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Stretched exponential decay and correlations from two-state single molecule trajectories

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Single molecule techniques offer a unique tool studying the dynamical behaviour of individual molecules, and provide the possibility to construct distributions from individual events rather than from a signal stemming from an ensemble of molecules. Observing the activity of individual lipase molecules for extended periods of time (hours), we get long trajectories, made of "*on*-state" and "*off*-state" events. The waiting time probability density function (PDF) of the *off*-state and the state-correlation function fit stretched exponentials, independent of the substrate concentration in a certain range. The data analysis unravels correlations between *off*-state events. These findings imply that the fluctuating enzyme model, which involves a spectrum of enzymatic conformations that interconvert on the timescale of the catalytic activity, best describes the observed enzymatic activity.

This contribution summarizes some of our recent published results.

Dynamics of chemical reactions are conventionally investigated by ensemble measurements. Recent advances in single molecule spectroscopy have enabled the study in of biophysical processes (*1 - 11*). These studies have demonstrated that new information about such processes can be extracted from single molecule measurements.

Noise in Complex Systems and Stochastic Dynamics III, edited by Laszlo B. Kish, Katja Lindenberg, Zoltán Gingl, Proc. of SPIE Vol. 5845 (SPIE, Bellingham, WA, 2005) · 0277-786X/05/$15 · doi: 10.1117/12.610133

Motivated by this, the enzymatic activity of individual 33 kDa lipase B from *Candida antarctica* (CALB) molecules has been examined (*12* - *13*) using confocal fluorescence microscopy. This lipase catalyzes the hydrolysis of esters in aqueous solution following the same reaction mechanism as that of a serine protease (*14*). In order to study the catalysis by single CALBs, a fluorogenic substrate was used, which upon hydrolysis forms a highly fluorescent carboxylic acid product (*15*, *16*). This enabled to probe the enzymatic activity by monitoring the fluorescence emission from single enzymes. The fluorescence emission displayed blinking of "*on*" and "*off*" events depending on the presence (and absence) of the fluorescent product in the confocal focus (*17*). Using this approach, long trajectories (for time periods of hours) have been obtained that allow reliable statistical analysis while varying the concentration of the substrate. This enabled studying the effect of changing the substrate concentration on the observed trajectories. By performing new methods of analyses on the data, we obtained information that is used to build a microscopic kinetic scheme most suitable to account for the experimental findings; that is, the fluctuating enzyme model, which involves both conformational changes and enzymatic activity.

Single Enzyme Two-State Trajectories

To analyse the recorded trajectories of the photon counts (*17*-*18*), the enzymatic turn over cycle (ETOC) is viewed as a two-state process. The two distinct states are naturally defined by the absence (*off*-state) and the presence (*on*-state) of fluorescence emission. The two-state ETOC dynamics is basically described by the waiting time PDFs of the *off*-state, $\phi_{off}(t)$, and of the *on*-state, $\phi_{on}(t)$. Namely, $\phi_{off}(t)dt$ ($\phi_{on}(t)dt$) is the probability that a given *off* (*on*) event along the trajectory lasts between t and $t + dt$. In principle, these functions do not *fully* describe the process (see the discussion below). However, they do give its basic characteristics, primarily the number of sub-states in each of the two states of the kinetic scheme. Based on our findings we suggest below a kinetic scheme for the enzymatic activity.

To get $\phi_{off}(t)$ and $\phi_{on}(t)$, the photon count trajectory is transformed into a two-state (digital) trajectory. This is done by applying a floating threshold method (*18*). $\phi_{off}(t)$ and $\phi_{on}(t)$ can be constructed by building a histogram from the time durations of the corresponding events along the trajectory. Figure 1A shows $\phi_{off}(t)$ on a ln-linear scale for a set of three different substrate

concentrations, $[S] = 0.6, 0.9, \text{and} 1.4 \mu M$, demonstrating that the relaxation patterns are non-exponential. All the three curves correspond to the kinetic behaviour of the same lipase molecule, obtained by the sequential addition of substrate while monitoring the enzymatic activity for ~30 *min* for each [*S*]. The best fit for these curves is found to be a normalized stretched exponential function,

$$\phi_{off}(t) = \phi_0 e^{-(t/\tau)^{\alpha}} \quad ; \quad \phi_0 = 1/\int_0^{\infty} e^{-(t/\tau)^{\alpha}} dt = \frac{\alpha/\tau}{\Gamma(1/\alpha)} \qquad (1)$$

where $\Gamma(\cdot)$ is the gamma function (*19*), and the value of the normalization factor, ϕ_0, is valid when assuming that the stretched behaviour characterizes the process for all times (namely, for $t \to \infty$). The stretching exponent α and the parameter τ are independent of [*S*] within the investigated range, $\alpha = 0.15$ and $\tau = 1.15 \mu s$, see Figs. 1B-1D. This observation supports the argument that the stretched exponential decay pattern does not originate, for these concentrations, from anomalous diffusion of the substrate to the enzyme vicinity but reflects an intrinsic feature of the enzymatic activity.

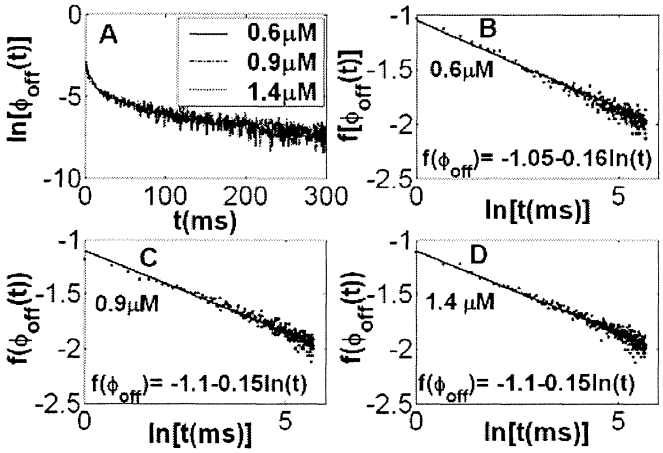

FIG 1 A - A ln-linear scale plot of the *off*-state waiting time PDF emphasises its non-exponential relaxation pattern. **B – D -** $\phi_{off}(t)$ for a set of three different substrate concentrations [*S*] = 0.6, 0.9, and 1.4 μM, shown in **B**, **C**, and **D**, respectively. Also shown are the corresponding fitting functions. Here $f[\phi_{off}(t)] = -\ln[-\ln(\phi_{off}(t))]$ is plotted versus $\ln(t)$, where the slope corresponds to the exponent $-\alpha$, the stretching exponent. For all curves the normalization used such that $\phi_{off}(t=0) = 1$, chosen for a convenient parameters extraction.

The waiting time PDF of the *on*-state $\phi_{on}(t)$, displayed a fast decay spanning only a few *ms*, independent of [*S*] (data not shown). Being [*S*]-independent is the expected behaviour, as each *on* event is terminated when the product diffuses away from the enzyme vicinity, a process which should indeed be [S]-independent. We approximated $\phi_{on}(t)$ by $\phi_{on}(t) \approx \kappa e^{-\kappa t}$ with $\kappa^{-1} = 1ms$. Deviations from exponential decay are possible, but the fast decay ($\kappa^{-1} = \Delta t$) prevented a more accurate fitting.

Having obtained $\phi_{on}(t)$ and $\phi_{off}(t)$ from the experiment, we wish to find a kinetic scheme compatible with these functions. However, as $\phi_{off}(t)$ is a stretched exponential, several models will fulfil this requirement. The question that arises is whether one can discriminate between kinetic schemes that lead to the same $\phi_{on}(t)$ and $\phi_{off}(t)$ by looking at the trajectory in more detail (*20*).

A trajectory is completely described by $\phi_{on}(t)$ and $\phi_{off}(t)$ only when waiting times along the trajectory are uncorrelated. Trajectories with no correlations between waiting-times do not contain information about the connectivity of substates within the two states, which is a consequence of a specific connectivity between substates of the different states (*20*). We say that kinetic schemes that produce uncorrelated waiting times trajectories are *reducible* to a two-state-semi-Markovian (TSSM). A TSSM process is one where the *on* [*off*] waiting times are drawn randomly and independently out of a non-exponential $\phi_{on}(t)$ [$\phi_{off}(t)$]. In the literature, the term non-Markovian is often used for any process with non-exponential waiting time PDFs. However, here we reserve this term to describe a trajectory of correlated waiting times, where the corresponding kinetic schemes are called *irreducible* (*20*).

A test for correlations between waiting times in the trajectory compares the bulk relaxation function (the state-correlation function), obtained directly from the trajectory, with the corresponding theoretical result for a TSSM process (*18*). The expression for the bulk relaxation function for a stationary TSSM is known, in Laplace space, for arbitrary waiting time PDFs (*21*),

$$\overline{C}(s) = \frac{1}{s}\left[1 - \frac{N}{s}\frac{[1-\overline{\phi}_{on}(s)][1-\overline{\phi}_{off}(s)]}{1-\overline{\phi}_{on}(s)\overline{\phi}_{off}(s)}\right]; \quad N = \left(< t_{off} > + < t_{on} >\right) / < t_{off} >< t_{on} >, \qquad (2)$$

so one can plug in the Laplace transforms of the experimental $\phi_{on}(t)$ and $\phi_{off}(t)$ into this expression, and invert the result, either analytically or numerically, back into the time domain. If the experimental bulk relaxation function and the theoretical one for a TSSM process with the experimental $\phi_{on}(t)$ and $\phi_{off}(t)$ coincide, the scheme is reducible, and no further analysis is required. By performing this test, we find that the theoretical and the experimental state-correlation functions do not coincide (Figs. 2A-2B). This means that the appropriate kinetic scheme is irreducible.

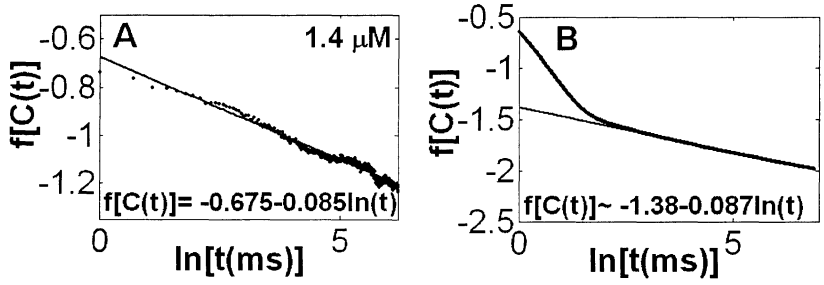

FIG 2A & 2B - Both the experimental (**A**) and theoretical (**B**) autocorrelation function $C(t)$ display a stretched exponential decay pattern ($f(\cdot)$ is defined in Fig. 1), with similar stretching exponent values but different $\tilde{\tau}$ values. See text for discussion.

Indeed, we find by considering a different trajectory that displays the chronologically ordered time duration of the *off*-state events (Fig. 3) that correlations between waiting times exist. On this trajectory, the height of the i^{th} line corresponds to the time duration of the i^{th} *off* event, where the events appear in the same order of their occurrence in the original time-series. Observing the ordered *off* waiting times trajectory, we noticed local trends (for all three concentrations) where events of similar short durations follow each other.

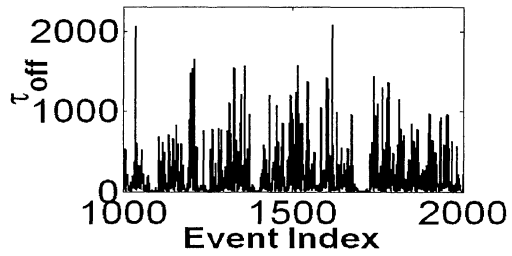

FIG 3 The trajectory of the time durations of the *off*-state events as a function of chronological event index for $[S]=1.4\mu M$. Noticeable along this trajectory are groups of successive fast events (each event in the group has τ_{off} value smaller than 35ms).

Namely, along this trajectory fast events (each event is faster than 35*ms*) are clustered together, with an average of 3.5 (± 1.1) events per group, and an average duration time of 10 (± 2) *ms* per event in the group. When considering these findings, along with our theory in (*20*) that gives a full description of the topology of reducible kinetic schemes, the most natural choice for a kinetic model for characterizing the enzymatic activity is the fluctuating enzyme model shown in Fig. 4.

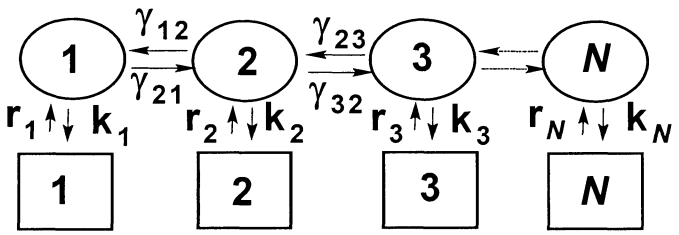

FIG 4 A schematic model of the enzymatic process. The *off*-state consists of a spectrum of N coupled sub-states. Also indicated are the coupling rates between the conformations and the enzymatic reaction rates.

Concluding Remarks

Non-exponential behaviour of biomolecules has been obtained in several systems (*10*, *22*, *23*). Here we report on a direct observation on the single molecule level of a stretched exponential *off* waiting time PDF in an enzymatic catalytic activity of lipase B from *Candida antarctica*, $\phi_{off}(t) \sim e^{-(t/\tau)^{0.15}}$ ($\tau \approx 1\mu s$). Searching for the origin of the stretched behaviour, we ruled out the possibility of an external source such as the substrate concentration. By confronting the experimental state-correlation function, $C(t)$, with a theoretical state-correlation function for a two-state semi-Markov process with the experimental $\phi_{on}(t)$ and $\phi_{off}(t)$, we concluded from the different behaviour of these that the underlying mechanism cannot be described as a two-state semi-Markov process, and that correlations between events exist. Indeed, the trajectory of the *off* time durations exhibits local trends of bunched fast events. We, thus, described the observed enzymatic activity by the fluctuating

enzyme model (Fig. 4). Other details about the relationships between the underling kinetic scheme and features of the trajectory, as well as other methods of analyses, are given in (*20*).

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