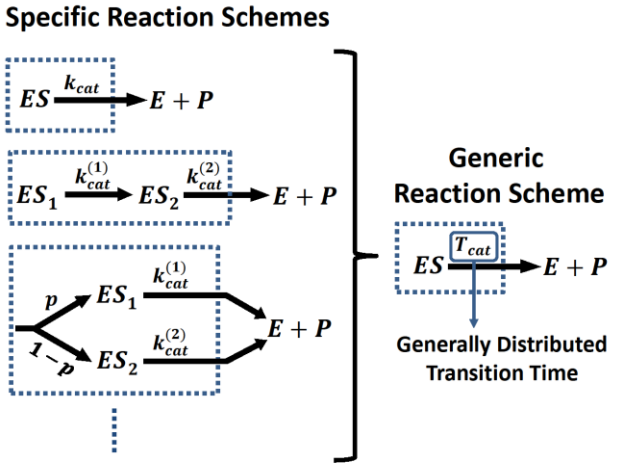

Figure 2. Kinetic intermediates and multiple reaction pathways could complicate the description of a reaction or various parts of it. When all intermediates and rates are known, these complications could, in principle, be addressed on a case by case basis. Alternatively, one could account for the non-Markovian nature of transitions between coarse grained states by allowing for generally, rather than exponentially, distributed transition times. The main advantage of this approach is that it allows for progress to be made even when the underlying reaction schemes are not known in full, i.e., in the absence of perfect information.

Competitive Inhibition at the Single-Enzyme Level. To concretely exemplify the approach proposed above we consider a generic, not necessarily Markovian, scheme for competitive inhibition at the single-enzyme level (Fig. 3). As

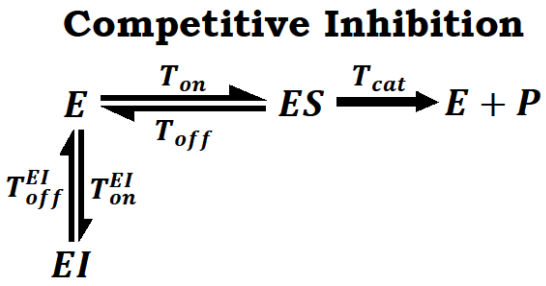

Figure 3. A generic scheme for competitive inhibition at the single enzyme level in which transition rates were replaced by generally distributed transition times.

usual in this mode of inhibition, the inhibitor can bind reversibly to the enzyme to form an enzyme-inhibitor complex which in turn prevents substrate binding and product formation. However, and in contrast to the Markovian approach, here we do not assume that the catalysis time T_{cat} is taken from an exponential distribution with rate k_{cat}, but rather let this time come from an arbitrary distribution. Since the enzyme is single but the substrate and inhibitor are present in Avogadro numbers, we assume that the binding times T_{on} and T_{on}^{EI} are taken from exponential distributions with rates $k_{on}[S]$ and $k_{on}^{EI}[I]$ correspondingly, but the distributions of the off times T_{off} and T_{off}^{EI} are once again left unspecified. We then find that the turnover rate of a single enzyme obeys (SI)

$$\frac{1}{k_{turn}} = \frac{K_m\left(1+\frac{[I]}{K_{EI}}\right)}{v_{max}}\frac{1}{[S]} + \frac{1}{v_{max}}. \quad (2)$$

Note that despite the fact that it is much more general, Eq. (2) shows the exact same dependencies on the substrate and inhibitor concentrations as in the classical theory (Eq. (1) in the limit $K_{\mathrm{ESI}} \to \infty$). This result is non-trivial, and turns out to hold irrespective of the mechanisms which govern the processes of catalysis and unbinding. However, and in contrast to Eq. (1), the constants K_{EI}, v_{max} and K_m, which enter Eq. (2), can no longer be expressed in terms of simple rates, and are rather given by (SI): $K_{EI} = \left(\langle T_{off}^{EI}\rangle k_{on}^{EI}\right)^{-1}$, $v_{max} = \Pr\left(T_{cat} < T_{off}\right)/\langle W_{ES}^0\rangle$ and $K_m = (k_{on}\langle W_{ES}^0\rangle)^{-1}$, where $\Pr\left(T_{cat} < T_{off}\right)$ is the probability that catalysis occurs prior to substrate unbinding, $\langle W_{ES}^0\rangle = \langle \min\left(T_{cat}, T_{off}\right)\rangle$ is the mean life time of the *ES* state, and $\langle T_{off}^{EI}\rangle$ is the mean life time of the *EI* state. Concluding, we see that while particular microscopic details of the reaction do enter Eq. (2), they only do so to determine various effective constants, and that the functional dependencies on [S] and [I] are unaffected by this and are in this sense completely universal.

Uncompetitive Inhibition at the Single-Enzyme Level. The situation is very different for the generic case of uncompetitive inhibition (Fig. 4 top) where the multi-conformational nature of enzymes may lead to strong deviations from the classical behavior. To see this, we follow a similar path to that taken above and obtain a generalized equation for the turnover rate of a single enzyme in the presence of uncompetitive inhibitors (SI)

$$\frac{1}{k_{turn}} = \frac{K_m}{v_{max}}\frac{A([I])}{[S]} + \frac{\left(1+\frac{[I]}{K_{ESI}}\right)B([I])}{v_{max}}, \quad (3)$$

where $K_{ESI} = \left(\langle T_{off}^{ESI}\rangle k_{on}^{ESI}\right)^{-1}$. Equation (3) should be compared to Eq. (1) in the limit $K_{\mathrm{EI}} \to \infty$, and we once again see that both exhibit the same characteristic 1/[S] dependence. Dependence on inhibitor concentration is, however, different from that in Eq. (1) as Eq. (3) also includes two additional factors, $A([I])$ and $B([I])$, which could be understood in terms of average life times and transition probabilities, but are otherwise complicated functions of [I] (Methods). Specifically, we find that $A(0) = B(0) = 1$ in all cases; and moreover note that in the special case where transition times are exponentially distributed, i.e., when the schemes presented in Fig. 4 (top) and Fig. 1 (middle) coincide, $A([I]) = B([I]) = 1$ for all [I]. As expected, Eq. (3) then reduces to Eq. (1) in the limit $K_{\mathrm{EI}} \to \infty$, but in all other cases analyzed

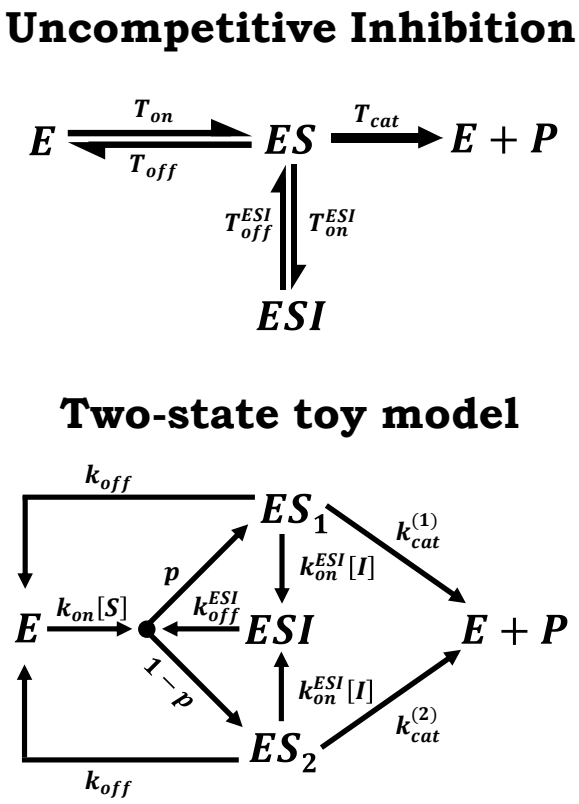

Figure 4. Top. A generic scheme for uncompetitive inhibition at the single enzyme level. Transition rates were once again replaced with generally distributed transition times. **Bottom.** A two-state toy model that is a particular instance of the generic scheme above. Binding of a substrate to the enzyme can occur in one of two ways with probabilities p and $(1-p)$, each leading to a different enzyme substrate complex (ES_1 or ES_2), that is furthermore equipped with a distinct catalytic rate ($k_{cat}^{(1)}$ or $k_{cat}^{(2)}$). The inhibitor binds each of the enzyme-substrate complexes with the same rate, $k_{on}^{ESI}[I]$, and when it unbinds these states are once again reached with probabilities p and $(1-p)$. Taking the stochastic transition times T_{on}, T_{off}, T_{on}^{ESI} and T_{off}^{ESI} to be exponentially distributed with rates $k_{on}[S]$, k_{off}, $k_{on}^{ESI}[I]$ and k_{off}^{ESI} respectively, and the PDF of the catalysis time T_{cat} as specified in the main text, the top and bottom schemes can be shown equivalent (SI).

this is no longer true and the classical theory simply breaks down.

To start and understand the reasons for this breakdown, and demonstrate the type of novel phenomena that may resultantly emerge, we consider—for illustration purposes—a model which is a simple generalization of the classical kinetic scheme for uncompetitive inhibition. Namely, we take $f_{T_{cat}}(t) = pk_{cat}^{(1)}\exp\left(-k_{cat}^{(1)}t\right) + (1-p)k_{cat}^{(2)}\exp\left(-k_{cat}^{(2)}t\right)$ with $0 \le p \le 1$, for the PDF of the catalysis time in Fig. 4 (top), and keep all other transitions times exponential. When written down explicitly (Fig. 4 bottom), the reaction scheme for this model can be seen to include two different enzyme-substrate complexes, ES_1 and ES_2, hence the two-state model, and we find that whenever $k_{cat}^{(1)} \neq k_{cat}^{(2)}$ the multi-conformational nature of the enzyme renders $A([I])$ and $B([I])$ monotonically decreasing functions of [I] (Fig. 5A & SI for explicit expressions). Specifically, this means that $A([I]), B([I]) \le 1$ for all [I], and we find that deviations from unity are linear with $[I]$ when inhibitor concentrations are low; and that for high inhibitor concentrations both $A([I])$ and $B([I])$ eventually plateau at a certain level. Since this level could be much lower than unity, the variation in $A([I])$ and $B([I])$ may strongly affect the turnover rate in Eq. (3).

The fact that $A([I])$ and $B([I])$ are predicted to depend on the concentration of the inhibitor in all but the simplest of cases has far reaching consequences. Consider, for example, Eq. (3) in the limit where substrate concentrations are very high and note that we then have $k_{turn}^{-1}([S] \to \infty) \simeq \left(1 + \frac{[I]}{K_{ESI}}\right) B([I])/v_{max}$. Any deviation from the classical linear relation between k_{turn}^{-1} and [I] is then due to $B([I])$ (Fig. 5b), and is thus a measurable telltale sign of non-Markovian kinetics.

Another important ramification is illustrated in Fig. 5C, where we plot the turnover rate from Eq. (3) directly. The classical theory predicts that turnover should be a monotonically decreasing function of inhibitor concentration, but in contrast to the case of competitive inhibition, here we find that this behavior is not universal. Specifically, in the two state model, we see that when $k_{cat}^{(1)} \gg k_{cat}^{(2)}$ (but also when

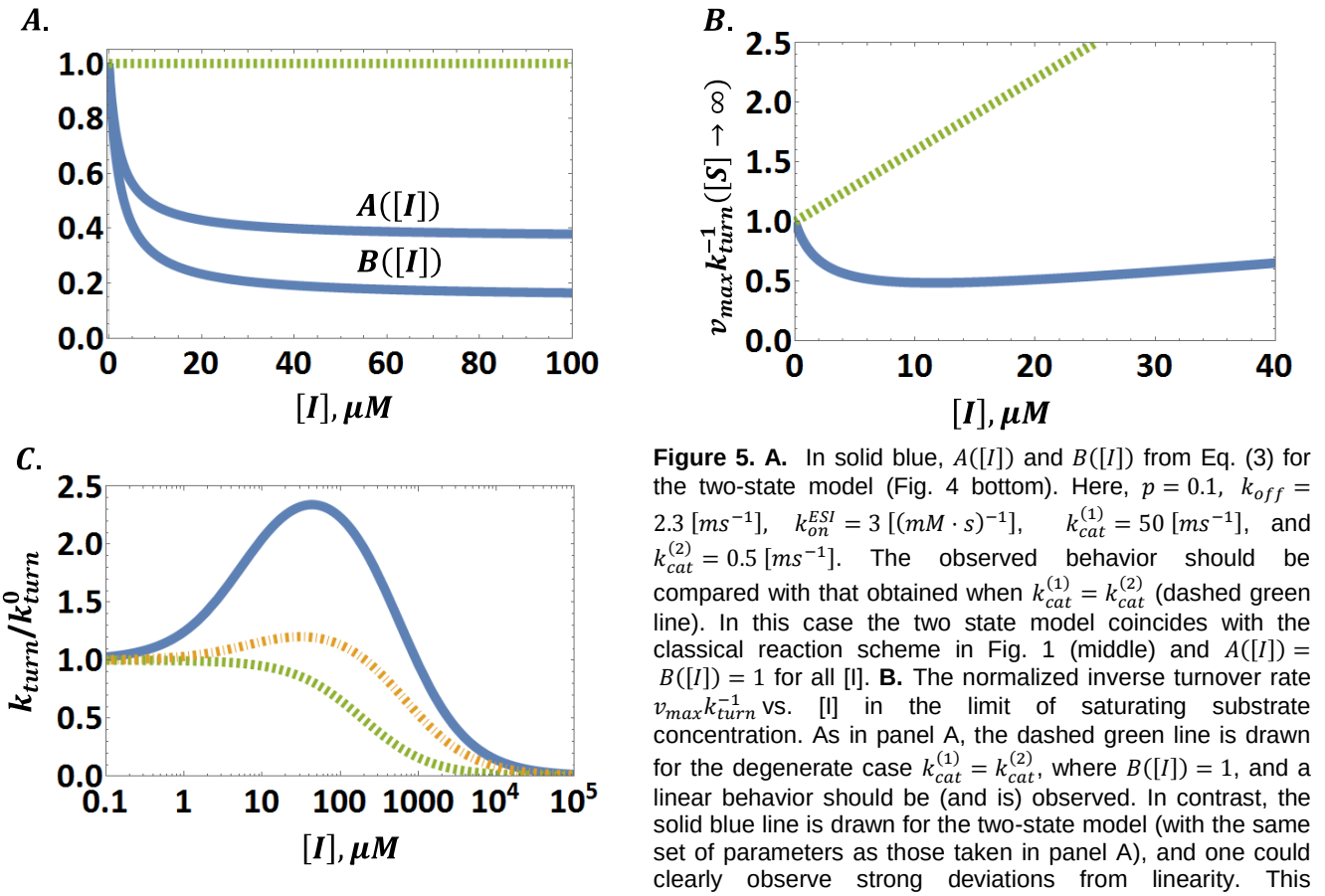

Figure 5. A. In solid blue, $A([I])$ and $B([I])$ from Eq. (3) for the two-state model (Fig. 4 bottom). Here, $p = 0.1$, $k_{off} = 2.3\ [ms^{-1}]$, $k_{on}^{ESI} = 3\ [(mM \cdot s)^{-1}]$, $k_{cat}^{(1)} = 50\ [ms^{-1}]$, and $k_{cat}^{(2)} = 0.5\ [ms^{-1}]$. The observed behavior should be compared with that obtained when $k_{cat}^{(1)} = k_{cat}^{(2)}$ (dashed green line). In this case the two state model coincides with the classical reaction scheme in Fig. 1 (middle) and $A([I]) = B([I]) = 1$ for all [I]. **B.** The normalized inverse turnover rate $v_{max}k_{turn}^{-1}$ vs. [I] in the limit of saturating substrate concentration. As in panel A, the dashed green line is drawn for the degenerate case $k_{cat}^{(1)} = k_{cat}^{(2)}$, where $B([I]) = 1$, and a linear behavior should be (and is) observed. In contrast, the solid blue line is drawn for the two-state model (with the same set of parameters as those taken in panel A), and one could clearly observe strong deviations from linearity. This characteristic signature of non-Markovian kinetics is directly measurable. **C.** The turnover rate, normalized by its value in the absence of inhibition, vs. [I] for the two-state model with: (i) $k_{cat}^{(1)} = k_{cat}^{(2)} = 0.5\ [ms^{-1}]$ (dashed green); (ii) $k_{cat}^{(1)} = 50\ [ms^{-1}]$, $k_{cat}^{(2)} = 2.5\ [ms^{-1}]$ (dash-dot orange); and (iii) $k_{cat}^{(1)} = 50\ [ms^{-1}]$, $k_{cat}^{(2)} = 0.5\ [ms^{-1}]$ (solid blue). Other parameters (common to all lines drawn) were taken to be $p = 0.1$, $k_{off} = 2.3\ [ms^{-1}], k_{on} = 0.2\ [(mM \cdot s)^{-1}], [S] = 1.2\ [\mu M], k_{on}^{ESI} = 3\ [(mM \cdot s)^{-1}]$ and $k_{off}^{ESI} = 50\ [ms^{-1}]$. In sharp contrast to what is predicted by the classical theory, we observe that turnover may exhibit a non-monotonic dependence on inhibitor concentration.

differences between catalytic rates are not as drastic) the presence of an "inhibitor" may surprisingly facilitate enzymatic activity. This effect is most pronounced at low-to-moderate inhibitor concentrations, and we see that at higher inhibitor concentrations—where $A([I])$ and $B([I])$ have reached their asymptotic values—further increasing [I] decreases the turnover rate. Thus, depending on its concentration—and the inner workings of the enzyme—the same molecule could act either as an inhibitor or as an activator.

Binding an inhibitor prevents the formation of a product, but in the two state model it could also act as an effective switch between fast and slow catalytic states. If one state is characterized by a catalytic rate that is much higher than that of the other ($k_{cat}^{(1)} \gg k_{cat}^{(2)}$), the time scale separation allows for inhibitor binding to be just as frequent so as to quickly terminate the slow catalytic pathway, but just as infrequent (hence the need for relatively low inhibitor concentrations) so as not to interrupt catalysis when it occurs rapidly enough (often through the fast pathway). When an inhibitor molecule binds it is then usually to the "slow state" (ES_2 above), but when it unbinds there is some chance that the system instead returns to the "fast state" (ES_1 above). If the *ESI* complex is

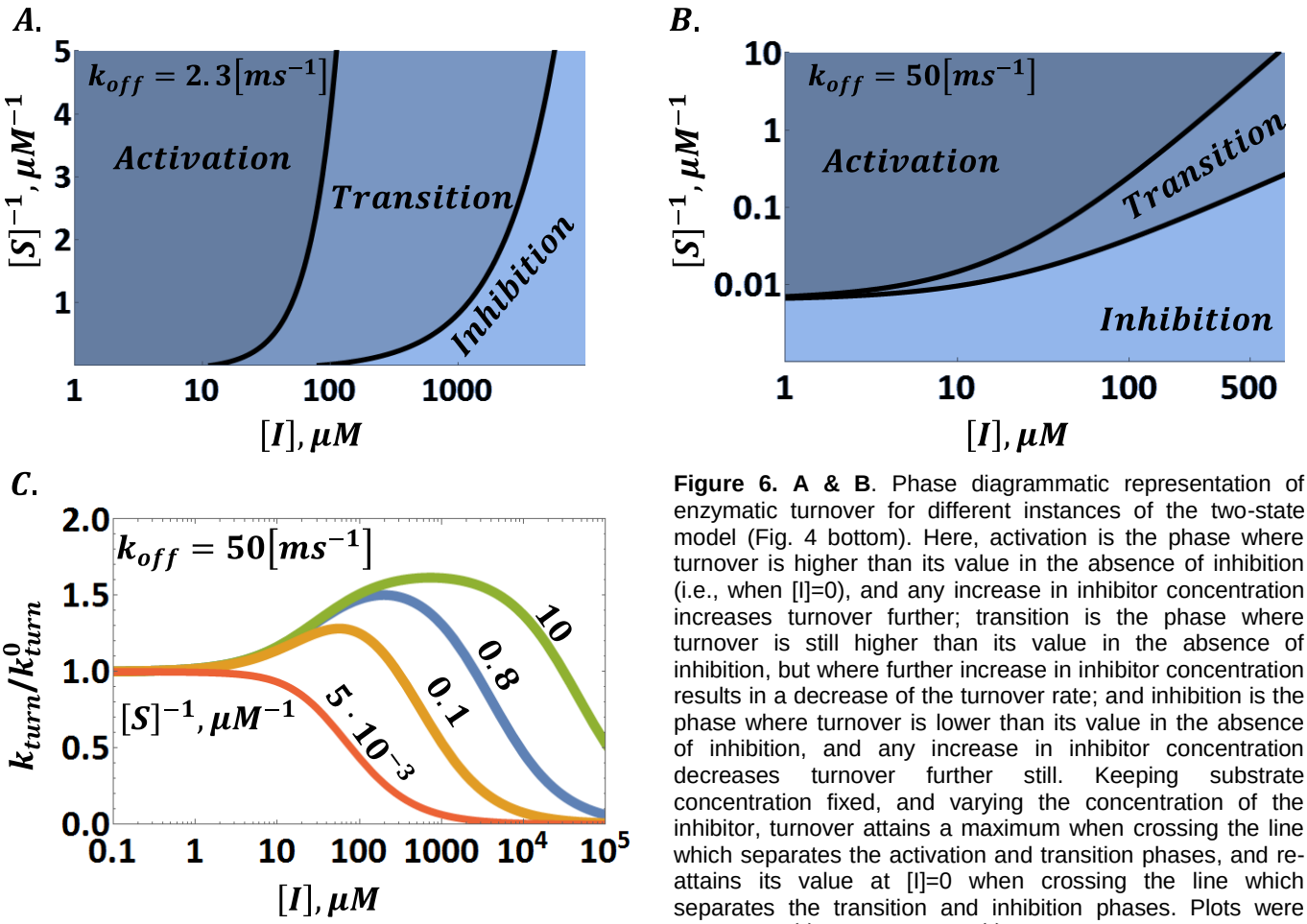

Figure 6. A & B. Phase diagrammatic representation of enzymatic turnover for different instances of the two-state model (Fig. 4 bottom). Here, activation is the phase where turnover is higher than its value in the absence of inhibition (i.e., when [I]=0), and any increase in inhibitor concentration increases turnover further; transition is the phase where turnover is still higher than its value in the absence of inhibition, but where further increase in inhibitor concentration results in a decrease of the turnover rate; and inhibition is the phase where turnover is lower than its value in the absence of inhibition, and any increase in inhibitor concentration decreases turnover further still. Keeping substrate concentration fixed, and varying the concentration of the inhibitor, turnover attains a maximum when crossing the line which separates the activation and transition phases, and re-attains its value at [I]=0 when crossing the line which separates the transition and inhibition phases. Plots were made for $k_{cat}^{(1)} = 50\ [ms^{-1}]$, $k_{cat}^{(2)} = 0.5\ [ms^{-1}]$, $p = 0.1$, $k_{on} = 0.2\ [(mM \cdot s)^{-1}]$, $k_{on}^{ESI} = 3\ [(mM \cdot s)^{-1}]$ and $k_{off}^{ESI} = 50[ms^{-1}]$. The value of k_{off} is listed in the top left corner of each panel. **C.** Lateral cross-sections through panel B showing the turnover rate, normalized by its value in the absence of inhibition, as a function of [I]. The activation phase in panel B corresponds to the ascending branch of the curves in panel C, whereas the transition and inhibition phases correspond to the part of the descending branch of the curves which respectively lies above, and below, unity. Substrate concentrations, corresponding to where cross-sections in panel B were taken, are indicated next to each curve.

moreover relatively short lived, this type of switching could greatly facilitate turnover.

The net effect resulting from uncompetitive inhibition also depends on substrate concentrations as is demonstrated in Figs. 6A & 6B where we dissect the {[I],1/[S]} plane into three, qualitatively distinct, phases. As before, we observe that as inhibitor concentrations increase an activator-inhibitor transition may take place, but it can now also be seen that the manner in which this transition unfolds depends on the concentration of the substrate (Fig. 6A), and that in some cases a transition only occurs when this concentration is low enough (Figs. 6B & 6C), or not at all (see discussion above). The challenge is then to provide a general condition asserting the onset of inhibitor-activator duality in enzymatic catalysis.

Enzymatic reactions may involve a large number of intermediate states and reaction pathways and could thus be markedly more complex than the two-state model considered above. However, rather than analyzing additional case studies one at a time, the approach developed herein allows us to treat an infinite collection of reaction schemes in a joint

and unified manner, and without making any additional assumptions. Analyzing the generic reaction scheme for uncompetitive inhibition (Fig. 4 top) we find (SI) that a condition asserting the emergence of inhibitor-activator duality (i.e., asserting that $dk_{turn}/d[I]|_{[I]=0} > 0$), can be written in terms of experimentally measurable quantities as

$$\overbrace{\frac{\langle T_{off}^{ESI}\rangle}{\langle W_{ES}^{0}\rangle}}^{\substack{ratio\ of\ mean\\ life\ times}} < \overbrace{\frac{1}{2}\left[CV_{W_{ES}^{0}}^{2}-1\right]}^{\substack{contribution\ from\\ statistical\ fluctuations\ in\\ life\ time\ of\ ES\ complex}} \qquad (4)$$

$$+\overbrace{\left[1-\frac{\langle W_{ES}^{0}\mid ES\rightarrow E+P\rangle}{\langle W_{ES}^{0}\rangle}\right]}^{\substack{contribution\ from\\ bias\ in\ breakdown\\ of\ ES\ complex}} / \overbrace{\left[\frac{[S]}{K_m+[S]}\right]}^{\substack{fraction\ of\ time\\ spent\ at\ ES\\ complex}} .$$

Here, $\langle T_{off}^{ESI}\rangle$, on the left hand side, is the mean life time of the *ESI* complex, but all other terms which enter Eq. (4) depend only on the kinetics of the uninhibited enzyme. Specifically, $\langle W_{ES}^{0}\rangle$ is the mean life time of the *ES* complex in the absence of inhibition, and we see that the ratio between $\langle T_{off}^{ESI}\rangle$ and $\langle W_{ES}^{0}\rangle$ should be small enough for the condition in Eq. (4) to hold.

The right hand side of Eq. (4) is composed of two terms. The first, accounts for statistical fluctuations in the life time of the *ES* complex in the absence of inhibition. Specifically, $CV_{W_{ES}^{0}}^{2} = \sigma^2(W_{ES}^{0})/\langle W_{ES}^{0}\rangle^2$ is the normalized variance of this life time, and we see that the contribution coming from this term is positive when $CV_{W_{ES}^{0}}^{2} > 1$. Moreover, we see that when $CV_{W_{ES}^{0}}^{2}$ is large enough the inequality in Eq. (4) will most certainly hold and the emergence of an activator-inhibitor transition is guaranteed. The second term in Eq. (4) accounts for a possible bias in the breakdown of the *ES* complex in the absence of inhibition. Here, $\langle W_{ES}^{0}\mid ES\rightarrow E+P\rangle$ stands for the mean life time of this complex given that its breakdown resulted in product formation. This mean conditional lifetime could be shorter, or longer, than the (unconditional) mean lifetime $\langle W_{ES}^{0}\rangle$ and we see that when it is shorter, i.e., when a product formation event also implies a shorter lifetime on average, the contribution coming from this term is positive. Low enough substrate concentrations will then assert the emergence of an activator-inhibitor transition, and an expression for the exact critical concentration at which this happens can be readily attained by rearrangement. A probabilistic derivation of Eq. (4) and thorough explanation of the intuition and rational behind it are given in the methods section.

Conclusions & Outlook. How would the average rate at which an enzyme converts substrate into product change in the presence of a molecule whose binding to the enzyme *completely* shuts down its ability to catalyze? As we have shown, the answer to this question is not as simple and straightforward as it seems and curiously depends on the mode of inhibition, the molecular inner workings of the enzyme, and is further subject to a delicate interplay between substrate and inhibitor concentrations. The classical theory of inhibition provides no clue to this, but the single-enzyme approach taken herein shows that a molecule whose binding prevents enzymatic activity will act as an inhibitor when in high concentrations, but may change its skin and act as an activator when its abundance is low. This finding not only exposes fundamental flaws in our current understanding of enzymatic inhibition, but also has direct practical implications as inhibitors are in widespread commercial use.

To illustrate this, we take for example the case of DAPT, a compound tested and verified to act as an inhibitor of γ-secretase. Developed and researched for over a decade, this once promising treatment of Alzheimer's disease was eventually abandoned when it was discovered that when administered at low concentrations,

and when substrate concentrations were also low, it acted as an activator[43,44,45,46]. More awareness to this issue would have surely resulted in earlier discovery of the biphasic response, saving precious time and money, but our findings suggest that this may be the tip of the iceberg. Inhibitor-activator duality is inherent to the uncompetitive mode of inhibition, and while it has so far been explained using specially tailored reactions schemes, we have shown that these are not needed. Moreover, the emergence of the effect could be predicted based on the stochastic kinetics of the enzyme in the absence of inhibition, and Eq. (4) further implies that the effect may even be observed in enzymes exhibiting multi-conformational (non-Markovian) kinetics from the kind that has already been documented in the past[15,16,17].

Concluding, we note that the mixed mode of inhibition is subject to the same type of analysis applied above. In this case we find (SI)

$$\frac{1}{k_{turn}} = \frac{K_m\left(1+\frac{[I]}{K_{EI}}\right)}{v_{max}}\frac{\mathrm{A}([\mathrm{I}])}{[S]} + \frac{\left(1+\frac{[I]}{K_{ESI}}\right)B([I])}{v_{max}}, \quad (5)$$

and an equation analogous to Eq. (4) could also be obtained (SI). In fact, all of the results in this paper could be derived starting from Eq. (5), which generalizes Eq. (1) and should moreover replace it in future discussions of enzymatic inhibition. Specifically, note that the structure of Eq. (5), and that of Eqs. (2) & (3) as special cases, casts doubt on the ability of classical methods, e.g., that of Lineweaver & Burk[47], to reliably discriminate between different modes of enzymatic inhibition, and suggests that these should also be revised. Finally, we note that while the framework considered herein allows for arbitrary, rather than exponentially, distributed transition times between kinetic states, it still retains the common assumption (also used in the stochastic derivation of Eq. (1)) that the system "forgets" the state of origin after leaving it[48]. Accounting for memory of past states could be important in certain cases, but the incorporation of a general form of such memory into the framework presented herein currently seems out of reach. Progress in this direction is an important future challenge and is anticipated to advance both theory and practice.

Methods.

I. Definition of $A([I])$ and $B([I])$ in Eqs. (3) and (5). $A([I])$ and $B([I])$ are defined using two auxiliary functions

$$\tilde{f}_M(s) = \int_0^\infty e^{-st} K_m k_{on}\, \bar{F}_{T_{cat}}(t)\bar{F}_{T_{off}}(t)dt\,, \quad (M1)$$

and

$$\tilde{f}_P(s) = \int_0^\infty e^{-st}\frac{K_m k_{on}}{v_{max}} f_{T_{cat}}(t)\bar{F}_{T_{off}}(t)dt\,. \quad (M2)$$

Here, v_{max} and K_m are defined as they were right after Eq. (2) in the main text, $\bar{F}_{T_{cat}}(t) = \int_t^\infty f_{T_{cat}}(t)\,dt$, and $\bar{F}_{T_{off}}(t) = \int_t^\infty f_{T_{off}}(t)\,dt$. It can then be shown that (SI)

$$A([I]) = \frac{1-\frac{k_{on}^{ESI}[I]}{K_m k_{on}}\tilde{f}_M\left(k_{on}^{ESI}[I]\right)}{\tilde{f}_P\left(k_{on}^{ESI}[I]\right)} = \frac{1-\langle W_{ES}\rangle/\langle T_{on}^{ESI}\rangle}{\Pr\left(T_{cat}<T_{off},T_{on}^{ESI}\right)/\Pr\left(T_{cat}<T_{off}\right)}, \quad (M3)$$

and

$$B([I]) = \frac{\tilde{f}_M\left(k_{on}^{ESI}[I]\right)}{\tilde{f}_P\left(k_{on}^{ESI}[I]\right)} = \frac{\langle W_{ES}\rangle \Pr\left(T_{cat}<T_{off}\right)}{\langle W_{ES}^0\rangle \Pr\left(T_{cat}<T_{off},T_{on}^{ESI}\right)}, \quad (M4)$$

where $\langle W_{ES}^0\rangle = \left\langle \min\left(T_{cat},T_{off}\right)\right\rangle$ and $\langle W_{ES}\rangle = \left\langle \min\left(T_{cat},T_{off},T_{on}^{ESI}\right)\right\rangle$ are correspondingly the mean life times of the *ES* complex with and without inhibition.

II. Probabilistic derivation of Eq. (4). When will the *introduction* of an uncompetitive inhibitor increase the turnover rate? Consider the difference between a scenario where inhibitor molecules are not present, and a

scenario where they are present at exceedingly low concentrations. Any interaction between the *ES* complex and an inhibitor molecule would then be very rare but will eventually happen, at some point in time, and we would like to determine the effect this has on the average time it takes the reaction cycle to complete.

After the inhibitor binds, an *ESI* complex is formed. It then takes the inhibitor $\langle T_{off}^{ESI} \rangle$ units of time, on average, to unbind, and for the enzyme another $\langle T_{turn}^{0} \rangle - \langle T_{on} \rangle$ units of time to form a product after having just returned to the *ES* state. Here, the mean turnover time in the absence of inhibition, $\langle T_{turn}^{0} \rangle = \frac{1}{k_{turn}^{0}} = \frac{K_m}{v_{max}} \frac{1}{[S]} + \frac{1}{v_{max}}$, was used since inhibitor concentrations were assumed to be exceedingly low. This allows us to safely neglect the probability the enzyme encounters an inhibitor again within the remaining span of the turnover cycle, and one then only needs to note that the mean substrate binding time $\langle T_{on} \rangle$ was subtracted from $\langle T_{turn}^{0} \rangle$ because the reaction continues from the *ES* state rather than starts completely anew. In total, a product will then be formed after $\langle T_{remain}^{1} \rangle = \langle T_{off}^{ESI} \rangle + \langle T_{turn}^{0} \rangle - \langle T_{on} \rangle$ units of time on average.

Suppose now that instead of having the inhibitor bind the *ES* complex as described above, the reaction would have simply carried on uninterruptedly from that point onward, i.e., as it would in the absence of inhibition. How much time would it then take it to complete? To answer this, we observe that the inhibitor encountered the *ES* complex at a random point in time, as opposed to immediately after its formation. Having already spent some amount of time at the *ES* state, the mean time remaining before the system exits this state need not necessarily be identical to the mean life time, $\langle W_{ES}^{0} \rangle$, of a freshly formed *ES* complex in the absence of inhibition. Indeed, the time we require here is the mean *residual* life time of the *ES* complex, i.e., starting from the random point in time at which it encountered the inhibitor and onward. A key result in renewal theory then asserts that, when averaged over all possible encounter times, the mean residual life time is given by $\frac{1}{2}\langle W_{ES}^{0} \rangle + \frac{1}{2}\frac{\sigma^2(W_{ES}^{0})}{\langle W_{ES}^{0} \rangle}$ [49], where $\sigma^2(W_{ES}^{0})$ denotes the variance in W_{ES}^{0}. This time could be larger, or smaller, than the mean life time $\langle W_{ES}^{0} \rangle$, and the two are equal only when $\sigma^2(W_{ES}^{0}) = \langle W_{ES}^{0} \rangle^2$—as happens, for example, in the case of the exponential distribution.

After the system exits the *ES* state two things could happen. If a product is formed the reaction there ends. Otherwise, the enzyme reverts back to its free state, and the reaction takes, on average, another $\langle T_{turn}^{0} \rangle$ units of time to complete. When the enzyme first enters the *ES* state the probability that a product is formed is $\Pr(T_{cat} < T_{off})$. What is, however, the probability that a product is formed from an *ES* complex that is first observed at some random point in time as in the scenario described above? Looking at the total time an enzyme spends at the *ES* state across many turnover cycles, this probability should coincide with the relative time fraction taken by *ES* visits which end in product formation, and this is given by $\Pr(T_{cat} < T_{off}) \langle W_{ES}^{0} \mid T_{cat} < T_{off} \rangle / \langle W_{ES}^{0} \rangle = \Pr(T_{cat} < T_{off}) \langle W_{ES}^{0} \mid ES \to E + P \rangle / \langle W_{ES}^{0} \rangle$. Summing the contributions above we see that when the reaction is left to proceed in an uninterrupted manner a product will be formed, on average, after $\langle T_{remain}^{0} \rangle = \langle T_{turn}^{0} \rangle \big(1 - \Pr(T_{cat} < T_{off}) \langle W_{ES}^{0} \mid ES \to E + P \rangle / \langle W_{ES}^{0} \rangle\big) + \frac{1}{2}\langle W_{ES}^{0} \rangle + \frac{1}{2}\frac{\sigma^2(W_{ES}^{0})}{\langle W_{ES}^{0} \rangle}$ units of time.

Concluding, we observe that for the introduction of an inhibitor to facilitate turnover one must have $\langle T_{remain}^{0} \rangle > \langle T_{remain}^{1} \rangle$, or equivalently

$$\langle T_{off}^{ESI} \rangle < \langle T_{on} \rangle + \frac{\langle W_{ES}^{0} \rangle}{2}\left[1 + \frac{\sigma^2(W_{ES}^{0})}{\langle W_{ES}^{0} \rangle^2}\right] \quad \text{(M5)}$$

$$- \langle T_{turn}^0 \rangle \frac{\Pr\left(T_{cat} < T_{off}\right) \langle W_{ES}^0 \mid ES \to E + P \rangle}{\langle W_{ES}^0 \rangle}.$$

Recalling that $\langle T_{on} \rangle = (k_{on}[S])^{-1}$, $v_{max} = \Pr\left(T_{cat} < T_{off}\right)/\langle W_{ES}^0 \rangle$ and $K_m = (k_{on}\langle W_{ES}^0 \rangle)^{-1}$, Eq. (M7) can be rearranged and shown equivalent to Eq. (4) in the main text. An alternative derivation of Eq. (4) is given in the SI.

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References

[1] Jeremy M. Berg, John L. Tymoczko, Gregory J. Gatto, Lubert Stryer (2015). Biochemistry (8th ed.). San Francisco: W.H. Freeman. ISBN 1-4641-2610-0.

[2] David L. Nelson, Michael M. Cox (2017). Lehninger Principles of Biochemistry (7th ed.). San Francisco: W.H. Freeman. ISBN 1-4641-2611-9.

[3] Vetter, J., 1998. Toxins of Amanita phalloides. Toxicon, 36(1), pp.13-24.

[4] Holmes, C.F.B., Maynes, J.T., Perreault, K.R., Dawson, J.F. and James, M.N.G., 2002. Molecular enzymology underlying regulation of protein phosphatase-1 by natural toxins. Current medicinal chemistry, 9(22), pp.1981-1989.

[5] Poehlsgaard, J. and Douthwaite, S., 2005. The bacterial ribosome as a target for antibiotics. Nature Reviews Microbiology, 3(11), pp.870-881.

[6] Drawz, S.M. and Bonomo, R.A., 2010. Three decades of β-lactamase inhibitors. Clinical microbiology reviews, 23(1), pp.160-201.

[7] Endo, A., 1992. The discovery and development of HMG-CoA reductase inhibitors. Journal of lipid research, 33(11), pp.1569-1582.

[8] Wlodawer, A. and Vondrasek, J., 1998. Inhibitors of hiv-1 protease: A major success of structure-assisted drug design 1. Annual review of biophysics and biomolecular structure, 27(1), pp.249-284.

[9] Duke, S.O., 1990. Overview of herbicide mechanisms of action. Environmental health perspectives, 87, p.263.

[10] Tan, S., Evans, R. and Singh, B., 2006. Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino acids, 30(2), pp.195-204.

[11] House, J.E., 2007. Principles of chemical kinetics. Academic Press.

[12] Baici, A., 2015. Kinetics of Enzyme-Modifier Interactions: Selected Topics in the Theory and Diagnosis of Inhibition and Activation Mechanisms. Springer.

[13] Funatsu, T., Harada, Y., Tokunaga, M., Saito, K. and Yanagida, T., 1995. Imaging of single fluorescent molecules and individual ATP turnovers by single myosin molecules in aqueous solution. Nature 374, pp. 555 – 559.

[14] Lu, H.P., Xun, L. and Xie, X.S., 1998. Single-molecule enzymatic dynamics. Science, 282(5395), pp.1877-1882.

[15] Edman, L. and Rigler, R., 2000. Memory landscapes of single-enzyme molecules. Proceedings of the National Academy of Sciences, 97(15), pp.8266-8271.

[16] Flomenbom, O., Velonia, K., Loos, D., Masuo, S., Cotlet, M., Engelborghs, Y., Hofkens, J., Rowan, A.E., Nolte, R.J., Van der Auweraer, M. and de Schryver, F.C., 2005. Stretched exponential decay and correlations in the catalytic activity of fluctuating single lipase molecules. Proceedings of the National Academy of Sciences of the United States of America, 102(7), pp.2368-2372.

[17] English, B.P., Min, W., Van Oijen, A.M., Lee, K.T., Luo, G., Sun, H., Cherayil, B.J., Kou, S.C. and Xie, X.S., 2006. Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revisited. Nature chemical biology, 2(2), pp.87-94.

[18] Min, W., Gopich, I.V., English, B.P., Kou, S.C., Xie, X.S. and Szabo, A., 2006. When does the Michaelis-Menten equation hold for fluctuating enzymes?. The Journal of Physical Chemistry B, 110(41), pp.20093-20097.

[19] Wiita, A.P., Perez-Jimenez, R., Walther, K.A., Gräter, F., Berne, B.J., Holmgren, A., Sanchez-Ruiz, J.M. and Fernandez, J.M., 2007. Probing the chemistry of thioredoxin catalysis with force. Nature, 450(7166), pp.124-127.

[20] Lomholt MA, Urbakh M, Metzler R, Klafter J (2007) Manipulating single enzymes by an external harmonic force. Phys Rev Lett 98(16):168302.

[21] Gumpp, H., Puchner, E.M., Zimmermann, J.L., Gerland, U., Gaub, H.E. and Blank, K., 2009. Triggering enzymatic activity with force. Nano letters, 9(9), pp.3290-3295.

[22] Alegre-Cebollada, J., Perez-Jimenez, R., Kosuri, P. and Fernandez, J.M., 2010. Single-molecule force spectroscopy approach to enzyme catalysis. Journal of Biological Chemistry, 285(25), pp.18961-18966.

[23] Moffitt, J.R., Chemla, Y.R. and Bustamante, C., 2010. Mechanistic constraints from the substrate concentration dependence of enzymatic fluctuations. Proceedings of the National Academy of Sciences, 107(36), pp.15739-15744.

[24] Puchner, E.M. and Gaub, H.E., 2012. Single-molecule mechanoenzymatics. Annual review of biophysics, 41, pp.497-518.
[25] Claessen, V.I., Engelkamp, H., Christianen, P.C., Maan, J.C., Nolte, R.J., Blank, K. and Rowan, A.E., 2010. Single-biomolecule kinetics: the art of studying a single enzyme. Annual Review of Analytical Chemistry, 3, pp.319-340.
[26] Van Oijen, A.M., 2011. Single-molecule approaches to characterizing kinetics of biomolecular interactions. Current opinion in biotechnology, 22(1), pp.75-80.
[27] Wu, J. and Cao, J., 2011. Generalized Michaelis–Menten Equation for Conformation-Modulated Monomeric Enzymes. Single-Molecule Biophysics: Experiment and Theory, Volume 146, pp.329-365.
[28] Choi, Y., Moody, I.S., Sims, P.C., Hunt, S.R., Corso, B.L., Perez, I., Weiss, G.A. and Collins, P.G., 2012. Single-molecule lysozyme dynamics monitored by an electronic circuit. Science, 335(6066), pp.319-324.
[29] Chaudhury, S., Cao, J. and Sinitsyn, N.A., 2013. Universality of Poisson indicator and Fano factor of transport event statistics in ion channels and enzyme kinetics. The Journal of Physical Chemistry B, 117(2), pp.503-509.
[30] Lu, H.P., 2014. Sizing up single-molecule enzymatic conformational dynamics. Chemical Society Reviews, 43(4), pp.1118-1143.
[31] Moffitt, J.R. and Bustamante, C., 2014. Extracting signal from noise: kinetic mechanisms from a Michaelis–Menten-like expression for enzymatic fluctuations. FEBS Journal, 281(2), pp.498-517.
[32] Barato, A. C. and Seifert, U. Skewness and kurtosis in statistical kinetics, Phys. Rev. Letters, (2015) 115, 188103.
[33] Barato, A. C., and Seifert, U. Universal bound on the Fano factor in enzyme kinetics, J. Phys. Chem. B (2015) 119, 6555−6561.
[34] Gorris, H.H., Rissin, D.M. and Walt, D.R., 2007. Stochastic inhibitor release and binding from single-enzyme molecules. Proceedings of the National Academy of Sciences, 104(45), pp.17680-17685.
[35] Piwonski, H.M., Goomanovsky, M., Bensimon, D., Horovitz, A. and Haran, G., 2012. Allosteric inhibition of individual enzyme molecules trapped in lipid vesicles. Proceedings of the National Academy of Sciences, 109(22), pp.E1437-E1443.
[36] Chaudhury, S.Poisson indicator and fano factor for probing dynamic disorder in single-molecule enzyme inhibition kinetics, J. Phys. Chem. B (2014) 118, 10405−10412.
[37] Saha, S., Sinha, A. and Dua, A., 2012. Single-molecule enzyme kinetics in the presence of inhibitors. The Journal of chemical physics, 137(4), p.045102.
[38] Pelz, B., Žoldák, G., Zeller, F., Zacharias, M. and Rief, M., 2016. Subnanometre enzyme mechanics probed by single-molecule force spectroscopy. Nature communications, 7.
[39] Reuveni, S., Urbakh, M. and Klafter, J., 2014. Role of substrate unbinding in Michaelis–Menten enzymatic reactions. Proceedings of the National Academy of Sciences, 111(12), pp.4391-4396.
[40] Rotbart, T., Reuveni, S. and Urbakh, M., 2015. Michaelis-Menten reaction scheme as a unified approach towards the optimal restart problem. Physical Review E, 92(6), p.060101.
[41] Reuveni, S., 2016. Optimal stochastic restart renders fluctuations in first passage times universal. Physical review letters, 116(17), p.170601.
[42] Pal, A. and Reuveni, S., 2016. First Passage Under Restart. arXiv preprint arXiv:1607.06048.
[43] Lanz, T.A., Karmilowicz, M.J., Wood, K.M., Pozdnyakov, N., Du, P., Piotrowski, M.A., Brown, T.M., Nolan, C.E., Richter, K.E., Finley, J.E. and Fei, Q., 2006. Concentration-dependent modulation of amyloid-β in vivo and in vitro using the γ-secretase inhibitor, LY-450139. Journal of Pharmacology and Experimental Therapeutics, 319(2), pp.924-933.
[44] Burton, C.R., Meredith, J.E., Barten, D.M., Goldstein, M.E., Krause, C.M., Kieras, C.J., Sisk, L., Iben, L.G., Polson, C., Thompson, M.W. and Lin, X.A., 2008. The amyloid-β rise and γ-secretase inhibitor potency depend on the level of substrate expression. Journal of Biological Chemistry, 283(34), pp.22992-23003.
[45] Svedružić, Ž.M., Popović, K. and Šendula-Jengić, V., 2013. Modulators of γ-secretase activity can facilitate the toxic side-effects and pathogenesis of Alzheimer's disease. *PloS one*, *8*(1), p.e50759.
[46] Walsh, R., 2014. Are improper kinetic models hampering drug development?.*PeerJ*, *2*, p.e649.
[47] Lineweaver, H. and Burk, D., 1934. The determination of enzyme dissociation constants. Journal of the American Chemical Society, 56(3), pp.658-666.
[48] In this sense, the approach presented in this paper could be said to be semi-Markovian.
[49] Gallager, R.G., 2013. *Stochastic processes: theory for applications*. Cambridge University Press.