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Fluctuating Enzymes: Lessons from Single-Molecule Studies

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ABSTRACT

Recent single-molecule enzymology measurements with improved statistics have demonstrated that a single enzyme molecule exhibits large temporal fluctuations of the turnover rate constant at a broad range of time scales (from 1 ms to 100 s). The rate constant fluctuations, termed as dynamic disorder, are associated with fluctuations of the protein conformations observed on the same time scales. We discuss the unique information extractable from these experiments and the reconciliation of these observations with ensemble-averaged Michaelis−Menten equation. A theoretical model based on the generalized Langevin equation (GLE) treatment of Kramers' barrier crossing problem for chemical reactions accounts naturally for the observation of dynamic disorder and highly dispersed kinetics.

Introduction

Most biochemical reactions are facilitated by enzymes. Enzymology has benefited from tremendous advances in structural biology and proteomics, which have provided much information about structures and functions of enzymes. As the static pictures and interaction networks of enzymes become known, the quest for understanding enzyme dynamics, that is, how enzymes work in real-time, continues to be a central focus of many research activities.[1−6]

Ever since the celebrated work of Michaelis and Menten,[7] enzyme kinetics has been vital in characterizing biochemical mechanisms. The Michaelis−Menten equation, which describes the hyperbolic dependence of enzymatic velocity on substrate concentration, provides a highly satisfactory description for most ensemble-averaged enzyme kinetics.

Constantly fluctuating in time, however, enzyme molecules are not synchronized with each other in an ensemble-averaged kinetic measurement, which complicates extraction of dynamic information. Recent advances in room-temperature single-molecule spectroscopy circumvent this complication and allow for dynamic behaviors of individual molecules to be recorded in real-time.[8−12] The absence of ensemble averaging yields a wealth of dynamic information, which has profoundly changed the way enzymatic dynamics is studied.[13−26] New knowledge derived from the single-molecule approach continues to emerge.

Single-molecule experiments are particularly powerful in elucidating mechanisms of enzymes by capturing transient intermediates, of which many specific examples of biological importance can be given.[13−17] In this Account, however, we focus on a fundamental issue common to all enzymatic systems, that is, enzymes are dynamic entities exhibiting distributions and fluctuations of catalytic rate constants. This phenomenon, referred to broadly as dynamic disorder,[27,28] has been inferred previously from ensemble measurements[29] and quantitatively verified by recent single-molecule experiments.[14,18−25] Dynamic disorder is not described within the framework of conventional transition state theory[30] and Kramers' theory[31,32] of condensed phase chemical reactions.

The specific questions that we address in this Account are as follows:

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1. How is single-molecule enzymatic kinetics reconciled with the conventional Michaelis–Menten equation in steady-state and pre-steady-state assays?
2. What is the new information available from single-molecule enzyme turnover experiments, especially in the context of dynamic disorder?
3. What are the distinct features of conformational fluctuation of a protein molecule at time scales of enzymatic reactions, and what are their effects on dynamic disorder?
4. How can dynamic disorder be described theoretically in the framework of Kramers' barrier crossing theory?

Our interest in these questions was stimulated by two recent single-molecule experiments, one on enzymatic turnovers of β-galactosidase[25] and the other on conformational dynamics of a protein complex between fluorescein and anti-fluorescein.[23] As will be briefly described below, both the turnover and conformation dynamics exhibit fluctuations spanning multiple time scales, from 1 ms to 10 s. The first experiment led to the development of single-molecule Michaelis–Menten equations,[24] while the second led to a model of conformational fluctuations based on the generalized Langevin equation (GLE) as well as the experimental determination of the power-law memory kernel of one-dimensional coordinate. To connect conformational and enzymatic dynamics, we have developed a theoretical model by incorporating the GLE with the power-law memory kernel of the reaction coordinate into the Kramers' barrier crossing problem.[33] Numerical simulations based on this model provide a quantitative description of dynamic disorder that can account for the single-molecule observations.[33]

Single-Molecule Enzymatic Turnover Dynamics

We first describe a recent single-molecule experiment by English et al.[25] on β-galactosidase, an enzyme that catalyzes the hydrolysis of lactose to glucose and galactose. A fluorogenic substrate, resorufin β-D-galactopyranoside (RGP), is also hydrolyzed by the enzyme, yielding a fluorescent product, resorufin. When the enzyme molecule is immobilized, each product molecule generates a detectable fluorescence burst before it quickly diffuses out of the laser focal volume (Figure 1A). In this way, each enzymatic turnover event can be recorded in real time. As expected, the higher the RGP concentration, the higher the frequency of the fluorescence burst. The advantage of this assay is that extremely long trajectories can be obtained for statistical analyses because trajectory lengths are not limited by photobleaching of the fluorophore.[21,22,34]

On a single-molecule basis, turnover events are intrinsically stochastic. We obtain the sequence of the time intervals between successive turnover events, the waiting times, τ, as a function of the turnover index number (Figure 1B). This waiting time trajectory contains rich dynamic information, extractable by statistical analyses.[35] Of particular interest, as described below, are four of its properties: the mean waiting time $\langle\tau\rangle$, the probability

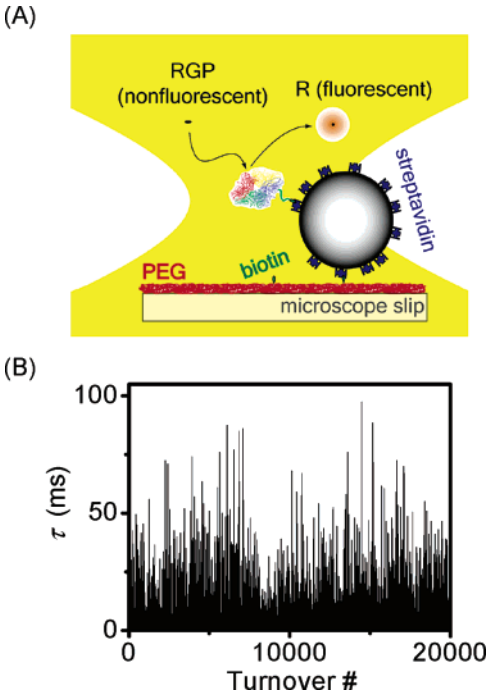

FIGURE 1. (A) Schematic representation of the experimental setup (not to scale). One β-galactosidase enzyme is linked to a streptavidin-coated polystyrene bead via a flexible PEG linker. The bead binds to the biotin–PEG surface of a cover slip. The photogenic substrate RGP is catalyzed, and the fluorescent product resorufin (R) is monitored in the diffraction-limited confocal volume. (B) A segment of the chronological waiting time trajectory as a function of turnover events index numbers at [S] = 100 μM. (Adapted from ref 25.)

density of waiting time $f(\tau)$ and its concentration dependence, the distribution of the turnover rate, and the autocorrelation function of the waiting times.

According to the Michaelis–Menten mechanism, a substrate S binds and unbinds reversibly with the enzyme E to form an enzyme–substrate complex ES that yields the product P and recovers the original enzyme quickly ($k_3 \to \infty$):

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_2} \mathrm{E}^0 + \mathrm{P}, \quad \mathrm{E}^0 \xrightarrow{k_3} \mathrm{E} \tag{1}$$

The classic Michaelis–Menten equation,

$$\frac{v}{[\mathrm{E}^0]} = \frac{k_2[\mathrm{S}]}{[\mathrm{S}] + K_\mathrm{M}} \tag{2}$$

where $[\mathrm{E}^0] = [\mathrm{E}] + [\mathrm{ES}]$ is the total enzyme concentration and $K_\mathrm{M} = (k_{-1} + k_2)/k_1$ is the Michaelis constant, gives explicitly the hyperbolic dependence of the enzyme velocity v on substrate concentration [S] in an ensemble experiment. In a conventional Lineweaver–Burke plot, $1/v$ vs $1/[\mathrm{S}]$ follows a linear relation.[6]

Our recent theoretical work has shown that single-molecule and ensemble-averaged measurements are consistent in that the reciprocal of the mean waiting time $1/\langle\tau\rangle = v/[\mathrm{E}^0]$ and obeys the same hyperbolic substrate concentration dependence.[24] In this way, we can reconcile single-molecule and ensemble-averaged Michaelis–Menten kinetics. Such a consistency arises from the equivalence

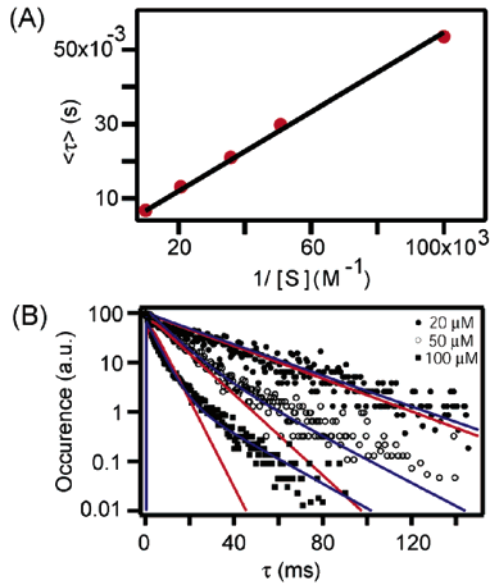

FIGURE 2. (A) Single-molecule Lineweaver–Burke plot of mean waiting times $\langle\tau\rangle$ vs 1/[S] for five different substrate concentrations. The best fit with the Michaelis–Menten rate equation gives two parameters, $\gamma_2 = 730 \pm 80\ s^{-1}$ and $C_M = 390 \pm 60\ \mu M$. These agree well with the ensemble measurement results $k_2 = 740 \pm 60\ s^{-1}$ and $K_M = 380 \pm 40\ \mu M$. (B) Waiting time histograms for three substrate concentrations, 20, 50, and 100 μM. Red curves are the fits from eq 3 with $k_{-1} = 62.4\ s^{-1}$, $k_2 = 730\ s^{-1}$, and $K_M = 380\ \mu M$. The deviation for the high substrate concentration is significant. Blue curves are the best fits from eq 5a assuming a phenomenological Γ distribution for $w(k_2)$. The fitting function is $w(k_2) = [1/(b^a\Gamma(a))]k_2^{a-1}\exp(-k_2/b)$ with $\Gamma(a)$ being the Γ function and $a = 4.2$, $b = 224\ s^{-1}$, $k_1 = 5 \times 10^7\ M^{-1}\ s^{-1}$, and $k_{-1} = 18\,300\ s^{-1}$. (Adapted from ref 25.)

between time averaging and ensemble averaging. Figure 2A depicts the Lineweaver–Burke plot, $\langle\tau\rangle$ vs 1/[S], for five single-molecule β-galactosidase experiments under five different substrate concentrations. The linear fit is in agreement with the conventional Michaelis–Menten equation (eq 2). As will be shown later, this relationship could hold even under the condition of dynamic disorder.

Single-molecule experiments can provide much more information than just the mean waiting time $\langle\tau\rangle$. For example, the whole waiting time distribution, $f(\tau)$, otherwise hidden in conventional ensemble-averaged steady-state assays, can be constructed. We have shown that for the Michaelis–Menten scheme in eq 1, $f(\tau)$ exhibits a single-exponential rise, followed by a single-exponential decay,

$$f(\tau) = \frac{k_1 k_2[\mathrm{S}]}{2\alpha}[\exp(\alpha+\beta)\tau - \exp(\beta-\alpha)\tau] \qquad (3)$$

where $\alpha = \sqrt{(k_1[\mathrm{S}]+k_{-1}+k_2)^2/4 - k_1k_2[\mathrm{S}]}$ and $\beta = -(k_1[\mathrm{S}] + k_{-1} + k_2)/2$.[26]

Figure 2B displays the experimental $f(\tau)$ data for three different substrate concentrations. Remarkably, the curves exhibit multiexponential decays at high substrate concentration and monoexponential decays at low substrate concentration. The long tails of $f(\tau)$ are evident due to the high dynamic range of the data, which is made possible by the long trajectories. These long tails should in principle be observable in some ensemble-averaged pre-steady-state assays, but in practice they remain hidden because of the limited dynamic range of ensemble kinetic data.

This multiexponential behavior of $f(\tau)$ *cannot* be described by eq 3 (red curves in Figure 2B). Physically, the concentration dependence of $f(\tau)$ can be understood as follows: The multiexponential decay of $f(\tau)$ at high substrate concentration is due to dynamic disorder, caused by the fluctuation of the rate constant of the catalytic step (k_2) at a time scale comparable to or longer than the waiting times. At low substrate concentration, however, the binding step becomes rate-limiting; the monoexponential decay indicates no fluctuation in k_1 since substrate concentration is held constant under the experimental condition. We did not observe evidence for k_{-1} fluctuation either.

For quantitative modeling, we assume that there are a large number, n, of interconverting conformers that possess different enzymatic reactivities. The Michaelis–Menten mechanism can then be represented by the following kinetic scheme:

$$\begin{array}{ccccccccc}
S + E_1 & \underset{k_{-11}}{\overset{k_{11}[S]}{\rightleftharpoons}} & ES_1 & \xrightarrow{k_{21}} & P + E_1^0 & \xrightarrow{k_{31}} & E_1 \\
\alpha_{12}\downarrow\uparrow\alpha_{21} & & \beta_{12}\downarrow\uparrow\beta_{21} & & \gamma_{12}\downarrow\uparrow\gamma_{21} & & \\
S + E_2 & \underset{k_{-12}}{\overset{k_{12}[S]}{\rightleftharpoons}} & ES_2 & \xrightarrow{k_{22}} & P + E_2^0 & \xrightarrow{k_{32}} & E_2 \\
\downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \\
\vdots & & \vdots & & \vdots & & \\
\downarrow\uparrow & & \downarrow\uparrow & & \downarrow\uparrow & & \\
S + E_n & \underset{k_{-1n}}{\overset{k_{1n}[S]}{\rightleftharpoons}} & ES_n & \xrightarrow{k_{2n}} & P + E_n^0 & \xrightarrow{k_{3n}} & E_n
\end{array} \qquad (4)$$

where $k_{11} = k_{12} = \ldots = k_{1n} \equiv k_1$ and $k_{-11} = k_{-12} = \ldots = k_{-1n} \equiv k_{-1}$ are assumed for convenience.[24]

We have shown that when interconversion among the ES_i conformers is slow compared to the enzymatic reaction, that is, $k_{2i} \gg \beta_{ij}$ (the quasi-static condition), $f(t)$ is the weighted average of that for each individual conformer. When n is large,

$$f(\tau) = \int_0^\infty \mathrm{d}k_2\, w(k_2)\frac{k_1k_2[\mathrm{S}]}{2\alpha}[\exp(\alpha+\beta)t - \exp(\beta-\alpha)t] \qquad (5a)$$

where $w(k_2)$ denotes the probability density of the catalytic rate constant k_2, and α and β are identical to the corresponding parameters for each conformer in eq 3. With $w(k_2)$ assumed to be a Γ distribution, the observed $f(t)$ histograms at all concentrations can be well fit globally to eq 5 (blue curves in Figure 2B).

Remarkably, the reciprocal of the mean waiting time, $1/\langle\tau\rangle$, of eq 5a still obeys a Michaelis–Menten-type equation:

$$\frac{1}{\langle\tau\rangle} = \frac{\chi_2[\mathrm{S}]}{[\mathrm{S}] + C_\mathrm{M}} \tag{5b}$$

where $C_\mathrm{M} = (\chi_2 + k_{-1})/k_1$. Thus, the hyperbolic concentration dependence is preserved even in the presence of dynamic disorder under the quasi-static condition. However, unlike their counterparts $v_{max}/[\mathrm{E}]_\mathrm{T}$ and K_M in eq 2, χ_2 and C_M in eq 6 carry distinctly different microscopic interpretations. Specifically, $\chi_2 = 1/[\int_0^\infty (w(k_2)/k_2)\, \mathrm{d}k_2]$, thus χ_2 is the weighted harmonic mean of k_2 for all conformers, depending not only on the mean but also on the distribution of k_2.

Equation 5b can be viewed as generalized single-molecule Michaelis−Menten equation for the situation of dynamic disorder.[24] Its implication is that the phenomenological constants k_2 (catalytic rate constant) and K_M (Michaelis constant) derived from ensemble-averaged steady-state assays may no longer hold their conventional interpretations. Only in the absence of dynamic disorder, that is, when $f(\tau)$ is monoexponential, $\chi_2 = k_2$ and $C_\mathrm{M} = K_\mathrm{M}$. The fit of the Lineweaver−Burke plot in Figure 2A with eq 5b yields $\chi_2 = 730 \pm 80\ \mathrm{s}^{-1}$ and $C_\mathrm{M} = 390 \pm 60\ \mu$M for β-galactosidase, consistent with the apparent k_2 and K_M from fitting the ensemble enzymatic velocity with the conventional eq 2.

It is evident from the trajectory in Figure 1B that the enzymatic velocity of a single enzyme molecule exhibits large fluctuations. Figure 3A shows the distribution of enzymatic velocities derived from the single-molecule trajectory at [S] = 100 μM by binning every 50 turnover events to calculate the velocity. The maximum and the minimum turnover rate differ by more than 10-fold. The broad distribution of turnover rates indicates that dynamic disorder is by no means a small effect.

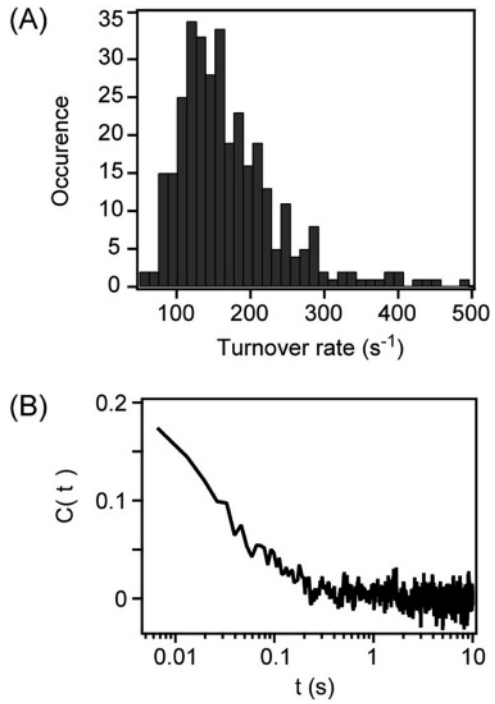

FIGURE 3. (A) Histogram of the enzymatic turnover velocity for [S] = 100 μM derived from a single-molecule trajectory after binning every 50 turnover events into one data point for the turnover velocity and (B) normalized autocorrelation functions of waiting times from eq 6 for [S] = 100 μM. This highlights the broad range of time scales of turnover rate fluctuations, spanning at least four decades from 10^{-3} to 10 s. (Adapted from ref 25.)

The time scale of the turnover rate fluctuation can be extracted from the autocorrelation function of the waiting times

$$C(m) = \frac{\langle\Delta\tau(0)\Delta\tau(m)\rangle}{\langle\Delta\tau^2\rangle} \tag{6}$$

where m is the index number for turnover events and $\Delta\tau(m) \equiv \tau(m) - \langle\tau\rangle$, the brackets denoting the time averaging along the trajectory.[14,25,26] In the absence of turnover rate fluctuation, $C(m) = 0$ for $m > 0$. The index number m can be further transformed to real-time t by multiplying by $\langle\tau\rangle$, namely, $t = m\langle\tau\rangle$.[35] Figure 3B depicts the striking $C(t)$ derived from the long trajectory of a single enzyme molecule. Every molecule under the same conditions exhibits similar behavior. The long time span of the $C(t)$ underscores the fact that the single enzyme molecule's catalytic velocity fluctuates over a broad range of time scales, from 10^{-3} to 10 s.

Similar enzymatic turnover rate fluctuations have been observed in other systems, such as cholesterol oxidase,[14] exonuclease,[19] and lipase.[21,22] We attribute these enzymatic rate fluctuations to fluctuations in the enzyme conformations, which is the subject of the following section.

Conformational Fluctuations within a Single Protein Molecule

We now describe another recent experiment[23] that has revealed conformational fluctuations within a single protein molecule spanning the time scales of milliseconds to hundreds of seconds, the same time scale at which the fluctuation of the enzymatic turnover rate constant takes place. This observation has provided a clue for the connection between enzymatic and conformational fluctuations.

The system is a protein complex formed between fluorescein (FL) and monoclonal anti-fluorescein (anti-FL),[23] whose crystal structure is shown in Figure 4A. Compared with our previous study on a flavin enzyme,[18] the FL/anti-FL complex is more stable with a low dissociation constant of 0.1 nM, allowing long-time observations at the single-molecule level. While free FL molecules in solution show a monoexponential fluorescence decay, the FL/anti-FL complex has a shorter fluorescence lifetime, γ^{-1}, with a multiexponential fluorescence decay (Figure 4B).

This results from photoinduced electron transfer (ET) from a nearby tyrosine residue (Tyr37 in anti-FL, the donor) to FL (the acceptor), which shortens γ^{-1} of FL, $\gamma^{-1} = (\gamma_0 + \gamma_\mathrm{ET})^{-1} \approx \gamma_\mathrm{ET}^{-1}$, where γ_0 denotes the fluorescence decay rate constant in the absence of quencher and γ_ET denotes the ET rate constant ($\gamma_\mathrm{ET} \gg \gamma_0$). γ_ET is exponentially dependent on and therefore extremely sensitive to

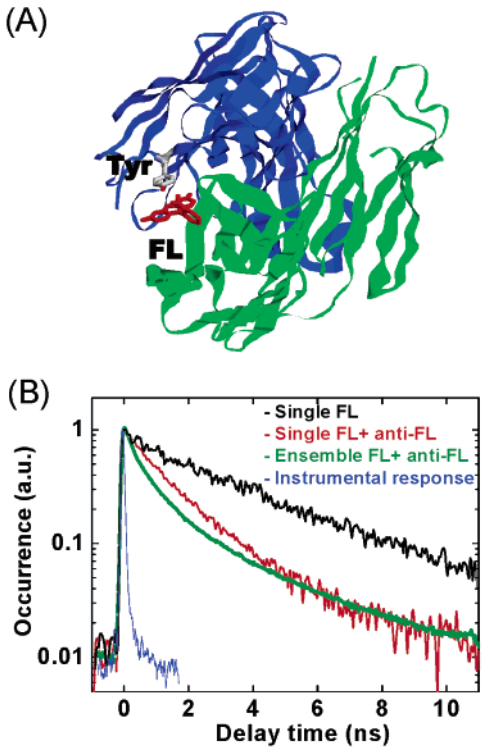

FIGURE 4. (A) Schematic of the structure of the FL and anti-FL complex. Tyr37 and FL are highlighted (adapted from ref 57) and (B) monoexponential fluorescence lifetime decay for a single FL molecule (black curve), multiexponential fluorescence decay for the FL/anti-FL complex at both ensemble (green curve) and single-molecule (red curve) levels, and the instrumental response function with 60-ps fwhm (blue curve) (adapted from ref 23).

the edge-to-edge distance between the ET donor and acceptor,[36]

$$\gamma_{ET}(t) = k_0\, e^{-\beta(x_{eq}+x(t))} \tag{7}$$

where k_0 is a constant, x_{eq} is the mean edge-to-edge distance between FL and Tyr37, $x(t)$ is the time-dependent distance fluctuation around x_{eq}, and $\beta \approx 1.4$ Å^{-1} for proteins. The multiexponential decay in Figure 4B is due to the spontaneous fluctuation of $x(t)$ during the course of the fluorescence lifetime measurement. By recording trajectories of $\gamma_{ET}^{-1}(t)$, that is, the sequence of the delay time of each detected photon with respect to its excitation pulse, we have been able to obtain trajectories of $x(t)$.[23,37] Figure 5A depicts a segment of the single-molecule $x(t)$ trajectory, along with the corresponding probability density of x, $P(x)$.

The potential of mean force (Figure 5B), which is determined by $U(x) = -k_BT \ln[P(x)]$, can be well fit to a harmonic potential, $U(x) = k_BTx^2/(2\theta)$, where $\theta = 0.22$ Å^2. The time scale of distance fluctuations can be extracted from the autocorrelation function of $x(t)$, $C_x(t) = \langle x(0)x(t)\rangle$. Figure 6A shows the $C_x(t)$ averaged over 13 molecules with fluctuations spanning a wide range of time scales, from 10^{-3} to 10^2 s, similar to the time scales of the turnover rate fluctuations described above.

We note that there has been considerable ensemble-averaged experimental evidence for the existence of long-lived conformers and multiple relaxations times in proteins. Most notable are the pioneering work of Frauenfelder et al. on dispersed kinetics of rebinding of ligands to heme protein upon photodissociation,[29] and the observation of spectral relaxation of the same system on multiple time scales (10^{-12} to 10^{-4} s).[38,39] The existence of long-lived protein conformers was also inferred by mass spectrometry.[40] NMR spectroscopy has also revealed multiple protein conformers with detailed structure information, as well as interconversion rates among the conformers.[41]

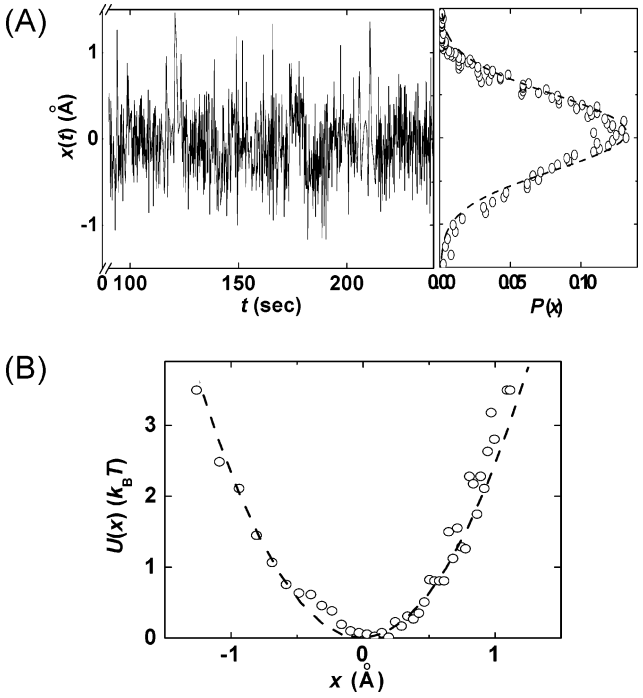

FIGURE 5. (A) A segment of the single-molecule $x(t)$ trajectory with the corresponding probability density function $P(x)$. The dashed line is the Gaussian fit for $P(x)$. (B) Potential of mean force $U(x)$ obtained from $U(x) = -k_BT \ln[P(x)]$. The dashed line is the best fit to a harmonic potential $U(x) = k_BTx^2/(2\theta)$, where $\theta = 0.22$ Å^2. (Adapted from ref 23.)

In the complex energy landscape of a protein, conformers at different local free energy minima can interconvert at room temperature due to thermal fluctuation.[42] Assuming an Arrhenius process with a prefactor of 10^{12} s^{-1}, the interconversion occurs either at the picosecond time scale if the barriers among them are 0.5 kcal/mol or at the second time scale if the barriers are 16 kcal/mol, which is by no mean unphysically high. The broad distributions of the barriers give rise to the large time span of conformational fluctuations. The single-molecule experiment allows for the characterization of the multiple time scale dynamics at an unprecedented level.[43]

In searching for a theoretical description of the observed 1D diffusion along the experimentally accessible coordinate, we first considered the Langevin equation for a particle diffusing with a reduced mass m within a harmonic well, $U(x) = m\omega^2x^2/2$,

$$m\frac{d^2x(t)}{dt^2} = -\zeta\frac{dx(t)}{dt} - \frac{dU(x)}{dx} + F(t) \tag{8}$$

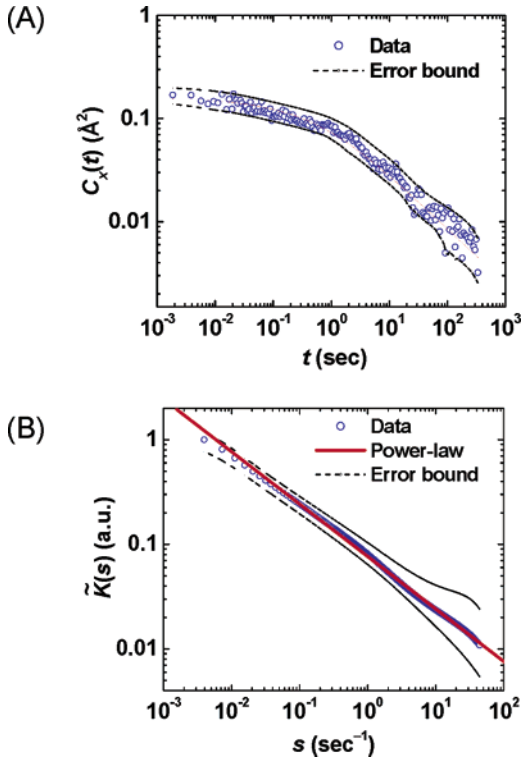

FIGURE 6. (A) Autocorrelation function of distance fluctuation $C_x(t)$ of the FL/anti-FL complex averaged over 13 molecules, determined with high time resolution, with $C_x(0) = \theta = 0.22$ Å^2 and (B) normalized $\tilde{K}(s)$ calculated from $C_x(t)$ in panel A using eq 11 (open circles). The full line is the fit of $s^{-0.49}$. Its inverse Laplace transform gives $K(t) \propto t^{-0.51}$. The dashed lines are error bounds carried over from the error bounds of $C_x(t)$ in panel A. (Adapted from ref 23.)

where ζ, the frictional constant, is related to the amplitude of the random fluctuating force, F, which is assumed to be white noise. However, eq 8 leads to the result $C_x(t) = \theta \exp(-m\omega^2 t/\zeta)$. The monoexponential decay fails to account for the observed multiexpoential $C_x(t)$, suggesting that the observed diffusion does not correspond to Brownian motion in a harmonic well.

The physical picture to explain the observation is that a protein molecule is a "sluggish" system in which one degree of freedom (say x) is strongly coupled to other degrees of freedom. A change in x requires collective motions of the other degrees of freedom. This results in a long time memory effect, a characteristic of non-Markovian behavior.

A mathematical reduction of this picture is provided by the generalized Langevin equation (GLE), which rigorously describes, in the linear response regime, the dynamics of a single degree of freedom of a general $(n + 1)$-dimensional Hamiltonian with $n \to \infty$ degrees of freedom of the bath.[44] The GLE is given by

$$m\frac{d^2x(t)}{dt^2} = -\zeta\int_0^t d\tau\, K(t-\tau)\frac{dx(\tau)}{d\tau} - \frac{dU(x)}{dx} + F(t) \quad (9)$$

where $\zeta K(t)$, the friction kernel, is the dynamic generalization of the static friction constant ζ in eq 8, and $F(t)$ is the random fluctuating force due to thermal motions of the bath and is no longer white but colored noise. For equilibrium fluctuations, $F(t)$ and $\zeta K(t)$ are related by the second fluctuation−dissipation theorem

$$\langle F(t)F(\tau)\rangle = k_B T\zeta K(t-\tau) \quad (10)$$

In the over-damped limit, where the inertial term $m\, d^2x(t)/dt^2$ can be neglected, we have shown that

$$\tilde{K}(s) = \frac{m\omega^2}{\zeta}\frac{\tilde{C}_x(s)}{C_x(0) - s\tilde{C}_x(s)} \quad (11)$$

where $\tilde{K}(s)$ and $\tilde{C}_x(s)$ are the Laplace transforms of $K(t)$ and $C_x(t)$, respectively.[23] When $C_x(t)$ is known from experiment, $\tilde{K}(s)$ can be calculated by numerical Laplace transform of $C_x(t)$.

We note that Berne and co-workers have extracted the memory kernel from velocity correlation functions derived from trajectories of molecular dynamics (MD) simulations on the femtosecond to picosecond time scales.[45] Equation 11 works for the over-damped regime (high ζ), allowing for the determination of the memory kernel at much longer time scales. The result, after normalization, is shown in Figure 6B. Over at least four decades of time, $\tilde{K}(s)$ exhibits a simple power-law decay, $\tilde{K}(s) \propto s^{\alpha}$, with $\alpha = -0.49 \pm 0.07$. The inverse Laplace transform of $\tilde{K}(s)$ gives $K(t) = t^{-\alpha-1} \propto t^{-1/2}$, which is remarkably simple.[23]

Is the existence of a power-law friction kernel on the 10^{-3} to 10^2 s time scale a common phenomenon for all proteins? The experiment on flavin adenine dinucleotide (FAD)/flavin oxidoreductase (Fre) complex also shows a power-law friction kernel with a similar power-law exponent.[20] The fact that these two systems are quite distinct both structurally and functionally and that the distances between ET donor and acceptor are different argues for the generality of power-law friction kernels common for all proteins. Similarly, MD simulations suggest a power-law behavior in the internal motion of lysozyme in solution on the picosecond to nanosecond time scales.[46] Single ion channel experiments have indicated that protein flexibility results in $1/f^{\alpha}$ noise in ion channel currents.[47,48]

More single-molecule experiments on additional systems are certainly needed to validate the generality of the power law phenomenon. Theoretically, however, we have recently proposed a microscopic model to account for the power law memory kernels based on the dynamics of a continuum semiflexible polymer chain.[49]

Kramer's Barrier Crossing Problem without Time Scale Separation

We shall now return to the observed multiexponential waiting time distributions (Figure 2B) and waiting time autocorrelations (Figure 3B) of enzyme catalysis. As discussed by Karplus,[50] this is a typical dynamic disorder phenomenon associated with protein biochemical reactions. Despite much progress in this field in the past two decades, the underpinnings are still not well understood theoretically. An insightful treatment of dynamic disorder has been given by Zwanzig in terms of a time-varying

control parameter, such as the area of the bottleneck.[27,28] Since then, various extensions and generalizations have been put forward along different lines.[51−53] Although this approach is conceptually straightforward, the control parameters are usually not experimentally accessible. As a result, their dynamics is often assumed empirically on an ad hoc basis. Instead, here we present an alterative model that employs the GLE for the Kramers' barrier crossing problem in conjunction with the experimentally determined power-law memory kernel.[33]

Kramers' approach to the problem of barrier crossing over a barrier is the corner stone for studies of chemical kinetics in the condensed phase.[31,32] The key idea is to describe a chemical reaction in terms of thermally activated barrier crossing events in a one-dimensional reaction coordinate. Despite its simplicity, the one-dimensional picture captures the essence of chemical dynamics, even for complicated systems such as proteins. In Kramers' original paper,[31] the bath friction is treated as being Markovian and the random fluctuating force is assumed to be Gaussian white noise (implying that the bath relaxes infinitely fast). As an important extension of Kramers' theory, Grote and Hynes introduced non-Markovian friction into the GLE for a bath with a finite time scale of thermal fluctuations.[54]

Both Kramers and Grote−Hynes theories give well-defined rate constants and therefore cannot account for dispersed kinetics or dynamic disorder, because the fluctuation of the reaction coordinate is always relatively fast compared to the reaction time scale[30,32] In other words, a clear separation of time scales is assumed in the theories of Kramers and Grote−Hynes at the outset.

However, what we have learned about single-molecule protein conformational fluctuations is that they span an extremely broad range of time scales and coincide with those of the enzymatic reaction.[23,20] Recent theoretical investigations have also suggested that the power-law friction kernel could be general for protein dynamics.[49,55] Our premise is that the same friction kernel might hold for the reaction coordinate in Kramers' escape problem for enzymatic reactions.

In our model, the dynamic disorder is not due to the fluctuation of barrier heights but is attributed to the non-Markovian random fluctuating force, $F(t)$. The attribution is less ad hoc in the context of GLE. With the phenomenological power law autocorrelation function, $F(t)$ fluctuates at time scales comparable to or longer than the time scale of barrier crossing. Such a lack of time scale separation has profound consequences for the reaction dynamics. In fact, rate constants no longer exist under this condition.

We have carried out stochastic simulations of generalized Langevin dynamics with a power-law memory kernel.[33] The equation that we numerically simulated is eq 9 in which $U(x)$ is a symmetric double well potential with a barrier height of E_a, a harmonic well frequency ω_a, and a barrier frequency ω_b (Figure 7, inset). The memory kernel function $K(t)$ was chosen to be $K(t - \tau) \propto |t - \tau|^{-1/2}$ according to the experimental findings described above.

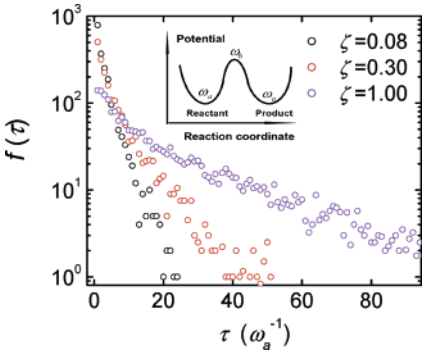

FIGURE 7. The waiting time distributions from the numeric simulation of Kramers' barrier crossing over a barrier (inset) with a power-law friction kernel described by GLE. The deviation of the decay from single-exponential behavior is greater when the coupling strength increases. In the simulation, $m = 1$, $k_BT = 1$, the barrier height $E_a = 2k_BT$, and all the times are given in units of $1/\omega_a$. (Adapted from ref 33.)

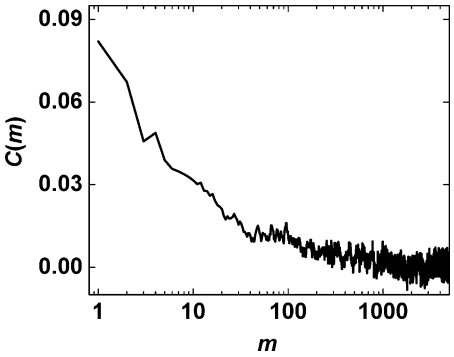

FIGURE 8. Autocorrelation function $C(m) = \langle \Delta\tau(0)\Delta\tau(m)\rangle/\langle \Delta\tau^2\rangle$ of a simulated waiting time trajectory that consists of 10 000 successive turnovers. The waiting time correlations span over a broad range of time scales, resembling the observed experimental results in Figure 3B. (Adapted from ref 33.)

The sampling algorithm for the random fluctuating force is based on a circulant matrix method that was originally designed for fractional Brownian motion.[56] We start the trajectory in the left well and record the waiting time when $x(t)$ crosses over the bottom of the product well. For a barrier height $E_a = 2k_BT$, barrier crossing events can be followed directly with reasonable computational times.

Figure 7 displays the simulated distribution of the first passage time, $f(\tau)$. For weak coupling strength (low friction coefficient, ζ), $f(\tau)$ shows single-exponential decay. However, for the large coupling strength limit ($\zeta = 1$), $f(\tau)$ exhibits multiexponential decay. For the latter situation, the same as the experimental data presented earlier, a rate constant is no longer well defined.[30,32]

We have also computed the autocorrelation function of the waiting times from a simulated repetitive enzymatic turnover trajectory.[33] For repetitive enzymatic turnovers with instantaneous reset from the product well to the reactant well, the random fluctuating force, $F(t)$, is continuously sampled following the power-law dynamics. Figure 8 depicts $C(m)$ of waiting times from a simulated 10 000 turnovers trajectory, calculated by eq 6. The multiexponential decay of $C(m)$ over three decades of time

scales clearly resembles the observed experimental results shown in Figure 3B.

The essence of our model is that by incorporating the experimentally determined power-law friction kernel into the GLE treatment of Kramers' barrier crossing formalism, the key experimental findings of multiexponential waiting time distributions and autocorrelations of enzymatic turnovers are accounted for naturally. The lack of a separation of time scales between the enzymatic turnover and conformational fluctuations results in no well-defined rate constant for enzymatic reactions.

In practice, only the mean enzymatic turnover rate, $1/\langle\tau\rangle$, holds rigorous physical meaning and could be well described by the Michaelis−Menten equation. The catalytic rate constants reported from ensemble-averaged assays are either oversimplifications due to low observable dynamic ranges and insufficient time spans of the data or associated with complicated functions reflecting dynamic disorder (eq 5b).

Conclusions

Our single-molecule experiments, together with theoretical and computational studies, have demonstrated that the single-molecule approach is extremely powerful in unraveling the conformational and enzymatic dynamics and have generated new insights about how enzymes' conformational fluctuations modulate catalytic activities. They not only have provided a qualitative picture that an enzyme molecule is a dynamic entity with fluctuations spanning a vast range of time scales but also have yielded quantitative information. In particular, we have shown that single-molecule Michaelis−Menten equations reconcile single-molecule and ensemble-averaged kinetics and that conformational fluctuations and dynamic disorder can be described by non-Markovian dynamics governed by the GLE.

The effects of enzymatic fluctuations would be less significant for a system comprising many enzyme molecules. However, they might have important implications for a living cell with a low copy number of enzymes in which the fluctuations might be readily manifested and have physiological consequences.

We hope this Account will add to our growing understanding of the working of enzymes and stimulate further experimental and theoretical investigations of enzyme dynamics in the future.

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