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ARTICLES

Ever-fluctuating single enzyme molecules: Michaelis-Menten equation revisited

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Enzymes are biological catalysts vital to life processes and have attracted century-long investigation. The classic Michaelis-Menten mechanism provides a highly satisfactory description of catalytic activities for large ensembles of enzyme molecules. Here we tested the Michaelis-Menten equation at the single-molecule level. We monitored long time traces of enzymatic turnovers for individual β-galactosidase molecules by detecting one fluorescent product at a time. A molecular memory phenomenon arises at high substrate concentrations, characterized by clusters of turnover events separated by periods of low activity. Such memory lasts for decades of timescales ranging from milliseconds to seconds owing to the presence of interconverting conformers with broadly distributed lifetimes. We proved that the Michaelis-Menten equation still holds even for a fluctuating single enzyme, but bears a different microscopic interpretation.

The quest to determine how enzymes work, essential to the understanding of life processes, continues to attract the fascination of enzymologists. Ever since the work of Michaelis and Menten[1], enzyme kinetics has had an essential role in the characterization of enzyme activities. The classic Michaelis-Menten equation provides a highly satisfactory description of enzymatic kinetics for large ensembles of enzyme molecules. However, recent advances in room-temperature single-molecule fluorescence studies[2–6] have allowed measurements of the distributions and fluctuations of molecular properties unattainable from ensemble data. Such measurements have shown that enzymes such as cholesterol oxidase[7], hairpin ribozyme[8], λ-exonuclease[9] and lipase[10,11] undergo temporal fluctuations of catalytic rates as a result of conformational fluctuations[12,13]. This finding has raised a number of intriguing questions. For example, how are single-molecule enzymatic measurements, which record stochastic waiting times of enzymatic reactions, reconciled with ensemble Michaelis-Menten kinetics? Why does the Michaelis-Menten equation work so well despite the broad distributions and dynamic fluctuations of single-molecule enzymatic rates? What new information is available from single-molecule experiments? In particular, what are the magnitude and timescales of the enzymatic rate fluctuations and what implication, if any, do these fluctuations have for cellular processes? Here we report a single-molecule assay of β-galactosidase that provides kinetic information over extended timescales (10^{-3}–10 s; 1–10^4 turnovers) and thereby allows us to address these questions.

According to the Michaelis-Menten mechanism, a substrate S binds reversibly with the enzyme E to form an enzyme-substrate complex ES. ES proceeds to product P, and E is regenerated for the next catalytic cycle.

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

The Michaelis-Menten equation

$$v = \frac{v_{\max}[S]}{[S] + K_M} \tag{2}$$

gives explicitly the hyperbolic dependence of the enzyme velocity v on substrate concentration $[S]$ in an ensemble experiment, where $K_M = (k_{-1} + k_2)/k_1$ is the Michaelis constant, $[E]_T = [E] + [ES]$ is the total enzyme concentration and $v_{\max}$ is the maximum enzyme velocity. Under the condition of large k_3, as in the case discussed below, $v_{\max} = k_2[E]_T$. In a conventional Lineweaver-Burke plot, $1/v$ versus $1/[S]$ follows a linear relation[14].

Distinctly different from ensemble experiments, a single-molecule turnover experiment records the stochastic time trace of repetitive reactions of an individual enzyme molecule, from which the probability density of the waiting time τ for an enzymatic reaction to occur, $f(\tau)$, can be determined[7,15,16]. It has been shown that for the kinetic scheme in equation (1), $f(\tau)$ shows a single exponential rise, followed by a single exponential decay[15,17].

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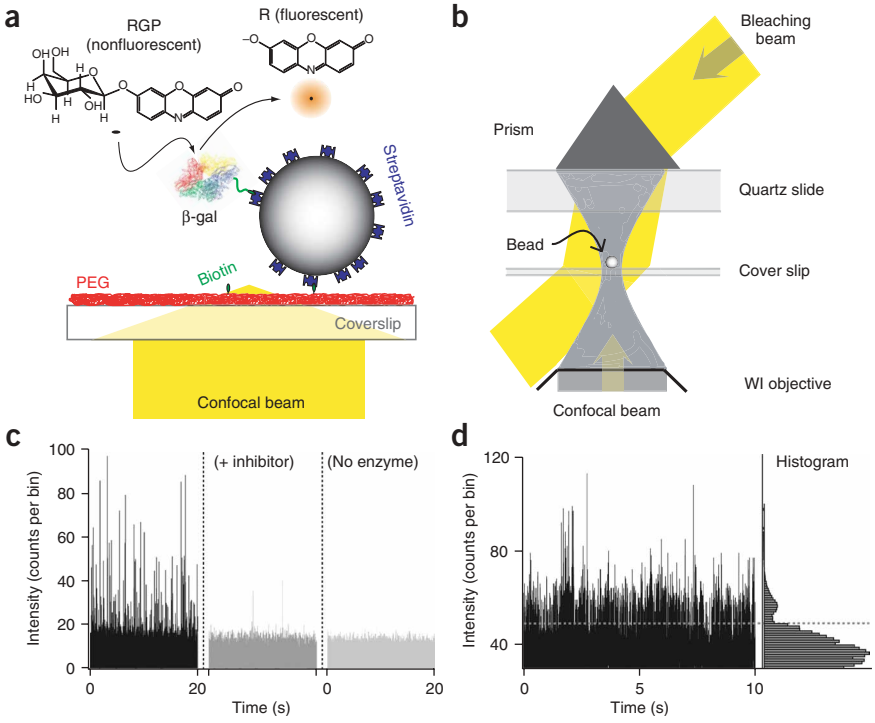


Figure 1 Single-molecule assay with fluorescent product. (**a**) Schematic representation of enzyme immobilization (not to scale). A single β-galactosidase molecule is linked to a streptavidin-coated polystyrene bead through a flexible PEG linker. The bead binds to the hydrophilic biotin-PEG surface of the glass coverslip. A photogenic resorufin-β-D-galactopyranoside (RGP, **1**) substrate in buffer solution is converted to a fluorescent resorufin (R, **2**) product by the single enzyme molecule, and detected one molecule at a time before it rapidly diffuses out of the confocal detection volume. (**b**) Schematic representation of the photobleaching and detection beams. A lens focuses a 550-mW, 560-nm photobleaching beam to a 200-μm-diameter spot surrounding the bead. The beam is coupled into the 100-μm-thick flow cell by a prism atop the quartz slide. The water immersion (WI) objective is used to focus a 1-mW, 560-nm detection beam onto a diffraction-limited spot around the bead and to collect the emission for detection with a photon-counting avalanche photodiode detector. (**c**) Turnover time trace of a single β-galactosidase molecule at 20 μM RGP. Left, fluorescence intensity as a function of time for a β-galactosidase molecule undergoing enzymatic turnovers, each giving a fluorescence burst. Middle, data for the same enzyme molecule after addition of 200 μM PETG inhibitor. Right, data for a bead without enzyme (no inhibitor). All time traces are obtained with 0.5-ms time bins. (**d**) Turnover time traces of a single β-galactosidase molecule at 100 μM RGP. Dashed line represents the threshold used to determine waiting times between two adjacent burst (see **Supplementary Methods** and **Supplementary Fig. 1** online). The intensity histogram of the enzymatic time trace is shown at right. The time trace has 0.5-ms time bins.

The concentration dependence of $f(\tau)$ is explicitly described in equation (3).

$$f(\tau) = \frac{k_1 k_2 [S]}{2A} [\exp(A+B)\tau - \exp(A-B)\tau] \quad (3)$$

where $A = \sqrt{(k_1[S] + k_{-1} + k_2)^2/4 - k_1 k_2 [S]}$ and

$B = -(k_1[S] + k_{-1} + k_2)/2$

The mean of τ is

$$\langle \tau \rangle = \int_0^\infty \tau f(\tau) d\tau$$

It follows from equation (3) that

$$\frac{1}{\langle \tau \rangle} = \frac{k_2[S]}{[S] + K_M} \quad (4)$$

which is termed the single-molecule Michaelis-Menten equation[17].

Comparing equation (4) with equation (2), it is evident that the reciprocal of the mean waiting time measured in a single-molecule experiment is related to the enzymatic velocity measured in an ensemble experiment by $1/\langle \tau \rangle = \nu/[E]_T$. This relation originates from the equivalence between averaging over a single molecule's long time trace and averaging over a large ensemble of identical molecules. There are two types of ensemble enzymatic experiments[18]: one reports enzymatic velocities under steady-state conditions, which can be compared with $1/\langle \tau \rangle$; the other reports time-dependent population kinetics under pre–steady-state conditions, which can be compared with $f(\tau)$.

We set out to test experimentally the validity of equations (2) and (4), that is, the validity of the fundamental Michaelis-Menten equation at the single-molecule level. Single-molecule enzymatic assays involving fluorescence detection have previously been limited to short observation times because of photobleaching of fluorophores. To circumvent this problem, an assay with continuously replenished fluorescent product molecules was proposed[19] and then adapted for lipase[10,11]. In order to acquire the statistics necessary for reliable data analyses, we conducted experiments on β-galactosidase with a fluorogenic substrate that allowed us to extend observation lengths to $\sim 2 \times 10^4$ total turnovers. The analysis of these time traces not only confirmed the validity of the single-molecule Michaelis-Menten equation but also revealed a memory effect that lasts for decades of timescales. The characterization of such a memory effect, hidden in ensemble experiments, has provided new insights into how enzymes work on a single-molecule level.

RESULTS

Single-molecule assay with fluorescent product

Escherichia coli β-galactosidase is a 465-kDa enzyme that catalyzes the hydrolysis of lactose. The enzyme's crystal structural and biochemical details have been described[20,21]. Containing four catalytic sites, β-galactosidase is active only as tetramer[22]. It is known to obey the Michaelis-Menten equation[23,24], suggesting that the four well-separated catalytic sites are independent of each other[25]. In our investigations, instead of lactose, we used fluorogenic resorufin-β-D-galactopyranoside (RGP, **1**) (Molecular Probes) as the substrate[26]. Upon hydrolysis this molecule generates resorufin (R, **2**) as a fluorescent product (**Fig. 1a**). Using a fluorescence microscope, we continuously monitored enzymatic turnovers of a single immobilized β-galactosidase molecule immersed in a buffer solution of a constant substrate concentration. Each turnover was characterized by a

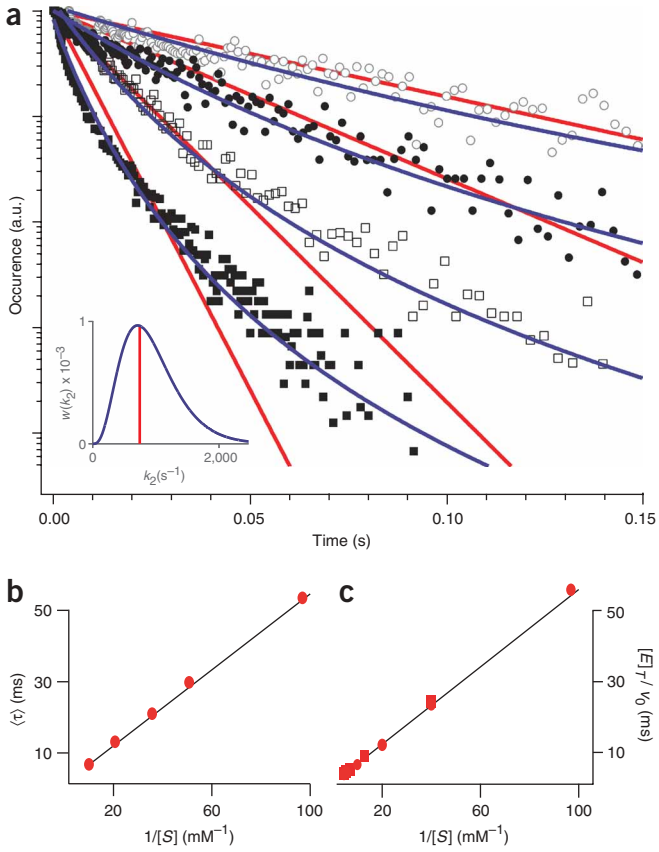


Figure 2 Concentration dependence of waiting time. (**a**) Histograms of the waiting time between two adjacent turnovers in a log-linear scale obtained from time traces of individual β-galactosidase molecules at four RGP concentrations, 10 μM (○), 20 μM (●), 50 μM (□) and 100 μM (■). The histograms change from monoexponential to multiexponential with increasing concentrations. Shown in blue are best global fits at all concentrations to $f(\tau)$ in the presence of dynamic disorder (equation (5)). We assume k_2 follows a gamma distribution, $w(k_2) = 1/\{b^a\Gamma(a)\}k_2^{a-1}\exp(-k_2/b)$, with $\Gamma(a)$ being the gamma function and a and b being the only adjustable parameters. The fitting parameters are $a = 4.2$ and $b = 220\ s^{-1}$, with $k_1 = 5 \times 10^7\ M^{-1}s^{-1}$ and $k_{-1} = 18{,}300\ s^{-1}$ (constrained by equation (8) with χ_2 and C_M obtained from **b**). The distribution (blue) and mean (red) of k_2 are shown in the inset. In contrast, shown in red are poor global fits of $f(\tau)$ in the absence of dynamic disorder (equation (3)) with the fitting parameter $k_2 = 730\ s^{-1}$, using the above k_1 and k_{-1}). (**b**) Single-molecule Lineweaver-Burke plot, $\langle\tau\rangle$ versus $1/[S]$ (red circles). Each data point is derived from one β-galactosidase molecule at the corresponding concentration. The black line depicts the fit with the single-molecule Michaelis-Menten equation (equation (6)), with $\chi_2 = 730 \pm 80\ s^{-1}$ and $C_M = 390 \pm 60$ μM. (**c**) Ensemble Lineweaver-Burke plot obtained at 11 pM (squares) and 53 pM (circles) biotin-β-galactosidase concentrations. The black line depicts the fit with the conventional Michaelis-Menten equation, equation (2), giving $v_{max}/[E]_T = 740 \pm 60\ s^{-1}$ and $K_M = 380 \pm 40$ μM, which are consistent with the χ_2 and C_M values, respectively, in **b**.

fluorescence burst from a resorufin molecule before it quickly diffused away from the probe volume of a tight laser focus (**Fig. 1b**). The residence times in the diffraction-limited confocal volume were less than the bin time of 500 μs. The burst intensities (**Fig. 1c,d**) vary because of different diffusion trajectories of the resorufin molecules out of the probe volume. Because resorufin is continuously produced by each turnover, observations could be made over long periods, limited only by the dissociation time of the β-galactosidase tetramer (several hours; see Methods and **Supplementary Fig. 2**).

We tethered single β-galactosidase molecules to polymer beads 490 nm in diameter, which were dispersed at an extremely low surface density onto a polymer-coated glass coverslip (**Fig. 1a**). This allowed us to locate enzyme molecules rapidly through difference interference contrast (DIC) images of the beads. The stoichiometry of bead-enzyme coupling (20:1) made it statistically unlikely that a bead would contain more than one β-galactosidase molecule. Special care was taken to avoid perturbation of the enzymatic activity by immobilization. The β-galactosidase molecules were biotinylated to the streptavidin-coated polystyrene beads via a flexible cysteine-reactive polyethylene glycol (PEG) linker. (As an important control, we measured the enzymatic velocities before and after coupling to the beads in an ensemble experiment with a fluorometer and found them to be the same, proving that enzyme activity is unperturbed by the beads; see **Supplementary Fig. 3**.) The beads then bound to the biotin-PEG surface of the coverslip, which has little affinity for both enzyme[27] and resorufin.

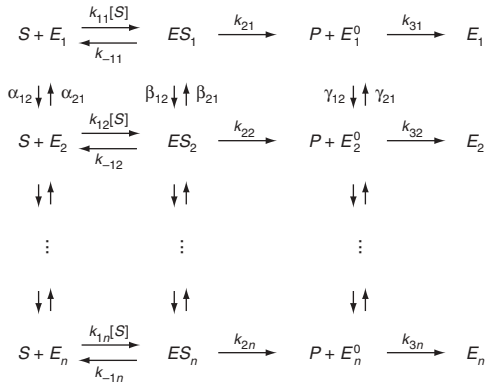
There existed a strong background signal caused by resorufin molecules that are continuously generated by autohydrolysis even in the absence of the enzyme (**Supplementary Fig. 4**) and that diffuse into the probe volume. In addition, resorufin molecules generated from previous or nearby enzymatic conversions can enter the probe volume. To circumvent this complication, we illuminated the area around the enzyme molecule with an intense laser beam (1,600 W cm^{-2}) (**Fig. 1b**) to bleach resorufin molecules diffusing into the probe volume, thus suppressing the background signal by at least two orders of magnitude (see **Supplementary Fig. 5**). However, a resorufin molecule generated by catalysis would not be photobleached during its short residence time within the probe volume.

Fluorescence-intensity time traces of enzymatic turnovers at 20 and 100 μM substrate concentrations at 22 °C are shown (**Fig. 1c,d**). As a control, we demonstrated that the signal disappeared when a competitive inhibitor, phenylethyl-β-D-thiogalactopyranoside (PETG), was introduced (**Fig. 1c**). As another control, we showed that no signal was visible when no β-galactosidase was bound to a bead (**Fig. 1c** and **Supplementary Methods** online for control experiments).

Michaelis-Menten equation with dynamic disorder

Plotting on a log-linear scale the waiting time between two adjacent fluorescence bursts obtained at substrate concentrations ranging from 10 to 100 μM RGP showed that at low substrate concentrations, the waiting-time histograms were monoexponential, as expected (see equation (3)) (**Fig. 2a**). At higher substrate concentrations, however, the waiting-time distributions became multiexponential, in disagreement with the prediction of monoexponential $f(\tau)$ (equation (3)) (compare the red curves of **Fig. 2a**). We note that the long tail in the multiexponential $f(\tau)$ is otherwise difficult to observe without a broad dynamic range and good statistics (>3,000 turnovers).

We attribute the multiexponential behavior of $f(\tau)$ to dynamic disorder[7,28,29], which refers to the fluctuations in the rate constants and is associated with conformational dynamics. It is well known that conformational changes, often triggered by substrate binding[30] and enzymatic reactions[31] within one catalytic cycle, are essential to enzyme functions. Nonetheless, it has long been inferred from ensemble-averaged experiments that a protein molecule undergoes conformational fluctuations on multiple timescales owing to rugged energy landscapes[32,33]. Fluctuations in single ion channels have been attributed to conformational changes[34]. Recently, such spontaneous



Scheme 1 Kinetic scheme of the multistate model involving n interconverting conformers. The multistate model is an extension of equation (1) where E, ES and E^0 are allowed to exist in any number n of mutually interconverting conformers. In this scheme, E_i not only interconverts with E_{i+1} and E_{i-1} but does so with all other conformers as well. This kinetic scheme does not include 'sequential' conformational intermediates induced by substrate binding or the enzymatic reaction within each catalytic cycle because their contributions to the long time fluctuation are less profound than those of the 'parallel' conformers.

conformational fluctuations have been directly observed and characterized at the single-molecule level on the timescale of 10^{-4}–10 s[12,13], a timescale longer than catalytic cycles. Although the microscopic characteristics of the conformers are yet unknown, they might be associated with the dynamic network of hydrogen bonds and electrostatic or ionic interactions within the protein. Mutation[35] and isotope[36] studies have indicated that either global or local conformational changes can affect enzymatic activity. Our premise is that the interconverting conformers possess different enzymatic reactivities. Therefore, the Michaelis-Menten mechanism can be represented by a kinetic scheme involving any number of interconverting conformers (**Scheme 1**).

Qualitatively, the concentration dependence of the multiexponential behavior of $f(\tau)$ can be understood as follows: at low substrate concentration, enzyme-substrate binding is rate limiting with a pseudo–first order rate constant, and hence $f(\tau)$ is a monoexponential decay. Indeed, the monoexponentiality of $f(\tau)$ at low $[S]$ argues for the lack of a broad distribution of k_{1i}. However, at high substrate concentrations, k_{2i} becomes rate-limiting, and the slow interconversion among the conformers ES_i results in a multiexponential decay of $f(\tau)$.

Quantitatively, when dynamic disorder is present only in k_2, that is when $k_{11} = k_{12} = \ldots = k_{1n} \equiv k_1$ (justified above) and $k_{-11} = k_{-12} = \ldots = k_{-1n} \equiv k_{-1}$ (to be justified later), and when interconversion among the ES_i conformers is slow compared to the enzymatic reaction, that is $\beta_{ij}/k_{2i} \rightarrow 0$, and α_{ij}/k_2 being small but nonzero (the quasi-static condition), $f(\tau)$ is the weighted average of k_{2i} conformers. Qualitatively, the quasi-static condition implies that the interconversion between the conformers is slow compared to the turnover time. We assume that k_2 has a continuous distribution, $w(k_2)$.

Under the condition of large number (n) of slowly interconverting conformers with different k_2 in a single enzyme molecule, it follows that

$$f(\tau) = \int_0^\infty dk_2 w(k_2) \frac{k_1 k_2 [S]}{2A} [\exp(A+B)t - \exp(B-A)t] \qquad (5)$$

where $w(k_2)$ denotes the probability density of k_2 for the conformers (see ref. 17). A and B are identical to the corresponding parameters in equation (3) for each conformer.

The observed histograms at all concentrations can be well fit globally to the predicted $f(\tau)$ assuming $w(k_2)$ is a gamma distribution using a maximum likelihood fitting procedure with $k_1 = 5 \times 10^7$ $M^{-1}s^{-1}$ and $k_{-1} = 18{,}300$ s^{-1} (**Fig. 2a**; blue curves). The resulting k_2 distribution (inset, **Fig. 2a**) highlights the extremely broad distribution of k_2, indicating the large number of conformers in a single enzyme molecule. Consistent with these findings, our Monte Carlo simulations have shown that the presence of even ten discrete conformers is not consistent with our experimental observations (see **Supplementary Methods** and **Supplementary Fig. 6**).

Notably, the reciprocal of the mean waiting time, $1/\langle\tau\rangle$, has been shown theoretically to obey a single-molecule Michaelis-Menten equation[17] under the quasi-static condition of dynamic disorder, a condition less stringent than the ones leading to equation (4).

$$\frac{1}{\langle\tau\rangle} = \frac{\chi_2 [S]}{[S] + C_M} \qquad (6)$$

Seemingly identical to the classic Michaelis-Menten equation (equation (2)), the hyperbolic concentration dependence is preserved even in the presence of dynamic disorder. However, unlike their counterparts $v_{max}/[E]_T$ and K_M, the parameters χ_2 and C_M carry distinctly different microscopic interpretations. Specifically,

$$\chi_2 = \frac{1}{\int_0^\infty \frac{w(k_2)}{k_2} dk_2} \qquad (7)$$

thus χ_2 is the weighted harmonic mean of k_2 for all conformers. Parallel with $K_M = (k_2 + k_{-1})/k_1$, the apparent Michaelis constant C_M is given by

$$C_M = (\chi_2 + k_{-1})/k_1 \qquad (8)$$

Hence χ_2 is dependent not only on the mean but also on the distribution of k_2. In other words, two mutations of an enzyme with an identical mean k_2 but different widths of the k_2 distributions would have different χ_2.

To verify the hyperbolic concentration dependence of the single-molecule Michaelis-Menten equation (equation (6)), we show a single-molecule Lineweaver-Burke plot, $\langle\tau\rangle$ versus $1/[S]$ (**Fig. 2b**), and compare it with the ensemble Lineweaver-Burke plot of

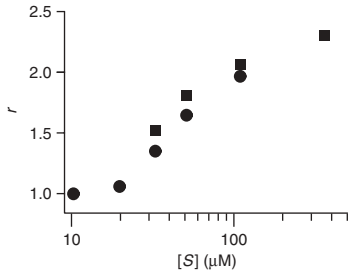


Figure 3 Concentration dependence of randomness parameter. Randomness parameter r as a function of substrate concentration. Circles, r obtained from waiting times of individual time traces: $r_\tau = (\langle\tau^2\rangle - \langle\tau\rangle^2)/\langle\tau\rangle^2$. Squares, r obtained from individual intensity time traces: $r_I = (\langle I^2\rangle - \langle I\rangle^2)/\langle I\rangle$ (see ref. 37, (equation (17)), consistent with r_τ at low concentrations, and obtainable even at the highest concentration (380 μM) when individual turnovers cannot be clearly resolved. That r is higher than unity at high substrate concentrations reveals the existence of dynamic disorder in k_2 but not in k_{-1}.

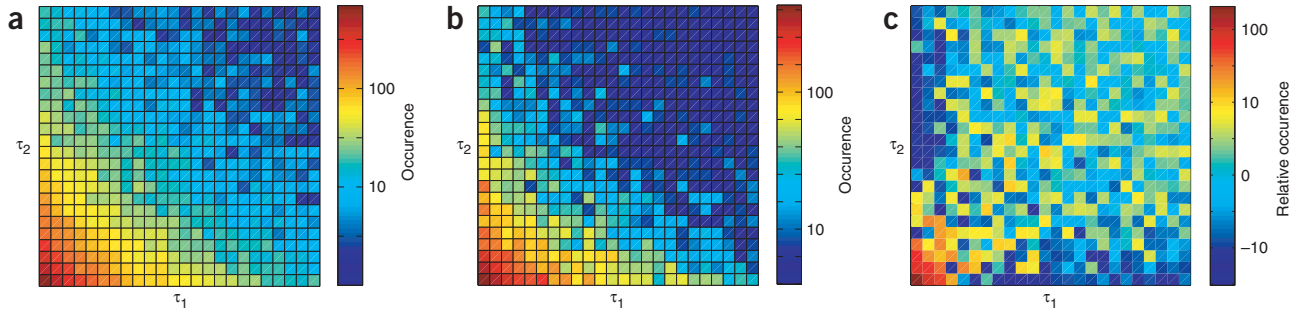


Figure 4 Two-dimensional joint-probability distributions of waiting times. (**a**) The 2D joint probability distribution of two adjacent waiting times (τ_1 and τ_2), $g(\tau_1,\tau_2)$, obtained from the time trace of a single β-galactosidase molecule at 100 μM substrate concentration. The τ_1 and τ_2 axes run from 0 to 12 ms. The color code represents the occurrence (*z* axis) from 500 (deep red) to 1 (dark blue). (**b**) The 2D joint probability distribution of two waiting times (τ_1 and τ_2) at a larger separation for same time trace as in **a**, which can be represented by $f(\tau_1)f(\tau_2)$ because of the lack of correlation between τ_1 and τ_2. The time axes are the same as in **a**. (**c**) The difference 2D histogram, $\delta(\tau_1, \tau_2) = g(\tau_1, \tau_2) - f(\tau_1)\, f(\tau_2)$. The time axes are the same as in **a**. The pixels in 'cold' colors (less probable) gather along two wings in the *x*-*y* plot, whereas those with 'warm' color (more probable) are mainly spread around the diagonal. A long waiting time tends to be followed by a long one, and a short waiting time tends to be followed by a short one.

biotin–β-galactosidase in solution (**Fig. 2c**). The least-squares fit in the former gives $\chi_2 = 730 \pm 80\ s^{-1}$ and $C_M = 390 \pm 60$ μM. The latter gives $v_{max}/E_T = 740 \pm 60\ s^{-1}$ and $K_M = 380 \pm 40$ μM (see **Supplementary Methods** online), which are consistent with the literature values for non-biotinylated enzymes[23,24,26]. The excellent agreement between χ_2 and v_{max}/E_T and between C_M and K_M, respectively, establishes the equivalence between $1/\langle\tau\rangle$ and the ensemble enzymatic velocity, and explains why the Michaelis-Menten equation is so widely applicable, even in the case of a multiexponential $f(\tau)$.

As a consistency check, under the condition that $w(k_2)$ is a gamma distribution with *a* and *b* values obtained from **Figure 2a**, equation (7) gives $\chi_2 = (a-1)b = 715\ s^{-1}$, which is consistent with the experimental χ_2 determined independently from the single-molecule and conventional Michaelis-Menten equations, respectively, as shown above.

Beyond just the consistency of the first moment of $f(\tau)$, $\langle\tau\rangle$, with ensemble measurements, the higher moments of $f(\tau)$ contain additional information. In particular, the second moment is related to the randomness parameter[37,38], $r = \{\langle\tau^2\rangle - \langle\tau\rangle^2\}/\langle\tau\rangle^2$. In the absence of dynamic disorder, *r* is unity at low or high substrate concentrations when there is only one rate-limiting step in equation (1), and less than unity at intermediate concentrations. We have shown theoretically that *r* becomes greater than unity for high concentrations in the presence of dynamic disorder in k_2 (ref. 17). Experimental measurement of *r* as a function of the substrate concentration (**Fig. 3**) showed that at low concentrations, *r* equals unity, as expected. Then parameter *r* rises above unity, however, as the dynamic disorder in k_2 becomes more apparent at increasing concentrations. We note that if the dynamic disorder were in k_{-1} instead of k_2, *r* would not be larger than unity[17], which corroborates the attribution of dispersed kinetics to dynamic disorder in k_2.

Memory effects of a single enzyme molecule

Besides the first and second moments of τ, a turnover time trace contains valuable information regarding temporal correlations between turnovers, which are inaccessible in ensemble data. To explore these correlations, we evaluate the two-dimensional (2D) joint probability $g(\tau_1, \tau_2)$ for two waiting times, τ_1 and τ_2, separated by a certain number of turnovers. Much discussed recently[39–43], this type of analysis was first introduced in analyzing time traces of cholesterol oxidase[7], for which only limited statistics were available. With much better statistics afforded by the long time traces in our experiment, we compared the 2D joint histogram for waiting times, τ_1 and τ_2, of two adjacent turnovers in a single-molecule time trace at 100 μM RGP concentration (**Fig. 4a**) with the 2D joint histogram of the same molecule for two turnovers with a large separation (**Fig. 4b**), which is described by $h(\tau_1, \tau_2) \equiv f(\tau_1)\, f(\tau_2)$ because of the loss of correlation. Were there no dynamic disorder, $g(\tau_1, \tau_2)$ and $h(\tau_1, \tau_2)$ would be the same. That these two histograms are different indicates the existence of a memory effect resulting from slow interconversion among the conformers. To highlight the memory effect, the difference histogram, $\delta(\tau_1, \tau_2) \equiv g(\tau_1, \tau_2) - h(\tau_1, \tau_2)$, is shown (**Fig. 4c**); this indicates that a short waiting time is less likely to be followed by a long waiting time, and vice versa.

Being a scrambled histogram, the difference histogram (**Fig. 4c**) does not explicitly reveal the timescales associated with the memory effect—that is, the fluctuation of the enzymatic velocity $k(t)$, defined as the number of turnovers per unit time. To extract this formation, we calculated the intensity autocorrelation function $C_I(t) = \langle\Delta I(0)\Delta I(t)\rangle/\langle\Delta I^2\rangle$, which characterizes the timescales of $k(t)$ fluctuations. With increasing substrate concentrations (**Fig. 5a**), $C_I(t)$ becomes more multiexponential as the dynamic disorder of k_2 is more apparent. As a control, we found that $C_I(t)$ is zero (**Fig. 5a**, in gray) when no enzyme molecule is in the probe volume. It should be noted that for $t > 0$, $C_I(t) = \langle\Delta I(0)\Delta I(t)\rangle/\langle\Delta I^2\rangle$ has no contribution from uncorrelated counting and background noise (see **Supplementary Methods** online).

However, $C_I(t)$ does not solely probe $k(t)$ fluctuations because it has a contribution from fluorophore diffusion[44], especially at low substrate concentrations (**Fig. 5a**, in red). To overcome this potential complication, we obtain the autocorrelation function of the waiting times

$$C_\tau(m) = \frac{\langle\Delta\tau(0)\Delta\tau(m)\rangle}{\langle\Delta\tau^2\rangle} \qquad (9)$$

as a function of m, the index number of turnovers and $\Delta\tau(m) \equiv \tau(m) - \langle\tau\rangle$. In the absence of dynamic disorder, $C_\tau(m) = 0$ for $m > 0$. The index number *m* is transformed to real time by $t = m\langle\tau\rangle$. It has been shown that $C_\tau(t)$ is approximately the normalized autocorrelation function of $k(t)$, $\langle\delta k(0)\delta k(t)\rangle/\langle\delta k^2\rangle$, under the limit of slow fluctuations of $k(t)$[45]. The blue trace in **Figure 5b** depicts $C_\tau(t)$ for the time trace with 100 μM RGP, which shows a decay similar to that of the $C_I(t)$ in **Figure 5a** (blue), only with a reduced time resolution (see **Supplementary Fig. 7** for a high time resolution). This

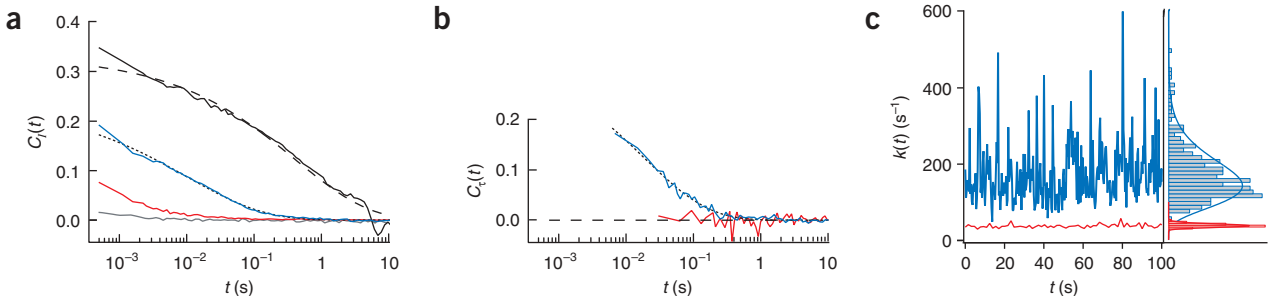


Figure 5 Fluctuations of turnover rate constants. (**a**) Intensity autocorrelation functions obtained from individual time traces of single β-galactosidase molecules at 20 μM (red), 100 μM (blue) and 380 μM (black) RGP. The intensity autocorrelation obtained 1 μm away from the enzyme is flat (in gray). At 100 μM, the dotted curve depicts the best fit to a stretched exponential: $C_I(t) = C_I(0)\ \exp[-(t/t_0)^\beta]$, with $\beta \sim 0.46$ and $t_0 \sim 0.017$ s. At 380 μM, the dashed curve depicts the best fit with $\beta \sim 0.4$ (more stretched) and $t_0 \sim 0.4$ s. We observed similar $C_I(t)$ for each of 23 individual enzyme molecules at 380 μM RGP ($\beta \sim 0.4 \pm 0.1$ and $t_0 \sim 0.4 \pm 0.1$ s). This $C_I(t)$ indicates that k_2 fluctuates at timescales spanning at least four decades (10^{-3}–10 s). (**b**) Waiting time autocorrelation functions, $C_\tau(t)$, obtained at 20 μM (red) and 100 μM (blue) RGP. The dotted curve depicts the best stretched exponential fit for **b** with parameters of $\beta = 0.45$ and $t_0 \sim 0.018$ s, identical to those for $C_I(t)$ at 100 μM. (**c**) Two time traces of the enzymatic velocities $k(t)$ of two single enzyme molecules at 20 μM (red) and 100 μM (blue) RGP concentration, respectively. Each data point is the average of 50 turnovers. The k histograms of the two time traces are shown on the right. The histograms are fit using gamma distributions as in **Figure 2a**, with $a = 71.5$ and $b = 0.5\ s^{-1}$ for the 20 μM, and with $a = 6.4$ and $b = 26.6\ s^{-1}$ for 100 μM RGP. The large amplitude of k fluctuation for the latter is evident.

consistency indicates that $C_I(t)$ reflects $\langle \delta k(0)\delta k(t)\rangle / \langle \delta k^2 \rangle$ at high concentrations. Unfortunately, $C_\tau(t)$ is not obtainable at an even higher concentration (380 μM) at which individual turnovers cannot be resolved and we revert to $C_I(t)$.

At 380 μM RGP, $C_I(t)$ is highly multiexponential (**Fig. 5a**, in black) and can be phenomenologically fit to a stretched exponential function $C_I(t) = C_I(0)\ \exp[-(t/t_0)^\beta]$ with $\beta = 0.4 \pm 0.1$ and $t_0 = 0.4 \pm 0.1$ s. $C_I(t)$ is monoexponential when $\beta = 1$ and shows a long tail in the decay when $\beta < 1$. We observed a low β value ($\beta \sim 0.4$) for each of the 23 enzyme molecules examined at the same substrate concentration. This highlights the broad range of timescales of $k(t)$ fluctuations, spanning at least four decades from 10^{-3}–10 s. Notably, this coincides with the same timescales at which conformational fluctuations within a single protein molecule have been observed[12,13]. This strongly argues that enzymatic fluctuations do indeed originate from conformational fluctuations.

We determined the distribution of $k(t)$ by binning every 50 turnovers along the turnover time trace. The $k(t)$ distributions of two enzyme molecules at 100 and 20 μM RGP (**Fig. 5c**) show that, as expected, the distribution at 20 μM was narrow because substrate binding is rate limiting. At 100 μM, the overall profile of the k distribution is well fit by a gamma distribution, which corroborates the assumption of gamma distribution made earlier. The broad width of the gamma distribution indicates that dynamic disorder is by no means a small effect.

DISCUSSION

The very large amplitude and broad timescales of enzymatic rate fluctuations at the single-molecule level, which are uncovered by analyses of long turnover time traces, highlight the fact that an enzyme molecule is an ever-fluctuating dynamic entity during catalysis. Despite its apparent generality[7–11], this phenomenon has been hidden in the ensemble enzymatic assays for two reasons. First, in pre–steady-state ensemble measurements, data often lack the dynamic range necessary to identify long tails in multiexponential kinetics. Second, in steady-state ensemble measurements, data are masked by the fact that the Michaelis-Menten equation holds not only for the simplistic case of a single conformer but also more generally for the case of a large number of slowly interconverting conformers with different enzymatic activities.

The effects of enzymatic fluctuations would be less significant for a system comprising many enzyme molecules. However, if a system contains a small number of enzyme molecules, as is often the case in a living cell, the enzymatic fluctuations could be readily manifested. Biologically important as they might be, such fluctuations might now be probed on a single-molecule basis in living cells.

METHODS

Substrate purification. The photogenic substrate solution has to be free of even trace amounts of resorufin before single-molecule turnovers can be observed. Resorufin-β-D-galactopyranoside (RGP) (Molecular Probes) was always newly purified at the start of each experiment using an anion-exchange FPLC column (HiTrap Q XL, Amersham) after most resorufin was removed by acidic chloroform extraction. A low-ionic-strength, pH 7.5 buffer containing 10 mM triethanolamine was used to elute the substrate at a concentration of 0.6 mM. Unless otherwise noted, all ensemble and single-molecule experiments were conducted in 25 mM dibasic phosphate buffer (pH adjusted to 7.5) containing 0.25 M NaCl, 5 mM $MgCl_2$, 0.05 mg ml^{-1} BSA (New England Biolabs), 0.005% Tween-20 (Sigma) and 10% (w/w) PEG (8,000 MW, Sigma).

Estimation of tetramer dissociation. It is well known that β-galactosidase is inactivated at very dilute concentrations owing to dissociation of the tetrameric enzyme. To determine the timescale of tetramer dissociation, we recorded the enzymatic activity, as a function of time, of 2 pM of biotin-linked β-galactosidase immobilized on 1-μm-diameter streptavidin-coated beads present in excess. At various time points, the beads were separated from solution by centrifugation and the amount of active enzyme measured using a fluorometer (**Supplementary Fig. 3**) with 200 μM RGP. In this experiment, the low concentration of the immobilized enzyme assured that no recombination occurred after tetramer dissociation. The enzyme activity decayed exponentially (**Supplementary Fig. 2**), giving a time constant of tetramer dissociation of 5.6 ± 0.7 h, which defined a time window in which the experiment had to be completed.

Linking enzyme to functionalized-glass surface. Owing to the high catalytic efficiency of β-galactosidase, the presence of large amounts of enzyme in the flow cell would markedly increase the background level of resorufin fluorophores. To avoid this, the surface concentration of immobilized enzyme was maintained at roughly one enzyme molecule per square millimeter (for β-galactosidase functionalization and characterization, see **Supplementary**

Methods online). To make it possible to rapidly locate an enzyme molecule at such dilute concentrations, the enzyme was coupled to streptavidin-coated polystyrene beads (490-nm diameter, Bangs) that were large enough to be visualized by DIC microscopy. The streptavidin-coated beads were chosen because they contribute no fluorescence signal under laser excitation and have little affinity for resorufin.

Biotin-linked enzyme (10 nM) and beads were incubated for 1 h at 4 °C. Because the tetrameric enzyme dissociates at very low concentrations, the incubation was conducted in a ten-fold excess of non-biotinylated β-galactosidase. Immediately before the single-molecule experiment, the beads were separated from unbound enzyme by centrifugation. The bead-enzyme coupling reaction was controlled such that most beads were without enzyme (>95%). This made it statistically unlikely (<0.25%) that an active bead would carry more than one β-galactosidase enzyme.

Before the start of the bead incubation, the flow cell was flushed with 0.1 mg ml^{-1} BSA and 0.01% Tween-20 to inhibit nonspecific binding of beads to the tubing and to the surfaces of the flow cell (see **Supplementary Methods** online for preparation of the flow chamber). The streptavidin beads bound specifically to the biotinylated PEG surface of the coverslip. The surface density of the beads was monitored through the eyepiece by DIC microscopy and the beads in solution were flushed out when a low surface density of several beads per 100 μm^2 was obtained.

Optical detection. An argon-ion laser (Innova 308, Coherent) provided 5 W of multiline power that was used to pump a rhodamine 110 dye laser (Model 599, Coherent). The dye laser was tuned to 560 nm and was filtered using a Z560/10-nm excitation filter (Chroma). The dye laser produced stable output power of 600 mW. A wedge was used to split the beam into a weak confocal beam and a strong bleaching beam (550 mW). An acousto-optical modulator (AOM 40, IntraAction) was used to stabilize the confocal beam to 1 mW. In combination with a Nikon TE 300 inverted microscope and a Nikon 60x water-immersion objective, a single-photon avalanche detector (SPAD, Perkin-Elmer SPCM-AQR-14) was used to detect fluorescence signals. A long-pass dichroic (568 nm cutoff) and two emission filters (LP 580, E 610/55, both from Chroma) separated resorufin fluorescence from excitation light. A telescope (×4 magnification) was used to match the diameter of the detected beam to the chip size of the SPAD, which was used as the confocal pinhole.

The more powerful bleaching beam continuously irradiated a large area around the immobilized enzyme. The bleaching beam was coupled into the flow cell by a prism on top of the quartz slide that was immersed in a thin layer of immersion oil (type FF, Cargille Laboratories) and was focused to a spot 200 μm in diameter. The bleaching angle was optimized so that the bleaching beam did not enter the objective to keep Raman and scattering background levels low (**Fig. 1b**).

***In situ* photobleaching.** To detect fluorescence from resorufin molecules generated by enzymatic reactions, we used a strong photobleaching beam to eliminate unwanted fluorescence signals from resorufin molecules arising from the following three processes: (i) both ion-exchange FPLC and reverse-phase HPLC chromatography leave residual resorufin at the nanomolar level; (ii) the RGP substrate has a low but measurable autohydrolysis rate (**Supplementary Fig. 4**); (iii) resorufin molecules from turnovers of neighboring immobilized β-galactosidase molecules enter the probe volume.

A strong and defocused laser beam illuminated and continuously photobleached the area within a 100-μm radius of the immobilized enzyme, which reduced the unwanted fluorescent signal by at least two orders of magnitude (**Supplementary Fig. 5**). The excitation intensity of the bleaching beam was high enough so that unwanted resorufin molecules were bleached before they diffused into the detection volume. Resorufin molecules created only within the diffraction-limited confocal volume were detected before photobleaching.

To assure efficient bleaching, all experiments were conducted in PEG (8,000 MW, Sigma) to prolong the residence time of a resorufin molecule (see **Supplementary Methods** online).

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Michaelis, L. & Menten, M.L. Kinetics of invertase action. *Biochem. Z.* **49**, 333–369 (1913).
2. Moerner, W.E. & Orrit, M. Illuminating single molecules in condensed matter. *Science* **283**, 1670–1676 (1999).
3. Xie, X.S. & Trautman, J.K. Optical studies of single molecules at room temperature. *Annu. Rev. Phys. Chem.* **49**, 441–480 (1998).
4. Ishijima, A. & Yanagida, T. Single molecule nanobioscience. *Trends Biochem. Sci.* **26**, 438–444 (2001).
5. Weiss, S. Fluorescence spectroscopy of single biomolecules. *Science* **283**, 1676–1683 (1999).
6. Bustamante, C., Bryant, Z. & Smith, S.B. Ten years of tension: single-molecule DNA mechanics. *Nature* **421**, 423–427 (2003).
7. Lu, H.P., Xun, L. & Xie, X.S. Single-molecule enzymatic dynamics. *Science* **282**, 1877–1882 (1998).
8. Zhuang, X. *et al.* Correlating structural dynamics and function in single ribozyme molecules. *Science* **296**, 1473–1476 (2002).
9. van Oijen, A.M. *et al.* Single-molecule kinetics of λ exonuclease reveal base dependence and dynamic disorder. *Science* **301**, 1235–1239 (2003).
10. Velonia, K. *et al.* Single-enzyme kinetics of CALB-catalyzed hydrolysis. *Angew. Chem. Intl. Edn.* **44**, 560–564 (2005).
11. Flomenbom, O. *et al.* Stretched exponential decay and correlations in the catalytic activity of fluctuating single lipase molecules. *Proc. Natl. Acad. Sci. USA* **102**, 2368–2372 (2005).
12. Yang, H. *et al.* Protein conformational dynamics probed by single-molecule electron transfer. *Science* **302**, 262–266 (2003).
13. Min, W., Luo, G., Cherayil, B.J., Kou, S.C. & Xie, X.S. Observation of a power-law memory kernel for fluctuations within a single protein molecule. *Phys. Rev. Lett.* **94**, 198302/1–198302/4 (2005).
14. Segel, I.H. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems* (Wiley, New York, 1993).
15. Xie, X.S. Single-molecule approach to enzymology. *Single Molecules* **2**, 229–236 (2001).
16. Qian, H. & Elson, E.L. Single-molecule enzymology: stochastic Michaelis-Menten kinetics. *Biophys. Chem.* **101–102**, 565–576 (2002).
17. Kou, S.C., Cherayil, B.J., Min, W., English, B.P. & Xie, S.X. Single-molecule Michaelis-Menten equations. *J. Phys. Chem. B* **109**, 19068–19081 (2005).
18. Fersht, A. *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding* (Freeman, New York, 1999).
19. Edman, L., Foldes-Papp, Z., Wennmalm, S. & Rigler, R. The fluctuating enzyme: a single molecule approach. *Chem. Phys.* **247**, 11–22 (1999).
20. Jacobson, R.H., Zhang, X.J., DuBose, R.F. & Matthews, B.W. Three-dimensional structure of β-galactosidase from *E. coli*. *Nature* **369**, 761–766 (1994).
21. Richard, J.P., Huber, R.E., Heo, C., Amyes, T.L. & Lin, S. Structure-reactivity relationships for β-galactosidase (*Escherichia coli*, lacZ). 4. Mechanism for reaction of nucleophiles with the galactosyl-enzyme intermediates of E461G and E461Q β-galactosidases. *Biochemistry* **35**, 12387–12401 (1996).
22. Marchesi, S.L., Steers, E., Jr. & Shifrin, S. Purification and characterization of the multiple forms of β-galactosidase of *Escherichia coli*. *Biochim. Biophys. Acta* **181**, 20–34 (1969).
23. Seong, G.H., Heo, J. & Crooks, R.M. Measurement of enzyme kinetics using a continuous-flow microfluidic system. *Anal. Chem.* **75**, 3161–3167 (2003).
24. Hadd, A.G., Raymond, D.E., Halliwell, J.W., Jacobson, S.C. & Ramsey, J.M. Microchip device for performing enzyme assays. *Anal. Chem.* **69**, 3407–3412 (1997).
25. Matthews, B.W. The structure of *E. coli* β-galactosidase. *C. R. Biol.* **328**, 549–556 (2005).
26. Hofmann, J. & Sernetz, M. Immobilized enzyme kinetics analyzed by flow-through microfluorimetry. Resorufin-β-D-galactopyranoside as a new fluorogenic substrate for β-galactosidase. *Anal. Chim. Acta* **163**, 67–72 (1984).
27. Ha, T. *et al.* Initiation and re-initiation of DNA unwinding by the *Escherichia coli* Rep helicase. *Nature* **419**, 638–641 (2002).
28. Zwanzig, R. Rate processes with dynamical disorder. *Acc. Chem. Res.* **23**, 148–152 (1990).

29. Karplus, M. Aspects of protein reaction dynamics: deviations from simple behavior. *J. Phys. Chem. B* **104**, 11–27 (2000).
30. Mesecar, A.D., Stoddard, B.L. & Koshland, D.E., Jr. Orbital steering in the catalytic power of enzymes: small structural changes with large catalytic consequences. *Science* **277**, 202–206 (1997).
31. Hammes, G.G. Multiple conformational changes in enzyme catalysis. *Biochemistry* **41**, 8221–8228 (2002).
32. Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. & Gunsalus, I.C. Dynamics of ligand binding to myoglobin. *Biochemistry* **14**, 5355–5373 (1975).
33. Frauenfelder, H., Sligar, S.G. & Wolynes, P.G. The energy landscapes and motions of proteins. *Science* **254**, 1598–1603 (1991).
34. Sakmann, B. & Neher, E. *Single-Channel Recording* 2nd edn. (Plenum, New York and London, 1995).
35. Benkovic Stephen, J. & Hammes-Schiffer, S. A perspective on enzyme catalysis. *Science* **301**, 1196–1202 (2003).
36. Kohen, A., Cannio, R., Bartolucci, S. & Klinman, J.P. Enzyme dynamics and hydrogen tunnelling in a thermophilic alcohol dehydrogenase. *Nature* **399**, 496–499 (1999).
37. Schnitzer, M.J. & Block, S.M. Statistical kinetics of processive enzymes. *Cold Spring Harb. Symp. Quant. Biol.* **60**, 793–802 (1995).
38. Svoboda, K., Mitra, P.P. & Block, S.M. Fluctuation analysis of motor protein movement and single enzyme kinetics. *Proc. Natl. Acad. Sci. USA* **91**, 11782–11786 (1994).
39. Yang, S. & Cao, J. Two-event echos in single-molecule kinetics: a signature of conformational fluctuations. *J. Phys. Chem. B* **105**, 6536–6549 (2001).
40. Lippitz, M., Kulzer, F. & Orrit, M. Statistical evaluation of single nano-object fluorescence. *ChemPhysChem* **6**, 770–789 (2005).
41. Lerch, H.-P., Rigler, R. & Mikhailov, A.S. Functional conformational motions in the turnover cycle of cholesterol oxidase. *Proc. Natl. Acad. Sci. USA* **102**, 10807–10812 (2005).
42. Flomenbom, O., Klafter, J. & Szabo, A. What can one learn from two-state single-molecule trajectories? *Biophys. J.* **88**, 3780–3783 (2005).
43. Barsegov, V., Chernyak, V. & Mukamel, S. Multitime correlation functions for single molecule kinetics with fluctuating bottlenecks. *J. Chem. Phys.* **116**, 4240–4251 (2002).
44. Magde, D., Elson, E. & Webb, W.W. Thermodynamic fluctations in a reacting system. Measurement by fluorescence correlation spectroscopy. *Phys. Rev. Lett.* **29**, 705–708 (1972).
45. Yang, S. & Cao, J. Direct measurements of memory effects in single-molecule kinetics. *J. Chem. Phys.* **117**, 10996–11009 (2002).