FEATURE ARTICLE

Single-Molecule Michaelis−Menten Equations

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Received: March 22, 2005; In Final Form: June 12, 2005

This paper summarizes our present theoretical understanding of single-molecule kinetics associated with the Michaelis−Menten mechanism of enzymatic reactions. Single-molecule enzymatic turnover experiments typically measure the probability density $f(t)$ of the stochastic waiting time t for individual turnovers. While $f(t)$ can be reconciled with ensemble kinetics, it contains more information than the ensemble data; in particular, it provides crucial information on dynamic disorder, the apparent fluctuation of the catalytic rates due to the interconversion among the enzyme's conformers with different catalytic rate constants. In the presence of dynamic disorder, $f(t)$ exhibits a highly stretched multiexponential decay at high substrate concentrations and a monoexponential decay at low substrate concentrations. We derive a single-molecule Michaelis−Menten equation for the reciprocal of the first moment of $f(t)$, $1/\langle t\rangle$, which shows a hyperbolic dependence on the substrate concentration [S], similar to the ensemble enzymatic velocity. We prove that this single-molecule Michaelis−Menten equation holds under many conditions, in particular when the intercoversion rates among different enzyme conformers are slower than the catalytic rate. However, unlike the conventional interpretation, the apparent catalytic rate constant and the apparent Michaelis constant in this single-molecule Michaelis−Menten equation are complicated functions of the catalytic rate constants of individual conformers. We also suggest that the randomness parameter r, defined as $\langle(t - \langle t\rangle)^2\rangle/\langle t\rangle^2$, can serve as an indicator for dynamic disorder in the catalytic step of the enzymatic reaction, as it becomes larger than unity at high substrate concentrations in the presence of dynamic disorder.

1. Introduction

The catalytic activity of enzymes has long been understood in terms of the Michaelis−Menten mechanism:[1] a substrate S binds reversibly with an enzyme E to form an enzyme−substrate complex ES that undergoes unimolecular decomposition to form a product P, regenerating the original enzyme E via E^0.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E^0 + P \qquad E^0 \xrightarrow{\delta} E \tag{1}$$

The rate of product formation v has a hyperbolic dependence on the substrate concentration [S], i.e., $v = k_2[E]_T[S]/([S] + K_M)$, where $K_M = (k_{-1} + k_2)/k_1$ and $[E]_T$ is the total enzyme concentration. This rate expression, the Michaelis−Menten equation, provides a highly satisfactory description of ensemble-averaged enzyme kinetics.

Recent advances in single-molecule spectroscopy and manipulation[2−16] have now made it possible to study enzymatic reactions at the level of *single* molecules, thus raising the question of whether the Michaelis−Menten equation remains an adequate description of single-molecule kinetics. It is therefore of both conceptual and practical importance to understand how single molecule and ensemble kinetics are reconciled, and what new information is available from single molecule data.

At the single-molecule level, an enzymatic reaction is a stochastic event, and a single-molecule experiment typically measures the waiting times for the completion of the enzymatic reaction. The probability density of these waiting times, $f(t)$, can be obtained by recording the histogram of many turnovers over a long period of time. Therefore, single-molecule kinetics cannot be formulated in terms of enzyme concentrations, but must be formulated instead in terms of the probabilities for the enzyme to be in one of the possible states in the reaction pathway.[17]

We will show that single-molecule and steady-state ensemble kinetics are consistent, in that the reciprocal of the first moment of $f(t)$, $1/\langle t\rangle$, has the same hyperbolic dependence on the substrate concentration as the enzymatic velocity described by the conventional Michaelis−Menten equation. However, $f(t)$ provides much more kinetic information, such as the existence of reaction intermediates and dynamic disorder, which are often obscured by ensemble-averaged measurements. In particular, multiexponentiality in $f(t)$ is a manifestation of dynamic disorder,[4,11,18−34] which refers to fluctuations in the rate constants of the reaction caused by transitions among different enzyme conformers. These fluctuations can occur on a time scale comparable to or longer than that of the enzymatic reaction, so the rate of product formation is no longer governed by a single rate constant, but effectively by a distribution of rate constants.[11,18,26,29]

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10.1021/jp051490q CCC: $30.25
Published on Web 08/02/2005

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A theory of the waiting time distribution $f(t)$ should account for two sets of single-molecule experimental results. The first is the initial rise and subsequent decay of $f(t)$ observed in experiments carried out by Lu et al.,[4] Asbury et al.,[35] and Yasuda et al.[7] Such rise and decay of $f(t)$ is generally attributed to the formation of one or more intermediates and is often characterized by the randomness parameter r introduced by Block and co-workers[36,37] as a measure of the relative magnitudes of the variance and the mean of the waiting time, $r \equiv \langle (t - \langle t \rangle)^2 \rangle / \langle t \rangle^2$. In the absence of dynamic disorder, it has been shown[36,37] that if the reaction has only one rate-limiting step, $r = 1$, whereas if the reaction has more than one rate-limiting step, $r < 1$.

The second result is the observation made by English et al.[16] that $f(t)$ is a highly stretched multiexponential decay at high substrate concentrations and a monoexponential decay at low substrate concentrations. The nonexponential decay of $f(t)$ is generally attributed to dynamic disorder.[4,11,18−34] Furthermore, Lu et al.,[4] Velonia et al.,[14] and English et al.[16] have observed dynamical correlations between successive enzymatic turnover events. Such memory effects are generally associated with slow conformational fluctuations of the enzyme during the course of the experiment.

In this article, we present models based on the Michaelis−Menten mechanism to account for these experimental observations.

Section 2 discusses the single-molecule Michaelis−Menten equation in the absence of dynamic disorder starting from the differential equations that define both the ensemble-averaged and single-molecule Michaelis−Menten kinetics. The conventional Michaelis−Menten equation is obtained from these equations by assuming a steady-state condition.[38] The corresponding single molecule differential equations, on the other hand, can be solved exactly for $f(t)$ without making this assumption. $f(t)$ itself exhibits a rise and decay due to the formation of an enzyme−substrate complex. The steady state condition actually corresponds to a very fast initial rise of $f(t)$. The substrate concentration dependence of the enzymatic rate $1/\langle t \rangle$, calculated from $f(t)$, obeys the Michaelis−Menten like equation.

Section 3 discusses the single-molecule Michaelis−Menten equation in the presence of dynamic disorder. We first consider the simplest case, in which each of the enzyme species in the reaction (E, ES, and E^0) exists in two interconverting conformers with different catalytic rate constants. Expressions for $f(t)$ and $1/\langle t \rangle$ are derived. Under the condition of slow interconversion between the conformers, the dependence of $1/\langle t \rangle$ on [S] is again found to be identical to the ensemble Michaelis−Menten equation, except that the apparent k_2 and K_M of the single-molecule Michaelis−Menten equation have meanings different from their conventional interpretations. Consistent with experimental findings,[16] $f(t)$ changes from a highly stretched multiexponential decay to a monoexponential decay as substrate binding becomes rate limiting. As a generalization of this model, we also consider the physically more realistic case of an arbitrary number of interconverting conformers, which leads to substantially the same conclusions as the two-state model. The slow interconversion among conformers results in the memory effect associated with the correlations between successive enzymatic turnover times.

Section 4 introduces a semi-Markovian (or memoryless) approximation to the kinetic scheme of the previous section in which the catalytic step is assumed to be non-Poissonian with a general multiexponential waiting time distribution. Again, we arrive at the important conclusion that $1/\langle t \rangle$ obeys the Michaelis−Menten equation. We also find, as before, that the kinetic parameters of the single-molecule Michaelis−Menten equation (corresponding to k_2 and K_M in ensemble measurements) have meanings different from their conventional interpretations.

Section 5 discusses the substrate concentration dependence of the randomness parameter r (which is related to the second moment of $f(t)$), with and without dynamic disorder, the treatment of dynamic disorder following the approach discussed in section 3. While it is known that r can be less than unity because of the existence of more than one rate-limiting step,[36,37] we show that r can also be larger than unity because of dynamic disorder. Thus, r can potentially serve as an indicator of dynamic disorder.

A summary of the main results is presented in the final section of the paper. Relevant mathematical details of the calculations are provided in the Appendices.

2. Single-Molecule Michaelis−Menten Kinetics in the Absence of Dynamic Disorder

The Michaelis−Menten mechanism for the enzymatic conversion of substrate S to product P by enzyme E is described

in eq 1. The rate equations for the concentrations of the chemical species in the first reaction are therefore given by

$$\frac{d[E]}{dt} = -k_1[E][S] + k_{-1}[ES] \quad (2)$$

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] \quad (3)$$

$$\frac{d[E^0]}{dt} = \frac{d[P]}{dt} = k_2[ES] \quad (4)$$

where t is the elapsed time from the onset of an ensemble-averaged experiment. The initial conditions are $[ES] = 0$ and $[E^0] = 0$ at $t = 0$. At early times, when very little substrate has been converted to product, the second reaction in eq 1 can be neglected. Because both [E] and [S] are time dependent, eqs 2−4 are nonlinear differential equations and cannot be solved exactly. However, an approximate solution for $v = d[P]/dt$ can be obtained if the concentration of the complex, [ES], is assumed to reach a steady state shortly after the onset of the reaction. This steady-state approximation corresponds to the condition $d[ES]/dt = 0$, and its application to eqs 2−4 is easily shown to lead to the classic Michaelis−Menten equation[38]

$$v = \frac{v_{max}[S]}{[S] + K_M} \quad (5)$$

where v_{max}, defined as $v_{max} = k_2[E]_T$, with $[E]_T = [E] + [ES]$ the total enzyme concentration, is the reaction velocity at saturating substrate concentration, and K_M, the Michaelis constant, defined as $K_M = (k_{-1} + k_2)/k_1$, is the substrate concentration at which the enzymatic velocity is half of v_{max}.

In a turnover experiment, a single enzyme molecule is monitored continuously as it cycles repetitively through the states E, ES, and E^0 in eq 1. The time for the first reaction to complete is now a stochastic variable that can be completely characterized by a waiting time distribution $f(t)$. To derive the rate equations that describe the corresponding single-molecule Michaelis−Menten kinetics, the concentrations in eqs 2−4 are replaced by the probabilities P of finding the single enzyme molecule in the states E, ES, and E^0, leading to the equations

$$\frac{dP_E(t)}{dt} = -k_1^0 P_E(t) + k_{-1}P_{ES}(t) \quad (6)$$

$$\frac{dP_{ES}(t)}{dt} = k_1^0 P_E(t) - (k_{-1} + k_2)P_{ES}(t) \quad (7)$$

$$\frac{dP_{E^0}(t)}{dt} = k_2 P_{ES}(t) \quad (8)$$

which must satisfy the initial conditions $P_E(0) = 1$, $P_{ES}(0) = 0$, and $P_{E^0}(0) = 0$ at $t = 0$ (the time of onset of the reaction), along with the constraint $P_E(t) + P_{ES}(t) + P_{E^0}(t) = 1$. Also, the rate constant for the forward step, k_1^0, is treated as a pseudo-first-order rate constant that can be written as $k_1^0 = k_1[S]$, with [S] assumed to be time-independent. This is reasonable, as there is essentially no depletion of substrate by a single enzyme molecule, and [S] can be considered as a constant. E^0 is converted back to E through the second half reaction in eq 1. Depending on the enzyme system, this can occur either instantaneously (E and E^0, thereby becoming effectively identical[16]), or through another chemical reaction via the ping-pong mechanism.[4]

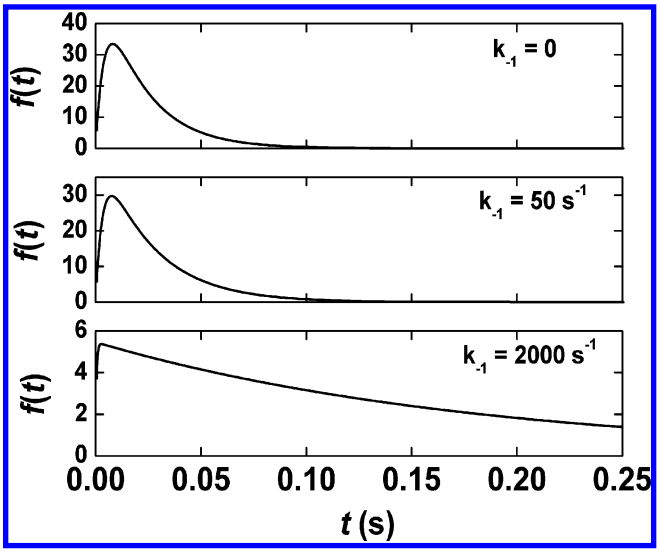

Figure 1. Probability density of the waiting time, $f(t)$, in the absence of dynamic disorder, as calculated from eq 10, for three different values of k_{-1} (0, 50, and 2000 s^{-1}) with $k_1 = 10^7$ M^{-1} s^{-1}, $k_2 = 250$ s^{-1}, and [S] = 0.005 mM.

Consequently, eqs 6−8 become a system of *linear* first-order differential equations, and they can be solved exactly for $P_E(t)$, $P_{ES}(t)$, and $P_{E^0}(t)$. Knowing $P_{E^0}(t)$, the waiting time distribution $f(t)$, which is normalized such that $\int_0^\infty dt\, f(t) = 1$, is obtained as follows: The probability that a turnover occurs between t and $t + \Delta t$ is $f(t)\Delta t$; $f(t)\Delta t$ is the same as the probability that the enzyme is in the state E^0 in the interval between t and $t + \Delta t$, which is $\Delta P_{E^0}(t) = k_2 P_{ES}(t)\Delta t$. Thus, in the limit of infinitesimal Δt,

$$f(t) = dP_{E^0}(t)/dt = k_2 P_{ES}(t) \quad (9)$$

From the solutions of eqs 6−8, and using the above relation for $f(t)$, it is easily shown that[4]

$$f(t) = \frac{k_1 k_2[S]}{2A}[\exp(A + B)t - \exp(B - A)t] \quad (10)$$

where $A = \sqrt{(k_1[S] + k_{-1} + k_2)^2/4 - k_1 k_2[S]}$ and $B = -(k_1[S] + k_{-1} + k_2)/2$, and the substrate concentration dependence [S] has been shown explicitly through the relation $k_1^0 = k_1[S]$.

A plot of $f(t)$ vs t at fixed values of [S] (0.005 mM), k_1 (10^7 M^{-1} s^{-1}), and k_2 (250 s^{-1}) is shown in Figure 1 for three different values of k_{-1} (0, 50, and 2000 s^{-1}.) These values of k_{-1} are illustrative of reactions in which (i) the dissociation of ES to E and S does not occur (top panel), (ii) the catalytic and dissociation rates are roughly comparable (middle panel), and (iii) the rate of dissociation of ES to E and S is significantly larger than the catalytic rate, leading to steady-state formation of ES (bottom panel).

The limit $k_{-1} \to 0$ of the top panel describes the sequential reaction $S + E \xrightarrow{k_1^0} ES \xrightarrow{k_2} E^0 + P$. The waiting time distribution $f(t)$ of such a reaction is the convolution of the waiting time distributions $f_1(t)$ and $f_2(t)$ of the two separate steps, i.e., $f(t) = (f_1 \otimes f_2)(t)$, or $f(t) = \int_0^t dt'\, f_1(t - t')f_2(t')$. If $f_1(t)$ and $f_2(t)$ are $k_1[S]\exp(-k_1[S]t)$ and $k_2\exp(-k_2 t)$, respectively, (implying that the steps $E + S \to ES$ and $ES \to E^0 + P$ are Poisson processes), then $f(t)$ is given exactly by

$$f(t) = \frac{k_1 k_2[S]}{k_2 - k_1[S]}(\exp(-k_1[S]t) - \exp(-k_2 t)) \quad (11)$$

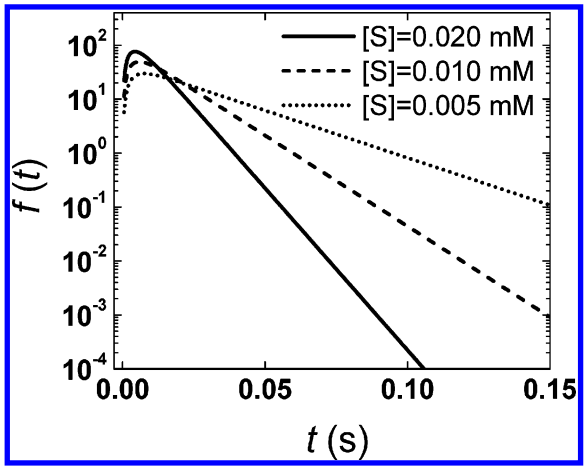

Figure 2. Probability density of the waiting time, $f(t)$, in the absence of dynamic disorder, as calculated from eq 10, for three different values of [S] (0.020 mM, 0.010 mM, and 0.005 mM) with $k_1 = 10^7$ M^{-1} s^{-1}, $k_2 = 250$ s^{-1}, and $k_{-1} = 50$ s^{-1}, respectively.

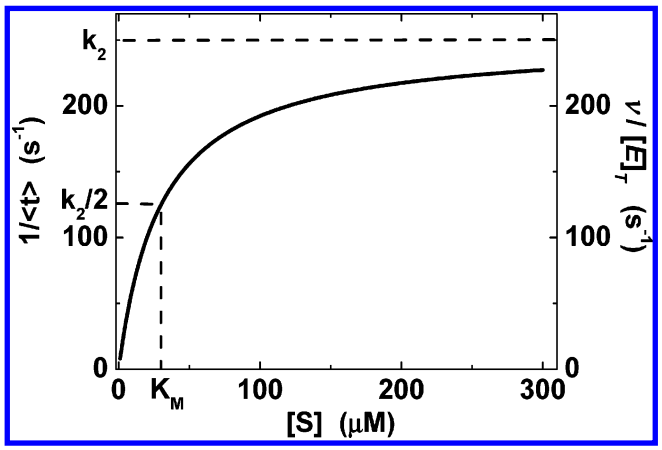

Figure 3. Average reaction rate $1/\langle t\rangle$ or its equivalent $v/[E]_T$, as calculated from eq 14b, the single-molecule Michaelis−Menten equation, as a function of substrate concentration [S] for $K_M = 30$ μM (the value corresponding to $k_{-1} = 50$ s^{-1}, $k_2 = 250$ s^{-1}, and $k_1 = 10^7$ M^{-1} s^{-1}).

and exhibits an exponential rise followed by an exponential decay, corresponding to the generation of the intermediate ES, with the faster of k_1 and k_2 being the rate constant of the rise, and the slower of k_1 and k_2 being the rate constant of the decay.[17]

Another limit of $f(t)$, shown in the bottom panel, exhibits only a single-exponential decay and corresponds to the steady-state limit in which ES is generated essentially immediately. In analogy with the ensemble steady-state approximation, this limit can be expressed analytically as $dP_{ES}(t)/dt = 0$, and typically holds when $k_2 \ll k_{-1}$. Combined with the constraint $P_E(t) + P_{ES}(t) + P_{E^0}(t) = 1$ and the initial condition $P_{E^0}(0) = 0$, the steady-state limit $dP_{ES}(t)/dt = 0$ applied to eqs 6−8 can be shown to lead to

$$P_{E^0}(t) = 1 - \exp\left[-\frac{k_1^{\,0} k_2 t}{k_1^{\,0} + k_{-1} + k_2}\right] \tag{12}$$

which, using eq 9, leads in turn to

$$f(t) = \frac{k_1 k_2[\mathrm{S}]}{k_1[\mathrm{S}] + k_{-1} + k_2} \exp\left[-\frac{k_1 k_2[\mathrm{S}] t}{k_1[\mathrm{S}] + k_{-1} + k_2}\right] \tag{13}$$

confirming the single-exponential decay of $f(t)$. Furthermore, in the limit of high concentration, $f(t)$ reduces to $k_2 \exp(-k_2 t)$, as expected.

The middle panel describes an intermediate case between the two limits described above. At such an intermediate value of k_{-1}, the dependence of $f(t)$ on [S] is illustrated in Figure 2 for fixed values k_1, k_{-1}, and k_2 (10^7 M^{-1} s^{-1}, 50 s^{-1}, and 250 s^{-1}, respectively.)

The first moment of $f(t)$, $\langle t\rangle = \int_0^\infty dt\, tf(t)$, gives the mean waiting time $\langle t\rangle$ for the reaction, from which the connection with the ensemble measurements under steady-state conditions can be made. The reciprocal of $\langle t\rangle$ can be interpreted as an average reaction rate.[39,40] Generally, this arises from the equivalence between time averaging and ensemble averaging. From eq 10, we deduce that

$$\frac{1}{\langle t\rangle} = -\frac{(A^2 - B^2)^2}{2Bk_1k_2[\mathrm{S}]} \tag{14a}$$

$$= \frac{k_2[\mathrm{S}]}{[\mathrm{S}] + K_M} \tag{14b}$$

A comparison of eqs 5 and 14b indicates that $v/v_{max} = 1/(k_2\langle t\rangle)$ or that $v/[E]_T = 1/\langle t\rangle$.

This is a gratifying result, indicating that the first moment of $f(t)$ does indeed recover the classic Michaelis−Menten equation, regardless of whether the steady-state approximation is used in the single-molecule probability calculation. We regard eq 14b as the single-molecule Michaelis−Menten equation.

A plot of $1/\langle t\rangle$ against [S] for the parameters used in the middle panel of Figure 1 is shown in Figure 3, exhibiting the characteristic hyperbolic profile of the classic Michaelis−Menten saturation curve. The fact that $1/\langle t\rangle$ calculated from eq 14b exactly coincides with eq 5 highlights the consistency between the single-molecule and ensemble-averaged kinetics. However, it is important to stress that $f(t)$ does provide more information than only the first moment, such as higher moments and the existence of intermediates. This is particularly true in the presence of dynamic disorder, as will be discussed next.

3. Single-Molecule Michaelis−Menten Kinetics in the Presence of Dynamic Disorder

The expressions for $f(t)$ derived in section 2 are not consistent with measurements on some enzyme systems,[14−16] which show significant multiexponentiality in the waiting time distribution at high substrate concentrations. This behavior can be attributed to dynamic disorder. One way to model dynamic disorder is to assume, as in the approach used by Zwanzig[20] and by Yang and Cao,[28] that the rate constant k_2, or the parameters on which it depends, are stochastic variables that fluctuate according to some prescribed statistics. However, the main goal of the rest of the paper is not to provide specific models for the fluctuations of these stochastic variables, but to explain the multiexponentiality of $f(t)$ and its concentration dependence, and to establish the general applicability of the Michaelis−Menten equation to single-molecule kinetics even in the presence of dynamic disorder.

(i) Two-State Model. To this end, we first consider the simplest extension of the Michaelis−Menten mechanism that incorporates the notion of dynamic disorder. This is the kinetic scheme in which the three states of the enzyme, E, ES, and E^0,

can each exist in two interconverting conformations, as shown below:

$$\begin{array}{ccccccc} S + E_1 & \underset{k_{-11}}{\overset{k_{11}[S]}{\rightleftharpoons}} & ES_1 & \xrightarrow{k_{21}} & P + E_1^{\,0} & \xrightarrow{\delta_1} & E_1 \\ \alpha\downarrow\uparrow\alpha & & \beta\downarrow\uparrow\beta & & \gamma\downarrow\uparrow\gamma & & \\ S + E_2 & \underset{k_{-12}}{\overset{k_{12}[S]}{\rightleftharpoons}} & ES_2 & \xrightarrow{k_{22}} & P + E_2^{\,0} & \xrightarrow{\delta_2} & E_2 \end{array} \quad (15)$$

Even this simple generalization presents theoretical challenges in the calculation of $f(t)$ and its first moment. An immediate complication is that over the course of a long time trajectory, each new reaction cycle begins from either E_1 or E_2 with a probability that reflects the steady-state populations of the various intermediates. This means that $f(t)$ must be calculated from the weighted average

$$f(t) = w_1 f_{T_{E_1}}(t) + w_2 f_{T_{E_2}}(t) \quad (16)$$

where $f_{T_{E_1}}(t)$ and $f_{T_{E_2}}(t)$ are the distributions of the waiting times T_{E_1} and T_{E_2} for the enzyme to complete the reaction starting from E_1 and E_2, respectively, and w_1 and w_2 are the corresponding steady-state probabilities for the enzyme to exist in one or other of these conformations. We find that while w_1 and w_2 can be calculated from the master equation formalism used to derive eqs 6−8, the calculation of $f(t)$ requires a different approach and can only be found in closed form in the Laplace domain. Details of the complete calculation are provided in Appendix A; here we state only the final result, which can be written as

$$\hat{f}(s) = (w_1, w_2, 0, 0)\hat{\mathbf{f}}(s) \quad (17)$$

where $\hat{f}(s)$ is the Laplace transform of $f(t)$, and $\hat{\mathbf{f}}(s) \equiv (\hat{f}_{T_{E_1}}(s), \hat{f}_{T_{E_2}}(s), \hat{f}_{T_{ES_1}}(s), \hat{f}_{T_{ES_2}}(s))^T$ is defined by

$$\hat{\mathbf{f}}(s) = (s\mathbf{I} - \mathbf{Q})^{-1}\mathbf{r} \quad (18)$$

where $\mathbf{I}$ is the identity matrix, $\mathbf{r} \equiv (0, 0, k_{21}, k_{22})^T$, $f_{T_{ES_1}}(t)$ and $f_{T_{ES_2}}(t)$ are the distributions of the waiting times T_{ES_1} and T_{ES_2} for the enzyme to complete the reaction starting from ES_1 and ES_2, respectively, and

$$\mathbf{Q} = \begin{pmatrix} -(\alpha + k_{11}[S]) & \alpha & k_{11}[S] & 0 \\ \alpha & -(\alpha + k_{12}[S]) & 0 & k_{12}[S] \\ k_{-11} & 0 & -(\beta + k_{-11} + k_{21}) & \beta \\ 0 & k_{-12} & \beta & -(\beta + k_{-12} + k_{22}) \end{pmatrix} \quad (19)$$

In the limit of the fast reset of E_1^0 and E_2^0 to E_1 and E_2, corresponding to the condition $\delta_1, \delta_2 \gg 1$, the steady-state weights w_1 and w_2, which satisfy

$$w_1 + w_2 = 1 \quad (20)$$

can be found from

$$\frac{w_1}{w_2} = \frac{k_{21}[\alpha(k_{11}k_{22} + k_{11}k_{-12}) + \alpha\beta(k_{11} + k_{12}) + \beta k_{11}k_{12}[S]]}{k_{22}[\alpha(k_{12}k_{21} + k_{12}k_{-11}) + \alpha\beta(k_{11} + k_{12}) + \beta k_{11}k_{12}[S]]} \quad (21)$$

Equations 16−21 provide the complete solution in Laplace space to the waiting time distribution of the two-state model of dynamic disorder. The first moment of $f(t)$, $\langle t \rangle$, is easily obtained from the formula $\langle t \rangle = -d\hat{f}(s)/ds|_{s=0}$. After lengthy but straightforward algebra, one can show that

$$\frac{1}{\langle t \rangle} = \frac{F^{-1}[S]}{[S] + \dfrac{G[S] + H}{FJ[S] + FK}} \quad (22)$$

where the constants F, G, H, and K are given by

$$F = \frac{2}{k_{21} + k_{22}} \quad (23a)$$

$$G = \alpha(k_{21} - k_{22})\frac{k_{12}(k_{21} + k_{-11}) - k_{11}(k_{22} + k_{-12})}{k_{21} + k_{22}} + \beta[k_{12}(k_{21} + k_{-11}) + k_{11}(k_{22} + k_{-12})] \quad (23b)$$

$$H = 2\alpha(k_{22} + k_{-12})(k_{21} + k_{-11}) + 2\alpha\beta(k_{21} + k_{22} + k_{-11} + k_{-12}) \quad (23c)$$

$$J = \beta k_{11}k_{12}(k_{21} + k_{22}) \quad (23d)$$

$$K = \alpha[k_{11}k_{21}(k_{22} + k_{-12}) + k_{12}k_{22}(k_{21} + k_{-11})] + \alpha\beta(k_{11} + k_{12})(k_{21} + k_{22}) \quad (23e)$$

Interpreting $1/\langle t \rangle$ as the ensemble rate (by the assumption of ergodicity), one sees from eq 22 that this rate does not always obey the Michaelis−Menten equation, which is characteristically hyperbolic in the substrate concentration [S]. However, there are a number of limiting conditions that do produce this hyperbolic relationship. In particular, a Michaelis−Menten-like equation is recovered if one of the following conditions (a−f) holds: (a) $k_{21} \gg \beta$, $k_{22} \gg \beta$, corresponding to the limit in which the catalytic rates k_{21} and k_{22} are much larger than the interconversion rate β between ES_1 and ES_2; (b) $\beta \rightarrow 0$, corresponding to the limit of slow interconversion between ES_1 and ES_2; (c) $\alpha \rightarrow 0$, corresponding to the limit of slow interconversion between E_1 and E_2; (d) $\alpha \rightarrow \infty$, corresponding to the limit of fast interconversion between E_1 and E_2; (e) $(k_{21} + k_{-11})/k_{11} = (k_{22} + k_{-22})/k_{12}$, corresponding to the case where the two channels $E_1 + S \leftrightarrow ES_1 \rightarrow E_1^0 + P$ and $E_2 + S \leftrightarrow ES_2 \rightarrow E_2^0 + P$ have identical Michaelis constants; and (f) $\beta \rightarrow \infty$ and $k_{11} = k_{12}$, corresponding to the limit of fast interconversion between ES_1 and ES_2, and an identical rate constant of interconversion for the steps E_1 to ES_1 and E_2 to ES_2.

Condition (a) is not very stringent, especially in light of recent observations of slow conformational fluctuations.[12,34] If after imposing this condition, one also takes α, the interconversion rate constant between E_1 and E_2, to be small, the disorder is effectively quasi-static. In this quasi-static disorder limit, there is a time scale separation between the fast catalytic reaction and the sluggish interconversions between the conformers of the enzyme and the enzyme−substrate complex. In this limit, it can be shown (see Appendix B) that the steady-state waiting time distribution is well approximated by

$$f(t) = \sum_{i=1}^{2} w_i \frac{k_{1i}k_{2i}[S]}{2A_i}[\exp(A_i + B_i)t - \exp(B_i - A_i)t] \quad (24)$$

where $A_i = \sqrt{(k_{1i}[S] + k_{-1i} + k_{2i})^2/4 - k_{1i}k_{2i}[S]}$, $B_i = -(k_{1i}[S] + k_{-1i} + k_{2i})/2$, and the weights w_1 and w_2 are

$$w_1 = \frac{k_{11}k_{21}(k_{22} + k_{-12})}{k_{11}k_{21}(k_{22} + k_{-12}) + k_{12}k_{22}(k_{21} + k_{-11})}$$

$$w_2 = \frac{k_{12}k_{22}(k_{21} + k_{-11})}{k_{11}k_{21}(k_{22} + k_{-12}) + k_{12}k_{22}(k_{21} + k_{-11})} \quad (25)$$

If $1/\langle t\rangle$ is calculated from eq 24, we arrive at the single-molecule Michaelis−Menten equation for the two conformer case,

$$\frac{1}{\langle t\rangle} = \frac{\chi_2'[S]}{[S] + C_M'} \quad (26)$$

where the apparent catalytic rate constant χ_2' and the apparent Michaelis constant C_M', unlike k_2 and K_M in eq 5, are found to be

$$1/\chi_2' = w_1/k_{21} + w_2/k_{22} \quad (27a)$$

$$C_M' = \chi_2'[w_1K_{M1}/k_{21} + w_2K_{M2}/k_{22}] \quad (27b)$$

with $K_{Mi} \equiv (k_{-1i} + k_{2i})/k_{1i}$. χ_2' is nothing but the weighted harmonic mean of the catalytic rate constants in the two channels, while C_M' is a more complex function of the catalytic and Michaelis constants of the two conformers.

The significance of eq 26 and eq 27 is that single molecule Michaelis−Menten equation holds even under the condition of dynamic disorder, though χ_2' and C_M' have different meanings from k_2 and K_M in the conventional Michaelis−Menten equation. We note that at the *ensemble* level, kinetic schemes involving multiple states similar to eq 15 have been shown[38] to lead to the Michaelis−Menten equation with redefined k_2 and K_M. However, we will show below that this is true for an arbitrary number of conformers.

(ii) Multistate Model. The two-state model of the foregoing section illustrates the effects on single molecule enzymatic trajectories of fluctuations between *pairs* of conformers, but real enzyme systems are likely to interconvert among a much larger number of conformational substates.[12,16,33,34] In this section we therefore consider a generalization of the two-state model in which each of the enzyme species in eq 1, E, ES, and E^0, is allowed to exist in any number n of mutually interconverting conformers. This n-state model of the Michaelis−Menten mechanism leads to the kinetic scheme shown below:

$$\begin{array}{ccccccc}
S + & E_1 & \underset{k_{-11}}{\overset{k_{11}[S]}{\rightleftharpoons}} & ES_1 & \xrightarrow{k_{21}} P + & E_1^0 & \xrightarrow{\delta_1} E_1 \\
 & \alpha_{12} \downarrow\uparrow \alpha_{21} & & \beta_{12} \downarrow\uparrow \beta_{21} & & \gamma_{12} \downarrow\uparrow \gamma_{21} & \\
S + & E_2 & \underset{k_{-12}}{\overset{k_{12}[S]}{\rightleftharpoons}} & ES_2 & \xrightarrow{k_{22}} P + & E_2^0 & \xrightarrow{\delta_2} E_2 \\
 & \vdots & & \vdots & & \vdots & \\
 & \downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & \\
 & \vdots & & \vdots & & \vdots & \\
S + & E_n & \underset{k_{-1n}}{\overset{k_{1n}[S]}{\rightleftharpoons}} & ES_n & \xrightarrow{k_{2n}} P + & E_n^0 & \xrightarrow{\delta_n} E_n
\end{array} \quad (28)$$

In this scheme, it should be understood that E_i not only interconverts with E_{i+1} or E_{i-1}, but does so with all the other conformers as well. And the same is true for the conformer ES_i. The calculation of the waiting time distribution for this scheme follows exactly the approach used earlier, except that many of the steps must be reformulated in terms of matrices. We first calculate the waiting time distributions through different channels; we then determine their steady-state average to obtain the overall waiting time distribution $f(t)$, the experimentally observed quantity. The details of the calculation are lengthy, and are provided in Appendix B. Here we point out only that when the reset of E_1^0, E_2^0, ... to E_1, E_2, ... is much faster than any of the other interconversion steps, it can be shown that under physically meaningful conditions the average enzymatic rate, $1/\langle t\rangle$, again follows a Michaelis−Menten-like equation in which the apparent catalytic rate constant and the apparent Michaelis constant are complicated functions of the various interconversion rates (see Appendix B).

The conditions leading to the Michaelis−Menten form include: (a) the limit in which the catalytic rates are much greater than the interconversion rates of the enzyme−substrate complex ES_i, (b) the limit of extremely slow interconversion between the enzyme−substrate complexes ES_i, (c) the limit of extremely slow interconversion between the enzymes E_i, (d) the limit in which interconversion rates between the E_i's are much greater than all the other rates, (e) the limit in which the Michaelis constants for a given reaction channel are nearly the same, $(k_{21} + k_{-11})/k_{11} = (k_{22} + k_{-22})/k_{12} = ... = (k_{2n} + k_{-1n})/k_{1n}$, and the interconversion rates between the different conformers are symmetric: $\alpha_{ij} = \alpha_{ji}$, $\beta_{ij} = \beta_{ji}$, and (f) the limit in which the interconversion rates β_{ij} and β_{ji} are equal, and are much faster than the other rates.

As in the two-state model, condition (a) is of direct relevance to real enzyme systems. If, after imposing this condition, the interconversion rates between the conformers of the enzyme are also made small, the disorder is effectively quasi-static, and as shown in Appendix B, the waiting time distribution $f(t)$ is then well approximated by

$$f(t) = \frac{1}{\sum_{i=1}^{n} w_i} \sum_{i=1}^{n} w_i \frac{k_{1i}k_{2i}[S]}{2A_i} [\exp(A_i + B_i)t - \exp(B_i - A_i)t] \quad (29)$$

where the w_i are the steady-state weights with which each reaction channel i contributes to the overall waiting time distribution, and the parameters A_i and B_i have the same definitions as the corresponding parameters in eq 24.

The use of eq 29 to calculate the mean enzymatic rate leads once again to the single-molecule Michaelis−Menten equation, in the form

$$\frac{1}{\langle t\rangle} = \frac{\chi_2[S]}{[S] + C_M} \quad (30)$$

where the apparent catalytic rate constant χ_2 and the apparent Michaelis constant C_M can be written as

$$1/\chi_2 = \sum_{i=1}^{n} w_i/k_{2i} \quad (30a)$$

$$C_M = \chi_2 \sum_{i=1}^{n} w_i K_{Mi}/k_{2i} = \chi_2 \sum_{i=1}^{n} w_i (k_{-1i} + k_{2i})/(k_{1i}k_{2i}) \quad (30b)$$

Thus, χ_2 and C_M have the same structure as the corresponding kinetic constants for the two-state model; i.e., χ_2 is the

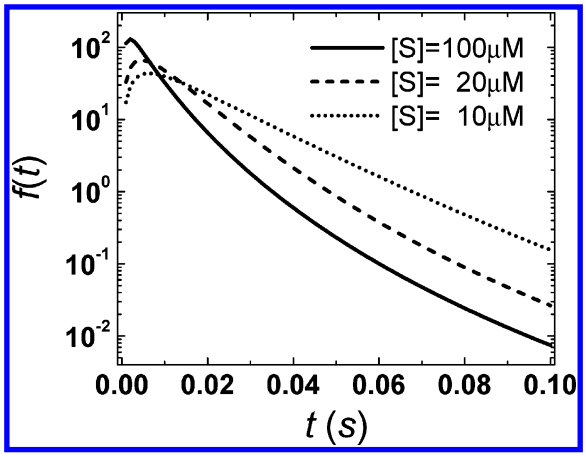

Figure 4. Probability density of the waiting time, $f(t)$, in the presence of dynamic disorder for three different concentrations (10, 20, and 100 μM), as calculated from eq 31, with $k_1 = 10^7$ M^{-1} s^{-1}, $k_{-1} = 50$ s^{-1}, and using the parameter values $a = 6$, $b = 35$ in the gamma distribution $w(k_2) = [1/b^a\Gamma(a)]k_2^{a-1}\exp(-k_2/b)$.

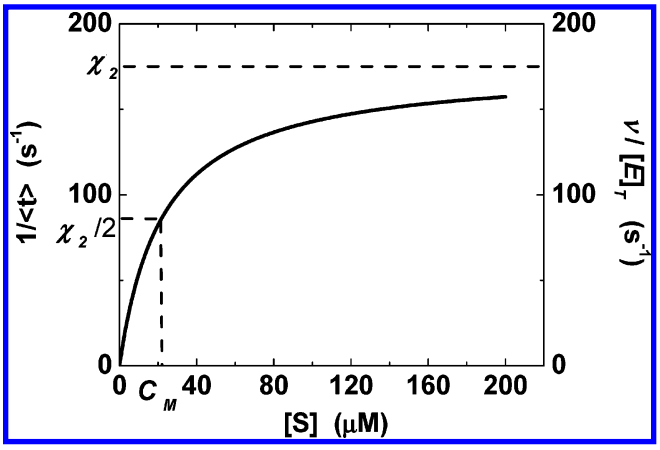

Figure 5. Substrate concentration dependence of the mean enzymatic velocity $1/\langle t\rangle$, or its equivalent $v/[E]_T$, according to the single-molecule Michaelis−Menten equation (eq 30), under the condition of the slow interconversion among conformers of a broad distribution of k_2. The parameters are the same as described in the caption of Figure 4.

weighted harmonic mean of the catalytic rate constants along the individual reaction channels, and C_M is a more complex function of the interconversion rates.

It is also interesting to note that the weights w_i can themselves be expressed in terms of the catalytic efficiency of individual conformers. The catalytic efficiency K_{Ei} of an individual conformer is defined as the ratio of its catalytic rate constant k_{2i} to its Michaelis constant K_{Mi}, i.e., $K_{Ei} = k_{2i}/K_{Mi} = k_{2i}k_{1i}/(k_{-1i} + k_{2i})$; w_i can be shown to be $w_i = K_{Ei}/\sum_{i=1}^{n} K_{Ei}$.

For the purpose of comparison with experiment, it is convenient to simplify eq 29 further by assuming that $k_{11} = k_{12} = ... = k_{1n} \equiv k_1$, and that $k_{-11} = k_{-12} = ... = k_{-1n} \equiv k_{-1}$. Additionally, if n is large (as is generally the case), a continuum approximation can be invoked. These simplifications then lead to

$$f(t) = \int_0^\infty \mathrm{d}k_2\, w(k_2)\frac{k_1k_2[\mathrm{S}]}{2A}[\exp(A+B)t - \exp(B-A)t] \tag{31}$$

where A and B are identical to the corresponding expressions that appear in eq 10. It is reasonable to further assume that the weight function $w(k_2)$ is a gamma distribution, such that $w(k_2) = [1/b^a\Gamma(a)]k_2^{a-1}\exp(-k_2/b)$, a and b being adjustable parameters. With this choice of weight function, the integral in eq 31 can be evaluated exactly. The resulting $f(t)$ is shown in Figure 4 as a function of t at different [S] for the following arbitrary parameter values: $a = 6$, $b = 35$, $k_1 = 10^7$ M^{-1} s^{-1}, and $k_{-1} = 50$ s^{-1}. The curves clearly illustrate how, as the substrate concentration increases, $f(t)$ increasingly departs from single-exponential decay behavior.

These trends are in complete qualitative agreement with experimental results,[16] and they may be explained as follows. At low substrate concentrations, the binding of the enzyme to the substrate is the rate-limiting step in the reaction, so $f(t)$ reflects the statistics of this Poissonian step, which is therefore governed by an exponential distribution. At high substrate concentrations, the dissociation of the enzyme−substrate complex to product is the rate-limiting step in the reaction, so $f(t)$ now reflects the statistics of this step, which is no longer Poissonian (because of dynamic disorder).

The calculation of $1/\langle t\rangle$ from eq 31 using the given expression for $w(k_2)$ readily establishes that $\chi_2 = (a-1)b$ and $C_M = (k_{-1} + \chi_2)/k_1$. Figure 5, showing the variation of $1/\langle t\rangle$ with [S] for the same set of parameters used in Figure 4, is characteristically hyperbolic, as is the ensemble enzymatic velocity.

Since the mean of the gamma distribution, $\bar{k}_2$, is ab, and the variance, Δ, is ab^2, the apparent catalytic rate constant can be written $\chi_2 = \bar{k}_2 - \Delta/\bar{k}_2$. Assuming $a = 6$, $b = 35$, if $\bar{k}_2$ is kept constant at 210 s^{-1} and Δ is increased by a factor of 4, χ_2 decreases from 175 s^{-1} to 70 s^{-1}. This result has interesting implications for the interpretation of different apparent catalytic constants, χ_2. A decrease in χ_2 need not be associated with an overall decrease in the mean catalytic constant, $\bar{k}_2$, but could arise from a larger variance, Δ.

The single-molecule Michaelis−Menten equation for the multiple conformer case, eq 30, explains why the conventional Michaelis−Menten equation, eq 5, is so widely applicable, since even in the presence of dynamic disorder for each single molecule, the hyperbolic concentration dependence of $1/\langle t\rangle$ almost invariably holds. In the presence of dynamic disorder, however, the constants k_2 and K_M in the ensemble Michaelis−Menten equation must be reinterpreted. In the slow interconversion limit, they are now seen to be weighted averages of the kinetic parameters characterizing individual conformers. Thus, the experimental observation of a hyperbolic dependence of the enzyme velocity on the substrate concentration does not imply that eq 1 accurately describes the underlying kinetic scheme, since a more complicated scheme, such as eq 28 can produce seemingly identical results. Pre-steady-state ensemble-averaged measurements can, in principle, distinguish the dispersed kinetics. However, in practice, they often do not have high enough dynamic range for accurate determination of multiexponential kinetics. We demonstrate that single-molecule measurements of $f(t)$ provide a sensitive measure of dynamic disorder.

4. Semi-Markovian (Memoryless) Approximation to Multistate Model

The model of single molecule kinetics based on conformational fluctuations introduced in the previous section provides detailed microscopic interpretations of ensemble rate expressions. However, these expressions [cf. Appendix B] are quite complex, so it is worthwhile to consider alternatives that capture key experimental observations without being algebraically

complex. This section introduces a semi-Markovian approximation to the multistate model that leads to simpler expressions for both the ensemble reaction rate and the waiting time distribution; but the approximation, by its Markovian nature, does not account for the memory effect. In this approximation, the distinct conformational states E_i, ES_i, and E_i^0 in the multistate model are collapsed into a set of effective states **E**, **ES**, and $\mathbf{E}^0$, and the corresponding reaction mechanism can then be written as $\mathbf{E} + S \leftrightarrow \mathbf{ES} \rightarrow \mathbf{E}^0 + P$. In this reduced description, the binding step $\mathbf{E} + S \rightarrow \mathbf{ES}$ and the dissociation step $\mathbf{ES} \rightarrow \mathbf{E} + S$ are assumed to be governed by monoexponential distributions with rate constants $k_1[S]$ and k_{-1} respectively. However, the catalytic step of the reaction, $\mathbf{ES} \rightarrow \mathbf{E}^0 + P$, is assumed to be governed not by a single well-defined rate constant k_2 but by a waiting time distribution $f_{T_C}(t)$, which can be specified arbitrarily. We shall refer to this scheme as a semi-Markovian approximation.[41] (In the context of the kinetic scheme described by eq 28, this semi-Markovian approximation becomes exact if the interconversion rate constants β_{ij} for the complexes ES_i are much larger than the catalytic rate constants k_{2i}, and the interconversion rate constants α_{ij} for the enzyme E_i approach infinity.)

The overall waiting time distribution $f(t)$ in this picture is now a function of $f_{T_C}(t)$ and is shown in Appendix C to be given by

$$\hat{f}(s) = \hat{f}_{T_C}(s + k_{-1}) \Big/ \left(\frac{k_1[S] + s}{k_1[S]} - \frac{k_{-1}}{k_{-1} + s}[1 - \hat{f}_{T_C}(s + k_{-1})] \right) \quad (32)$$

The semi-Markov approximation thus provides a quick way to obtain the waiting time distribution $f(t)$.

The Laplace inverse of eq 32 is not known in closed form for general $f_{T_C}(t)$, but given an expression for $f_{T_C}(t)$, $f(t)$ can be readily calculated from eq 32 numerically. As an example, if the distribution $f_{T_C}(t)$ were described by a sum of three exponentials:

$$f_{T_C}(t) = \sum_{i=1}^{3} a_i \kappa_i \exp(-\kappa_i t) \quad (33)$$

with the a_i satisfying $\sum_{i=1}^{3} a_i = 1$ to ensure normalization of $f_{T_C}(t)$, the calculated $f(t)$ as a function of [S], for some suitable set of parameters k_1, k_{-1}, a_i, and κ_i, is easily shown to reproduce the general trends depicted in Figure 4. In other words, $f(t)$ is a stretched multiexponential decay at high substrate concentration and a single-exponential decay at low concentrations, again in qualitative agreement with experiment.[16]

The average reaction rate $1/\langle t \rangle$ calculated from eq 32 is

$$\frac{1}{\langle t \rangle} = \frac{\gamma_2[S]}{[S] + \Delta_M} \quad (34)$$

where

$$\gamma_2 = \frac{k_{-1}\hat{f}_{T_C}(k_{-1})}{1 - \hat{f}_{T_C}(k_{-1})} \qquad \Delta_M = \frac{k_{-1}}{k_1[1 - \hat{f}_{T_C}(k_{-1})]} \quad (35)$$

Here $\hat{f}_{T_C}(k_{-1})$ means $\hat{f}_{T_C}(s + k_{-1})|_{s=0}$. Parallel with the definition of $K_M = (k_{-1} + k_2)/k_1$, it is readily shown that

$$\Delta_M = \frac{k_{-1} + \gamma_2}{k_1} \quad (36)$$

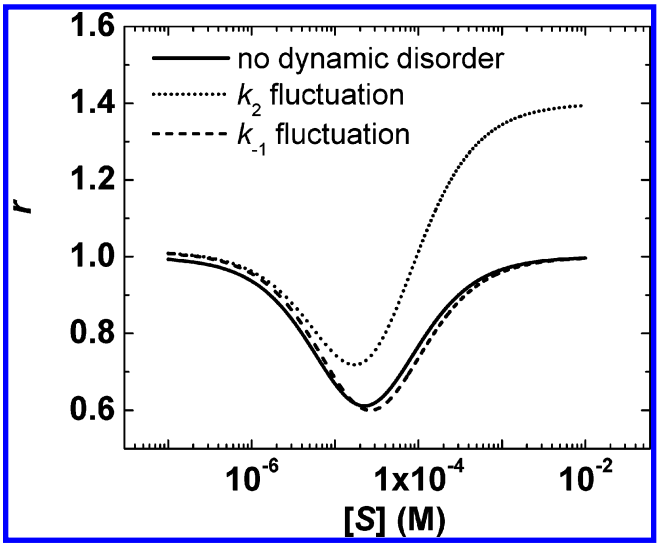

Figure 6. Randomness parameter r vs [S] under the following conditions: (i) no dynamic disorder (full line), calculated using eq 38 with $k_1 = 10^7\ M^{-1}\ s^{-1}$, $k_2 = 250\ s^{-1}$, and $k_{-1} = 50\ s^{-1}$; (ii) dynamic disorder present in the catalytic step (dotted line), calculated using eq 31, with $k_1 = 10^7\ M^{-1}\ s^{-1}$, $k_{-1} = 50\ s^{-1}$, and $a = 6$, $b = 35$ in $w(k_2)$, with the mean of k_2 assigned the value $175\ s^{-1}$; (iii) dynamic disorder present in the dissociation step (dashed line), calculated using eq 31 according to the method described in the text, with $k_1 = 10^7\ M^{-1}\ s^{-1}$, $k_2 = 175\ s^{-1}$, and $a = 6$, $b = 10$ in $w(k_2)$, with the mean of k_{-1} assigned the value $50\ s^{-1}$.

As in the microscopic model of dynamic disorder given in section 3, the semi-Markovian approximation also leads, in this case directly, without the imposition of additional constraints, to the Michaelis−Menten equation, with the ensemble parameters k_2 and K_M replaced by quantities (γ_2 and Δ_M, respectively) related to the waiting time distribution of the catalytic step. The classic Michaelis−Menten parameters are recovered when $f_{T_C}(t)$ is described by a single exponential, $k_2 \exp(-k_2 t)$.

It is worth noting again that since the above treatment invokes the semi-Markov approximation, successive enzyme turnover times are uncorrelated; i.e., they exhibit no memory effects.

5. Randomness Parameter

The probability density $f(t)$ characterizes the kinetics of single-molecule enzymatic reactions completely, with the nth moment in general given by $\langle t^n \rangle \equiv \int_0^\infty dt\, f(t) t^n$. While the first moment of $f(t)$ can be described by the single-molecule Michaelis−Menten equation, higher moments of $f(t)$ contain more information.[23] Often it is convenient to evaluate the second moment of $f(t)$, which is related to a randomness parameter r, defined as[36,37]

$$r = \frac{\langle t^2 \rangle - \langle t \rangle^2}{\langle t \rangle^2} \quad (37)$$

For a one-step Poisson process, $f(t) = k \exp(-kt)$, $\langle t \rangle = 1/k$, $\langle t^2 \rangle - \langle t \rangle^2 = 1/k^2$, therefore, $r = 1$. For multistep processes, assuming an identical rate constant k in n sequential rate-limiting steps, the variance of t in the numerator of eq 37 is n/k^2, while in the denominator variance is n^2/k^2. Hence $r = 1/n$. The greater the number of rate-limiting steps, the smaller is the value of r.

In eq 1, if the reaction steps are all characterized by exponentially distributed waiting time distributions (implying no dynamic disorder), r has been shown to be given by[36]

$$r = \frac{(k_1[S] + k_2 + k_{-1})^2 - 2k_1k_2[S]}{(k_1[S] + k_2 + k_{-1})^2} \quad (38)$$

which is drawn as the full line in Figure 6. This curve may be interpreted as follows. At low substrate concentrations [S], r is unity because substrate binding is the rate-limiting step. As [S] increases, r decreases, reflecting the formation of the enzyme−substrate complex as an intermediate. At still higher [S], r returns to unity when the catalytic step becomes rate-limiting.

The [S] dependence of r can be quite different when dynamic disorder is present. Assuming that dynamic disorder is manifested in the catalytic step of the reaction through the distribution $w(k_2)$ governing the range of possible values of k_2, the evaluation of r is carried out by using eq 31 to calculate the first and second moments of $f(t)$. These moments are found to be

$$\langle t^2\rangle = \frac{2}{k_1^{\,2}[\mathrm{S}]^2} + \frac{2}{(a-1)bk_1[\mathrm{S}]}\left(1+\frac{2k_{-1}}{k_1[\mathrm{S}]}\right) + \frac{2}{(a-1)(a-2)b^2}\left(1+\frac{k_{-1}}{k_1[\mathrm{S}]}\right)^2 \quad (39a)$$

$$\langle t\rangle^2 = \frac{1}{k_1^{\,2}[\mathrm{S}]^2} + \frac{2}{(a-1)bk_1[\mathrm{S}]}\left(1+\frac{k_{-1}}{k_1[\mathrm{S}]}\right) + \frac{1}{(a-1)^2b^2}\left(1+\frac{k_{-1}}{k_1[\mathrm{S}]}\right)^2 \quad (39b)$$

The variation of r with [S] as determined by the above expressions is shown in Figure 6 (dotted line) for the following parameter values: $a = 6$, $b = 35$, $k_1 = 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, and $k_{-1} = 50\ \mathrm{s}^{-1}$, with the mean of k_2 [which is given by $(a-1)b$] being $175\ \mathrm{s}^{-1}$. As is evident, r can exceed 1 at high substrate concentration. This is in agreement with recent experimental findings.[16] We should note that another reason for r being larger than unity is the existence of a reversible reaction in the catalytic step, which was previously reported,[42] but can only occur for enzymatic reactions close to equilibrium.

It is conceivable that dynamic disorder could be manifested in the dissociation step of the reaction, in which case the rate constant k_{-1}, rather than k_2, would have a range of different values, governed by a distribution function $w(k_{-1})$. The evaluation of r under these circumstances can be carried out, as before, by using eq 31 with $w(k_{-1})$ replacing $w(k_2)$, and the integration being performed over k_{-1}. If $w(k_{-1})$ is given by a gamma distribution, with $a = 6$, $b = 10$, (such that the mean of k_{-1} is $50\ \mathrm{s}^{-1}$) and the other parameters are assigned the values $k_1 = 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, and $k_2 = 175\ \mathrm{s}^{-1}$, the result of such a calculation is shown as the dashed line in Figure 6. Thus, when dynamic disorder is present in the dissociation step of the reaction only, r cannot exceed unity at high substrate concentrations. This discussion highlights the fact that if r is observed experimentally to be greater than 1 for an irreversible enzymatic reaction, then (i) dynamic disorder must be present, and (ii) it must be present in the catalytic step, because a constant k_2 cannot give rise to r larger than unity.

6. Conclusions

The classic Michaelis−Menten equation relates the enzymatic velocity to the substrate concentration. It was derived by solving the nonlinear differential eqs 2−4 under the steady-state approximation. Single-molecule enzymatic turnover experiments provide more dynamic information than the ensemble-averaged results through a different observable, $f(t)$, the probability density of the waiting time.

In the absence of dynamic disorder, a single-molecule Michaelis−Menten equation, eq 10, that explicitly describes the temporal behavior of $f(t)$ at any specified substrate concentration is easily derived. The $f(t)$ in eq 10 is exact and does not invoke the steady-state assumption, but it can be reduced to the steady-state case, i.e., to a fast rise followed by an exponential decay, when $k_2 \ll k_{-1}$. Irrespective of the use of the steady-state condition, the reciprocal of the first moment of $f(t)$ is always consistent with the ensemble Michaelis−Menten equation (eq 5), which is an important insight.

In the presence of dynamic disorder, the treatment of single-molecule Michaelis−Menten kinetics becomes considerably more complex. The existence of distinct conformational states of the enzyme that interconvert on time scales comparable to or longer than the time scales of the reaction results in disperse kinetics. The Michaelis−Menten mechanism of eq 1 is easily generalized to include these conformational states (eqs 15 and 28). The calculation of the waiting time distribution $f(t)$ and the mean enzymatic rate $1/\langle t\rangle$ for these multistate models of dynamic disorder can be carried out analytically. It has been found that $1/\langle t\rangle$ does not always exhibit the same substrate concentration dependence as the Michaelis−Menten equation. However, under many conditions, $1/\langle t\rangle$ does follow the single-molecule Michaelis−Menten equation. In these limits, the parameters k_2 and K_M that appear in the ensemble Michaelis−Menten equation are replaced by the weighted averages of distributions of the corresponding kinetic constants of the conformers. Although the first moment of $f(t)$ contains the same information as in the ensemble measurements, $f(t)$ itself provides new informantion about dynamic disorder, and it also exhibits multiexponential long tails under saturating substrate concentrations.

A semi-Markovian approximation to this description of single-molecule kinetics views the origin of dynamic disorder in terms of the occurrence of a non-Poisson distribution of reaction times in the catalytic step of eq 1. In this approximation, the calculated mean rate, $1/\langle t\rangle$, directly recovers the hyperbolic substrate concentration dependence of the ensemble Michaelis−Menten equation. As before, the parameters k_2 and K_M are replaced by generalized counterparts. At the same time, the waiting time distribution $f(t)$ is now found to show highly nonexponential decay at high substrate concentrations, as seen in experiments.

The first and second moments of $f(t)$ (when calculated with the model described by eq 31) can be used to find expressions for the randomness parameter, r, which provides a convenient characterization of single-molecule turnover trajectories. In the absence of dynamic disorder, if the reaction is dominated by one rate-limiting step, $r = 1$, whereas if the reaction has more than one rate-limiting step, $r < 1$. In the presence of dynamic disorder, however, r could be greater than 1.

We hope the results in this paper provide a theoretical framework for understanding the ever-expanding activities in single-molecule enzymology, and perhaps enzymology in general.

Acknowledgment. We are grateful to Attila Szabo and Hong Qian for their critical reading of the manuscript and stimulating comments. We also thank Liang Jiang for helpful comments, and Joseph Klafter for sending us preprints of refs 14 and 15. X.S.X. acknowledges NIH Roadmap for Medical Research/NIH Director's Pioneer Award Program for support, and S.C.K. acknowledges support from NSF grant DMS-02-04674 and the NSF Career Award.

Note Added in Proof. We give in Section 3(i) six limiting conditions (a−f) under which the single-molecule Michaelis−Menten equation holds for the two-state model (eq 15). To

be more precise, in condition (a) we mean $\beta/k_{21} \rightarrow 0$ and $\beta/k_{22} \rightarrow 0$, but α/k_{21} and α/k_{22} do not approach 0; in condition (b) we mean $\beta \rightarrow 0$, but α does not approach 0; in condition (c) we mean $\alpha \rightarrow 0$, but β does not approach 0. Correspondingly, the six limiting conditions (a−f) in Section 3(ii) for the single-molecule Michaelis−Menten equation to hold for the multistate model (eq 28) should be more precisely stated in a similar way.

Appendix A. Calculation of the Waiting Time Distribution for the Two-State Model

The total time to form the product P in the two-state kinetic scheme of eq 15 is a random variable T that is governed by the waiting time distribution $f(t)$. To calculate $f(t)$, the quantity of interest, we first seek expressions for the waiting time distributions that govern the times needed to complete the reaction starting from E_1, E_2, ES_1, or ES_2. These times, which are random variables, are denoted T_{E_1}, T_{E_2}, T_{ES_1}, and T_{ES_2}, respectively, and their corresponding waiting time distributions are denoted $f_{T_{E_1}}(t)$, $f_{T_{E_2}}(t)$, $f_{T_{ES_1}}(t)$, and $f_{T_{ES_2}}(t)$. Once these distributions are determined, $f(t)$ can be expressed as a weighted steady-state average of $f_{T_{E_1}}(t)$ and $f_{T_{E_2}}(t)$. To obtain the expressions for $f_{T_{E_1}}(t)$, $f_{T_{E_2}}(t)$, $f_{T_{ES_1}}(t)$, and $f_{T_{ES_2}}(t)$, we set up and solve a set of four linear simultaneous equations as follows.

Imagine that the system is initially in the state E_1, so that the total time to complete the reaction and form the product is the random variable T_{E_1}. From E_1 the system can proceed either to E_2 or to ES_1, from where the reaction is then completed in the time T_{E_2} or T_{ES_1}. The system reaches E_2 if the step $E_1 \rightarrow E_2$ occurs before the step $E_1 \rightarrow ES_1$ (or equivalently, if the time T_α to complete the former is less than the time T_{11} to complete the latter), and it reaches ES_1 if the step $E_1 \rightarrow ES_1$ occurs before the step $E_1 \rightarrow E_2$ (or equivalently, if $T_{11} < T_\alpha$.) Hence, the probability that T_{E_1} is realized within some time interval t, which we denote $P(T_{E_1} < t)$, can be written as

$$P(T_{E_1} < t) = P(T_{E_2} + T_\alpha < t)P(T_\alpha < T_{11}) + P(T_{ES_1} + T_{11} < t)P(T_{11} < T_\alpha) \quad \text{(A.1)}$$

The steps $E_1 \rightarrow E_2$ and $E_1 \rightarrow ES_1$ occur at random through a Poisson process with average rates α and $k_{11}[S]$, respectively. Hence, the times T_α and T_{11} are drawn from the following waiting time distributions:

$$f_{T_\alpha}(t) = \alpha \exp(-\alpha t) \quad \text{(A.2)}$$

$$f_{T_{11}}(t) = k_{11}[S] \exp(-k_{11}[S]t) \quad \text{(A.3)}$$

In general, for any random variable X, $f_X(x) = dP(X < x)/dx$, so eq A.1 can be differentiated with respect to t to produce

$$f_{T_{E_1}}(t) = f_{T_{E_2}+T_\alpha}(t)P(T_\alpha < T_{11}) + f_{T_{ES_1}+T_{11}}(t)P(T_{11} < T_\alpha) \quad \text{(A.4)}$$

Since the distribution of the sum of two random variables is the convolution of the distributions of the individual random variables, eq A.4 can be further written as

$$f_{T_{E_1}}(t) = \int_0^t dt_1\, f_{T_{E_2}}(t - t_1) f_{T_\alpha}(t_1)P(t_1 < T_{11}) + \int_0^t dt_1\, f_{T_{ES_1}}(t - t_1) f_{T_{11}}(t_1)P(t_1 < T_\alpha) \quad \text{(A.5)}$$

From eqs A.2 and A.3, we can show that

$$P(t_1 < T_{11}) = k_{11}[S]\int_{t_1}^{\infty} dt_2 \exp(-k_{11}[S]t_2) = \exp(-k_{11}[S]t_1) \quad \text{(A.6)}$$

$$P(t_1 < T_\alpha) = \alpha\int_{t_1}^{\infty} dt_2 \exp(-\alpha t_2) = \exp(-\alpha t_1) \quad \text{(A.7)}$$

Therefore, by taking the Laplace transform of eq A.5, and making use of eqs A.2 and A.3 and A.6 and A.7, we obtain

$$\hat{f}_{T_{E_1}}(s) = \frac{\alpha \hat{f}_{T_{E_2}}(s)}{s + \alpha + k_{11}[S]} + \frac{k_{11}[S]\hat{f}_{T_{ES_1}}(s)}{s + \alpha + k_{11}[S]} \quad \text{(A.8)}$$

where $\hat{f}_{T_{E_1}}(s)$, $\hat{f}_{T_{E_2}}(s)$, and $\hat{f}_{T_{ES_1}}(s)$ are Laplace transforms of $f_{T_{E_1}}(t)$, $f_{T_{E_2}}(t)$, and $f_{T_{ES_1}}(t)$, respectively. Equation A.8 is one equation connecting three of the unknown waiting time distributions, $f_{T_{E_1}}(t)$, $f_{T_{E_2}}(t)$ and $f_{T_{ES_1}}(t)$. An equation for the waiting time distribution of the random variable T_{E_2}, the time to complete the reaction starting from the state E_2, can be obtained immediately from eq A.8 by an interchange of labels. That is,

$$\hat{f}_{T_{E_2}}(s) = \frac{\alpha \hat{f}_{T_{E_1}}(s)}{s + \alpha + k_{12}[S]} + \frac{k_{12}[S]\hat{f}_{T_{ES_2}}(s)}{s + \alpha + k_{12}[S]} \quad \text{(A.9)}$$

We can also derive two more expressions involving $\hat{f}_{T_{ES_1}}(s)$ and $\hat{f}_{T_{ES_2}}(s)$ in a similar way. In outline, the procedure is as follows. Imagine the system to start in the state ES_1, so that T_{ES_1} is the time needed to complete the reaction and form the product. At ES_1, the system can either dissociate and return to E_1 at a rate constant k_{-11}, or it can isomerize to the conformer ES_2 at a rate constant β, or it can catalyze the substrate to the product at a rate constant k_{21}. The probability that the reaction time T_{ES_1} occurs within a time interval t (following the earlier reasoning) is therefore given by

$$P(T_{ES_1} < t) = P(T_{-11} + T_{E_1} < t)P(T_{-11} < T_\beta)P(T_{-11} < T_{21}) + P(T_\beta + T_{ES_2} < t)P(T_\beta < T_{-11})P(T_\beta < T_{21}) + P(T_{21} < t)P(T_{21} < T_{-11})P(T_{21} < T_\beta) \quad \text{(A.10)}$$

where T_{-11}, T_β, and T_{21} are the random times required to execute the steps $ES_1 \rightarrow E_1$, $ES_1 \rightarrow ES_2$, and $ES_1 \rightarrow E_1^0$, respectively. As before, these steps are Poisson processes, so T_{-11}, T_β, and T_{21} are drawn from the waiting time distributions

$$f_{T_{-11}}(t) = k_{-11} \exp(-k_{-11}t) \quad \text{(A.11)}$$

$$f_{T_\beta}(t) = \beta \exp(-\beta t) \quad \text{(A.12)}$$

$$f_{T_{21}}(t) = k_{21} \exp(-k_{21}t) \quad \text{(A.13)}$$

After differentiating eq A.10 with respect to t, making use of eqs A.11−A.13 and taking Laplace transforms, it is readily shown that

$$\hat{f}_{T_{ES_1}}(s) = \frac{k_{-11}\,\hat{f}_{T_{E_1}}(s)}{s + \beta + k_{-11} + k_{21}} + \frac{\beta\hat{f}_{T_{ES_2}}(s)}{s + \beta + k_{-11} + k_{21}} + \frac{k_{21}}{s + \beta + k_{-11} + k_{21}} \quad \text{(A.14)}$$

This is a third equation connecting the unknown waiting time

distributions. A fourth and final equation is obtained from eq A.14 by symmetry:

$$\hat{f}_{T_{ES_2}}(s) = \frac{k_{-12}\,\hat{f}_{T_{E_2}}(s)}{s+\beta+k_{-12}+k_{22}} + \frac{\beta\hat{f}_{T_{ES_1}}(s)}{s+\beta+k_{-12}+k_{22}} + \frac{k_{22}}{s+\beta+k_{-12}+k_{22}} \quad \text{(A.15)}$$

Equations A.8, A.9, A.14, and A.15 are conveniently represented in matrix form as

$$s\hat{\mathbf{f}}(s) = \mathbf{Q}\hat{\mathbf{f}}(s) + \mathbf{r} \quad \text{(A.16)}$$

where $\hat{\mathbf{f}}(s) = (\hat{f}_{T_{E_1}}(s), \hat{f}_{T_{E_2}}(s), \hat{f}_{T_{ES_1}}(s), \hat{f}_{T_{ES_2}}(s))^{\mathrm{T}}$, $\mathbf{r} = (0, 0, k_{21}, k_{22})^{\mathrm{T}}$, and

$$\mathbf{Q} = \begin{pmatrix} -(\alpha+k_{11}[\mathrm{S}]) & \alpha & k_{11}[\mathrm{S}] & 0 \\ \alpha & -(\alpha+k_{12}[\mathrm{S}]) & 0 & k_{12}[\mathrm{S}] \\ k_{-11} & 0 & -(\beta+k_{-11}+k_{21}) & \beta \\ 0 & k_{-12} & \beta & -(\beta+k_{-12}+k_{22}) \end{pmatrix} \quad \text{(A.17)}$$

Equation A.16 can be inverted to yield the following expression for $\hat{\mathbf{f}}(s)$ [cf. eq 18]:

$$\hat{\mathbf{f}}(s) = (s\mathbf{I}-\mathbf{Q})^{-1}\mathbf{r} \quad \text{(A.18)}$$

The relation between $\hat{\mathbf{f}}(s)$ and $f(t)$, the waiting time distribution that is actually measured in experiments, is now established as follows. At the end of each catalytic cycle, the enzyme exists either as the conformer $E_1{}^0$ or as the conformer $E_2{}^0$. $E_1{}^0$ is assumed to return to E_1 at a rate constant δ_1, while $E_2{}^0$ is assumed to return to E_2 at a rate constant δ_2. In single-molecule enzymatic turnover experiments, the successive reaction times are obtained over a long time interval so that many turnovers occur. But over the course of many such turnover cycles, the fraction of time that the enzyme resides in E_1 or E_2 attains a steady-state value. Therefore, during a long time trajectory, the waiting time distribution $f(t)$ observed in enzymatic turnover experiments corresponds to the steady-state weighted average of the two waiting time distributions $f_{T_{E_1}}(t)$ and $f_{T_{E_2}}(t)$, when the reaction starts from E_1 or E_2, respectively. This steady-state weight for $f_{T_{E_1}}(t)$, which accounts for how often the system starts from E_1 immediately after restarting the cycle, is proportional to the steady-state probability $P^0_{E_1{}^0}$ that the system is in $E_1{}^0$ multiplied by the rate constant δ_1 of $E_1{}^0$'s return to the E_1 state. Similarly, the steady-state weight for $f_{T_{E_2}}(t)$ is proportional to the steady-state probability $P^0_{E_2{}^0}$ that the system is in $E_2{}^0$ multiplied by the rate constant δ_2 of $E_2{}^0$'s return to the E_2 state. In other words

$$f(t) = \frac{\delta_1 P^0_{E_1{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}} f_{T_{E_1}}(t) + \frac{\delta_2 P^0_{E_2{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}} f_{T_{E_2}}(t) \quad \text{(A.19)}$$

where the denominator in this expression is introduced to ensure that $f(t)$ is properly normalized to unity.

Equation A.19 can be rewritten in Laplace space in terms of the vector $\hat{\mathbf{f}}(s)$ obtained earlier:

$$\hat{f}(s) = \left(\frac{\delta_1 P^0_{E_1{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}}, \frac{\delta_2 P^0_{E_2{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}}, 0, 0 \right) \hat{\mathbf{f}}(s) \quad \text{(A.20)}$$

The calculation of the equilibrium probabilities $P^0_{E_1{}^0}$ and $P^0_{E_2{}^0}$ is discussed next.

In the kinetic scheme shown in eq 15, the probabilities $P_\nu(t)$ that the enzyme is in one or other of the states $\nu = E_1, E_2, ES_1, ES_2, E_1{}^0$, or $E_2{}^0$ satisfy a master equation, i.e., a system of coupled linear first order differential equations analogous to eqs 6−8 that express the balance of probability into and out of ν. At long times, $t \to \infty$, when the system reaches equilibrium, the rates $\mathrm{d}P_\nu(t)/\mathrm{d}t$ vanish, and the above system of equations reduces to

$$\mathbf{SP} = 0 \quad \text{(A.21)}$$

where $\mathbf{P}$ is the vector $(P^0_{E_1}, P^0_{E_2}, P^0_{ES_1}, P^0_{ES_2}, P^0_{E_1{}^0}, P^0_{E_2{}^0})^{\mathrm{T}}$, the superscript 0 denoting the equilibrium value, and

$$\mathbf{S} = \begin{pmatrix} -(\alpha+k_{11}[\mathrm{S}]) & \alpha & k_{-11} & 0 & \delta_1 & 0 \\ \alpha & -(\alpha+k_{12}[\mathrm{S}]) & 0 & k_{-12} & 0 & \delta_2 \\ k_{11}[\mathrm{S}] & 0 & -(\beta+k_{-11}+k_{21}) & \beta & 0 & 0 \\ 0 & k_{12}[\mathrm{S}] & \beta & -(\beta+k_{-12}+k_{22}) & 0 & 0 \\ 0 & 0 & k_{21} & 0 & -(\gamma+\delta_1) & \gamma \\ 0 & 0 & 0 & k_{22} & \gamma & -(\gamma+\delta_2) \end{pmatrix} \quad \text{(A.22)}$$

From the solution to eq A.21 under the constraint

$$P^0_{E_1} + P^0_{E_2} + P^0_{ES_1} + P^0_{ES_2} + P^0_{E_1{}^0} + P^0_{E_2{}^0} = 1 \quad \text{(A.23)}$$

and in the limit $\delta_1, \delta_2 \gg 1$ (corresponding to fast reset of $E_1{}^0$ and $E_2{}^0$ to E_1 and E_2), it can be shown that

$$\frac{\delta_1 P^0_{E_1{}^0}}{\delta_2 P^0_{E_2{}^0}} = \frac{k_{21}[\alpha(k_{11}k_{22}+k_{11}k_{-12})+\alpha\beta(k_{11}+k_{12})+\beta k_{11}k_{12}[\mathrm{S}]]}{k_{22}[\alpha(k_{12}k_{21}+k_{12}k_{-11})+\alpha\beta(k_{11}+k_{12})+\beta k_{11}k_{12}[\mathrm{S}]]} \quad \text{(A.24)}$$

from which the steady-state fractions in eq A.20 can be calculated. Once we obtain the Laplace transform $\hat{f}(s)$, the mean waiting time $\langle t\rangle = \int_0^\infty \mathrm{d}t\, tf(t)$ is readily given by $\langle t\rangle = -\mathrm{d}/\mathrm{d}s\, \hat{f}(s)|_{s=0}$. Using eqs A.18 and A.20, we have

$$\langle t\rangle = \left(\frac{\delta_1 P^0_{E_1{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}}, \frac{\delta_2 P^0_{E_2{}^0}}{\delta_1 P^0_{E_1{}^0} + \delta_2 P^0_{E_2{}^0}}, 0, 0 \right) \mathbf{Q}^{-2}\mathbf{r} \quad \text{(A.25)}$$

which, after lengthy but straightforward algebra, can be rearranged to the form in eq 24.

Appendix B. Calculation of the Waiting Time Distribution for the Multistate Case

The approach introduced in Appendix A can also be applied to the analysis of the multistate model of dynamic disorder. In deriving an expression for the waiting time distribution $f(t)$ and its first moment, it is convenient to introduce some simplified

notation. Let A_i, B_i, and C_i stand for the states E_i, ES_i, and E_i^0, respectively. As diagrammed in eq 28, A_i can convert to B_i or to any of the A_j, B_i can convert to A_i, C_i, or to any of the B_j, and C_i can convert to A_i or to any of the C_j. If we introduce the matrix $\mathbf{Q}_{AA}$ to denote the transition rates between the A_i's and the matrix $\mathbf{Q}_{AB}$ to denote the transition rates between the A_i's and the B_i's, and likewise introduce $\mathbf{Q}_{BA}$, Q_{BB}, $\mathbf{Q}_{CC}$, and $\mathbf{Q}_{CA}$ to denote the transition rates between the corresponding states, then, following the diagram of eq 28, the transition matrices are $\mathbf{Q}_{AA} = [\alpha_{ij}]$, $\mathbf{Q}_{BB} = [\beta_{ij}]$, $\mathbf{Q}_{CC} = [\gamma_{ij}]$, $\mathbf{Q}_{AB} = \mathrm{diag}(k_{11}[S], k_{12}[S], ..., k_{1n}[S])$, $\mathbf{Q}_{BA} = \mathrm{diag}(k_{-11}, k_{-12}, ..., k_{-1n})$, $\mathbf{Q}_{BC} = \mathrm{diag}(k_{21}, k_{22}, ..., k_{2n})$, and $\mathbf{Q}_{CA} = \mathrm{diag}(\delta_1, \delta_2, ..., \delta_n)$. The entire network of interconversions can be described by a matrix $\mathbf{Q}$ given by

$$\mathbf{Q} = \begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} & \mathbf{0} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) & \mathbf{Q}_{BC} \\ \mathbf{Q}_{CA} & \mathbf{0} & \mathbf{Q}_{CC} - \mathbf{Q}_{CA} \end{pmatrix} \tag{B.1}$$

Following the method of Appendix A, one can derive the following relation, in Laplace space, between the distributions associated with the times needed to complete the reaction starting from different states of the network:

$$s\begin{pmatrix} \hat{\mathbf{f}}_{T_A}(s) \\ \hat{\mathbf{f}}_{T_B}(s) \end{pmatrix} = \begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) \end{pmatrix}\begin{pmatrix} \hat{\mathbf{f}}_{T_A}(s) \\ \hat{\mathbf{f}}_{T_B}(s) \end{pmatrix} + \begin{pmatrix} \mathbf{0} \\ \mathbf{Q}_{BC}\mathbf{1} \end{pmatrix} \tag{B.2}$$

where $\hat{\mathbf{f}}_{T_A}(s) = (\hat{f}_{T_{A_1}}(s), \hat{f}_{T_{A_2}}(s), ..., \hat{f}_{T_{A_n}}(s))^T$, $\hat{\mathbf{f}}_{T_B}(s) = (\hat{f}_{T_{B_1}}(s), \hat{f}_{T_{B_2}}(s), ..., \hat{f}_{T_{B_n}}(s))^T$, and $\mathbf{1} = (1, 1, ..., 1)^T$. Here $\hat{f}_{T_{A_i}}(s)$ and $\hat{f}_{T_{B_i}}(s)$ are the Laplace transforms of the waiting time distributions where the system starts from A_i and B_i, respectively. Equation B.2 can be solved by matrix inversion:

$$\begin{pmatrix} \hat{\mathbf{f}}_{T_A}(s) \\ \hat{\mathbf{f}}_{T_B}(s) \end{pmatrix} = \left[s\mathbf{I} - \begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) \end{pmatrix}\right]^{-1}\begin{pmatrix} \mathbf{0} \\ \mathbf{Q}_{BC}\mathbf{1} \end{pmatrix} \tag{B.3}$$

To calculate the mean waiting time $\langle t \rangle$, eq B.2 is differentiated with respect to s, and the result evaluated at $s = 0$. Together with eq B.3, this leads to

$$-\begin{pmatrix} \hat{\mathbf{f}}'_{T_A}(0) \\ \hat{\mathbf{f}}'_{T_B}(0) \end{pmatrix} = -\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) \end{pmatrix}^{-1}\begin{pmatrix} \hat{\mathbf{f}}_{T_A}(0) \\ \hat{\mathbf{f}}_{T_B}(0) \end{pmatrix}$$

$$= -\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) \end{pmatrix}^{-1}\begin{pmatrix} \mathbf{1} \\ \mathbf{1} \end{pmatrix} \tag{B.4}$$

where the last equality makes use of the results $\hat{f}_{T_{A_i}}(0) = \hat{f}_{T_{B_i}}(0) = 1$. The inverse in eq B.4 is calculated by block matrix inversion, producing

$$\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}) \end{pmatrix}^{-1} = \begin{pmatrix} \mathbf{L} & \mathbf{M} \\ \mathbf{N} & \mathbf{R} \end{pmatrix} \tag{B.5}$$

where

$$\mathbf{L} = [\mathbf{Q}_{AA} - \mathbf{Q}_{AB} - \mathbf{Q}_{AB}(\mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}))^{-1}\mathbf{Q}_{BA}]^{-1} \tag{B.6}$$

$$\mathbf{M} = -[\mathbf{Q}_{AA} - \mathbf{Q}_{AB} - \mathbf{Q}_{AB}(\mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}))^{-1}\mathbf{Q}_{BA}]^{-1}\mathbf{Q}_{AB}(\mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}))^{-1} \tag{B.7}$$

$$\mathbf{N} = -(\mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}))^{-1}\mathbf{Q}_{BA}[\mathbf{Q}_{AA} - \mathbf{Q}_{AB} - \mathbf{Q}_{AB}(\mathbf{Q}_{BB} - (\mathbf{Q}_{BA} + \mathbf{Q}_{BC}))^{-1}\mathbf{Q}_{BA}]^{-1} \tag{B.8}$$

$$\mathbf{R} = [\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC} - \mathbf{Q}_{BA}(\mathbf{Q}_{AA} - \mathbf{Q}_{AB})^{-1}\mathbf{Q}_{AB}]^{-1} \tag{B.9}$$

It then follows that

$$\begin{pmatrix} \hat{\mathbf{f}}'_{T_A}(0) \\ \hat{\mathbf{f}}'_{T_B}(0) \end{pmatrix} = \begin{pmatrix} (\mathbf{L} + \mathbf{M})\mathbf{1} \\ (\mathbf{N} + \mathbf{R})\mathbf{1} \end{pmatrix} \tag{B.10}$$

As before, since each new reaction cycle can start from any A_i, the overall waiting time distribution $f(t)$ is the steady-state weighted average of the distributions $f_{T_{A_i}}(t)$. The calculation of these steady-state probabilities is considered next. Let P_{A_i}, P_{B_i}, and P_{C_i} denote the steady-state probabilities of A_i, B_i, and C_i, respectively. To calculate them, we proceed as earlier from the stationary solution of the master equation, which is defined by

$$(\mathbf{p}_A^T \quad \mathbf{p}_B^T \quad \mathbf{p}_C^T)\mathbf{Q} = 0 \tag{B.11}$$

where $\mathbf{p}_A = (P_{A_1}, P_{A_2}, ..., P_{A_n})^T$, $\mathbf{p}_B = (P_{B_1}, P_{B_2}, ..., P_{B_n})^T$, $\mathbf{p}_C = (P_{C_1}, P_{C_2}, ..., P_{C_n})^T$. To solve eq B.11, $\mathbf{p}_A$ and $\mathbf{p}_B$ are first rewritten in terms of $\mathbf{p}_C$, so that an equation solely in $\mathbf{p}_C$ is obtained. This equation is then solved by standard matrix techniques. To implement the first step in this process, the definition of $\mathbf{Q}$ is substituted into eq B.11, producing

$$(\mathbf{p}_A^T \ \mathbf{p}_B^T \ \mathbf{p}_C^T)\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} & \mathbf{0} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC} & \mathbf{Q}_{BC} \\ \mathbf{Q}_{CA} & \mathbf{0} & \mathbf{Q}_{CC} - \mathbf{Q}_{CA} \end{pmatrix} = \mathbf{0} \tag{B.12}$$

Equation B.12 implies that

$$(\mathbf{p}_A^T \ \mathbf{p}_B^T)\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC} \end{pmatrix} + \mathbf{p}_C^T(\mathbf{Q}_{CA} \ \ \mathbf{0}) = \mathbf{0} \tag{B.13}$$

and

$$\mathbf{p}_B^T\mathbf{Q}_{BC} + \mathbf{p}_C^T(\mathbf{Q}_{CC} - \mathbf{Q}_{CA}) = \mathbf{0} \tag{B.14}$$

Equation B.13 leads to

$$(\mathbf{p}_A^T \quad \mathbf{p}_B^T) = -\mathbf{p}_C^T(\mathbf{Q}_{CA} \ \ \mathbf{0})\begin{pmatrix} \mathbf{Q}_{AA} - \mathbf{Q}_{AB} & \mathbf{Q}_{AB} \\ \mathbf{Q}_{BA} & \mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC} \end{pmatrix}^{-1}$$

$$= -\mathbf{p}_C^T(\mathbf{Q}_{CA} \ \ \mathbf{0})\begin{pmatrix} \mathbf{L} & \mathbf{M} \\ \mathbf{N} & \mathbf{R} \end{pmatrix}$$

$$= -(\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{L} \ \ \mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{M}) \tag{B.15}$$

Hence,

$$\mathbf{p}_A^T = -\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{L} \qquad \mathbf{p}_B^T = -\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{M} \tag{B.16}$$

Substituting eq B.16 into eq B.14, the sought for equation in $\mathbf{p}_C$ is obtained as

$$-\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{M}\mathbf{Q}_{BC} + \mathbf{p}_C^T(\mathbf{Q}_{CC} - \mathbf{Q}_{CA}) = \mathbf{0} \quad \text{(B.17)}$$

which can be solved for $\mathbf{p}_C$ up to a normalizing constant. This then provides expressions for $\mathbf{p}_A$ and $\mathbf{p}_B$ via eq B.16.

Having obtained the steady-state probabilities, the overall waiting time distribution $f(t)$ (in Laplace space) is calculated from

$$\hat{f}(s) = \frac{\mathbf{p}_C^T\mathbf{Q}_{CA}\hat{\mathbf{f}}_{T_A}(s)}{\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{1}} \quad \text{(B.18)}$$

Hence, the mean waiting time is calculated as

$$\langle t\rangle = \frac{-\mathbf{p}_C^T\mathbf{Q}_{CA}\hat{\mathbf{f}}'_{T_A}(0)}{\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{1}} = \frac{-\mathbf{p}_C^T\mathbf{Q}_{CA}(\mathbf{L}+\mathbf{M})\mathbf{1}}{\mathbf{p}_C^T\mathbf{Q}_{CA}\mathbf{1}} \quad \text{(B.19)}$$

Introducing a vector $\mathbf{v}^T$ defined as $\mathbf{v}^T = \mathbf{p}_C^T\mathbf{Q}_{CA}$, eqs B.18 and B.19 can be written in more compact notation as

$$\hat{\mathbf{f}}(s) = \frac{\mathbf{v}^T\hat{\mathbf{f}}_{T_A}(s)}{\mathbf{v}^T\mathbf{1}} \qquad \langle t\rangle = \frac{-\mathbf{v}^T(\mathbf{L}+\mathbf{M})\mathbf{1}}{\mathbf{v}^T\mathbf{1}} \quad \text{(B.20)}$$

Hence, the steady-state waiting time distribution $f(t)$ is the weighted average of the waiting time distributions associated with starting the reaction from E_i, with the weights given by the steady-state probability to be in E_i.

Now from eqs B.6−B.9, it follows that

$$\mathbf{L} = -\mathbf{M}(\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC})\mathbf{Q}_{AB}^{-1} \quad \text{(B.21)}$$

which leads to

$$\mathbf{L} + \mathbf{M} = -\mathbf{M}[(\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC})\mathbf{Q}_{AB}^{-1} - \mathbf{I}] \quad \text{(B.22)}$$

This equation, together with eq B.17, yields

$$\langle t\rangle = \frac{-\mathbf{v}^T(\mathbf{L}+\mathbf{M})\mathbf{1}}{\mathbf{v}^T\mathbf{1}} = \frac{1}{\mathbf{v}^T\mathbf{1}}\mathbf{v}^T\mathbf{Q}_{CA}^{-1}(\mathbf{Q}_{CC} - \mathbf{Q}_{CA})\mathbf{Q}_{BC}^{-1}[(\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC})\mathbf{Q}_{AB}^{-1} - \mathbf{I}]\mathbf{1} \quad \text{(B.23)}$$

In the limit when the δ_i are much larger than the other rates (so that at the end of the reaction, the system returns rapidly to the state E_i), eq B.23 reduces to

$$\langle t\rangle = -\frac{1}{\mathbf{v}^T\mathbf{1}}\mathbf{v}^T\mathbf{Q}_{BC}^{-1}[(\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC})\mathbf{Q}_{AB}^{-1} - \mathbf{I}]\mathbf{1} \quad \text{(B.24)}$$

with $\mathbf{v}$ satisfying (cf. eq B.17) $\mathbf{v}^T\mathbf{M}\mathbf{Q}_{BC} + \mathbf{v}^T\mathbf{I} = \mathbf{0}$, or equivalently

$$\mathbf{v}^T(\mathbf{I} + \mathbf{Q}_{BC}^{-1}\mathbf{M}^{-1}) = \mathbf{0} \quad \text{(B.25)}$$

Next we note that the mean enzymatic reaction rate, $1/\langle t\rangle$, calculated from eq B.24 will be of the Michaelis−Menten form (cf. eq 29)

$$\frac{1}{\langle t\rangle} = \frac{\chi_2[S]}{[S] + C_M} \quad \text{(B.26)}$$

if the equilibrium weight $\mathbf{v}$ does not depend on the substrate concentration. This can be demonstrated by first noting that only the transition rates associated with $A_i \rightarrow B_i$ involve [S]. The matrix, $\mathbf{Q}_{AB}$ can therefore be written $\mathbf{Q}_{AB} = [S]\tilde{\mathbf{Q}}_{AB}$, where $\tilde{\mathbf{Q}}_{AB} = \mathrm{diag}(k_{11}, k_{12}, ..., k_{1n})$. The use of this definition in eq B.24 followed by rearrangement leads to eq B.26, with both χ_2 and C_M independent of [S]. It is now straightforward to show that each of the conditions (a−f) in section 3 (ii) does indeed guarantee that the weights $\mathbf{v}$ do not depend on the concentration [S], and that each condition therefore leads to a Michaelis−Menten equation, as seen in eq 29.

To calculate the waiting time distribution $f(t)$ in the slow interconversion limit, we start from eq B.2, which can be written as

$$s\hat{\mathbf{f}}_{T_A}(s) = (\mathbf{Q}_{AA} - \mathbf{Q}_{AB})\hat{\mathbf{f}}_{T_A}(s) + \mathbf{Q}_{AB}\hat{\mathbf{f}}_{T_B}(s)$$
$$s\hat{\mathbf{f}}_{T_B}(s) = \mathbf{Q}_{BA}\hat{\mathbf{f}}_{T_A}(s) + (\mathbf{Q}_{BB} - \mathbf{Q}_{BA} - \mathbf{Q}_{BC})\hat{\mathbf{f}}_{T_B}(s) + \mathbf{Q}_{BC}\mathbf{1} \quad \text{(B.27)}$$

In the given limit, $\mathbf{Q}_{AA}$ and $\mathbf{Q}_{BB}$ are small, so eq B.27 effectively reduces to a set of equations for the individual components, i.e.,

$$s\hat{f}_{T_{A_i}}(s) = -k_{1i}[S]\hat{f}_{T_{A_i}}(s) + k_{1i}[S]\hat{f}_{T_{B_i}}(s)$$
$$s\hat{f}_{T_{B_i}}(s) = -k_{-1i}\hat{f}_{T_{A_i}}(s) - (k_{-1i} + k_{2i})\hat{f}_{T_{B_i}}(s) + k_{2i} \quad \text{(B.28)}$$

Solving for $\hat{f}_{T_{A_i}}(s)$, we obtain

$$\hat{f}_{T_{A_i}}(s) = \frac{k_{1i}k_{2i}[S]}{s^2 + s(k_{1i}[S] + k_{-1i} + k_{2i}) + k_{1i}k_{2i}[S]} \quad \text{(B.29)}$$

which can be inverted to

$$f_{T_{A_i}}(t) = \frac{k_{1i}k_{2i}[S]}{2\alpha_i}[\exp(A_i + B_i)t - \exp(B_i - A_i)t] \quad \text{(B.30)}$$

where A_i and B_i have been defined after eq 26. This expression, when weighted by w_i and summed over the reaction channels yields eq 30.

Appendix C. Calculation of the Waiting Time Distribution for the semi-Markovian Approximation

The general method of calculating $f(t)$ remains the same as the method described in Appendix A. The reaction is imagined to start from **E**. The total time to complete the reaction starting from **E** is a random variable T governed by a waiting time distribution $f(t)$. After a time T_1, which is drawn from the waiting time distribution $f_{T_1}(t)$, the system moves to ES, from where the reaction is completed in a total time T_2, which is drawn from the waiting time distribution $f_{T_2}(t)$. Thus, the probability that, starting from E, the reaction time T is realized within a time interval t is given by

$$P(T < t) = P(T_1 + T_2 < t) \quad \text{(C.1)}$$

The distribution $f_{T_1}(t)$ is assumed to be exponential, with a time constant of $k_1[S]$; hence $f_{T_1}(t) = k_1[S]\exp(-k_1[S]t)$. Differentiating eq C.1 with respect to t, we obtain

$$f(t) = k_1[S]\int_0^t dt_1 \exp[-k_1[S](t - t_1)]f_{T_2}(t_1) \quad (C.2)$$

The Laplace transform of eq C.2 yields

$$\hat{f}(s) = \frac{k_1[S]}{s + k_1[S]}\hat{f}_{T_2}(s) \quad (C.3)$$

Once the enzyme has reached **ES**, a total time of T_2 will elapse before the product is formed. In forming the product, **ES** can either move to the state $\mathbf{E}^0$ directly in a time T_C drawn from the unknown waiting time distribution $f_{T_C}(t)$, or it can return to **E** in a time T_{-1} drawn from the exponential waiting time distribution $f_{T_{-1}}(t) = k_{-1}\exp(-k_{-1}t)$. The first option is selected if $T_C < T_{-1}$, the second if $T_{-1} < T_C$. The probability that, starting from **ES**, the reaction time T_2 is realized within a time t is therefore given by

$$P(T_2 < t) = P(T_C < t)P(T_C < T_{-1}) + P(T_{-1} + T < t)P(T_{-1} < T_C) \quad (C.4)$$

After differentiating with respect to t, eq C.4 becomes

$$f_{T_2}(t) = f_{T_C}(t)P(t < T_{-1}) + \int_0^t dt_1\, f(t - t_1)f_{T_{-1}}(t_1)P(t_1 < T_C) \quad (C.5)$$

From the expression for $f_{T_{-1}}(t)$, $P(t < T_{-1})$ is given by $\exp(-k_{-1}t)$, while $P(t_1 < T_C)$ is given by $1 - \int_0^{t_1} dt_2\, f_{T_C}(t_2)$. Hence the Laplace transform of eq C.5 is

$$\hat{f}_{T_2}(s) = \hat{f}_{T_C}(s + k_{-1}) + \frac{k_{-1}}{s + k_{-1}}\hat{f}(s)[1 - \hat{f}_{T_C}(s + k_{-1})] \quad (C.6)$$

Substituting eq C.6 into eq C.3 and rearranging, we find

$$\hat{f}(s) = \hat{f}_{T_C}(s + k_{-1})/\left(\frac{k_1[S] + s}{k_1[S]} - \frac{k_{-1}}{k_{-1} + s}[1 - \hat{f}_{T_C}(s + k_{-1})]\right) \quad (C.7)$$

which is the expression shown in eq 32.

Note Added after ASAP Publication. The Note Added in Proof was included with this paper on September 22, 2005. The article was published ASAP on 8/2/05. The appended version was reposted on 9/23/05.

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