Single-Molecule Studies

Single-Enzyme Kinetics of CALB-Catalyzed Hydrolysis**

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Insight into the dynamic behavior of chemical processes is typically derived from ensemble measurements. Direct experimental information about the dynamics at the single-molecular level, however, is sparse and has until recently been primarily deduced from molecular-dynamics simulations. Current advances in single-molecule spectroscopy have paved the way for exploring the behavior of individual molecules in the course of a chemical reaction. Thus far it has proven possible to monitor in real time the dynamic behavior of single-biomolecular processes and observe the enzymatic turnovers of a few motor proteins,[1–5] an oxidase,[6] horseradish peroxidase,[7] and a nuclease.[8] More recently, structural fluctuations of a single flavin reductase[9] and of T4 lysozyme during the course of a reaction[10] have been detected. These few examples clearly demonstrate the tremendous potential of studying an enzymatic process at the single-molecular level.

Herein we report the direct observation of the real-time catalysis and substrate kinetics of a single-enzyme-catalyzed

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reaction, using a simple method, a robust enzyme, and confocal fluorescence microscopy (CFM). With the method described herein, we were able to monitor single enzyme turnovers for extended periods of time (hours) and use these measurements to unravel hidden characteristics in the catalytic behavior of individual enzymes. The lipase B from *Candida antarctica* (CALB) proved to be an excellent candidate for the present studies.[11,12] This 33 kDa workhorse enzyme catalyzes the hydrolysis of esters in aqueous solutions following the same reaction mechanism as a serine protease.[13]

A prerequisite for the imaging of single CALB molecules with CFM is that they are fluorescent and fixed onto a surface. To make it fluorescent the native lipase B from CALB was non-specifically labeled with Alexa Fluor^{488} dye molecules.[14,15] To fix the CALB molecules to a surface, a variety of immobilization techniques were examined. A commonly used protein-immobilization method is the trapping of enzyme molecules in agarose or polyacrylamide gels.[6] However, in the case of the CALB, this trapping method proved inadequate as it led to either inactivation of the protein, or cleavage of the Alexa Fluor^{488} markers, or to substrate-diffusion limitations. An alternative immobilization technique involves the deposition of enzymes on hydrophilic glass surfaces. Using this procedure, deposition of the protein in single-molecule concentrations again led to inactivation of CALB. The only solid support that allowed the study of the enzymatic conversion without inactivation or free movement (translational diffusion) of the protein, was a simple hydrophobic glass surface derivatized with dichlorodimethylsilane.

A scanning confocal microscope (CFM) with a focused laser spot was used to image the surface-immobilized CALB molecules and measure their individual emission time trajectories. At an optimum concentration of 3 nM Alexa^{488}–CALB, the enzymes were adsorbed onto the hydrophobic glass surface with a density sufficiently low that the fluorescence emission from a single labeled enzyme as a function of time could be observed. Blank experiments involving the imaging of the samples before and after the addition of water revealed that the enzymes remained immobilized and that the background emission level was negligible.

When a single Alexa^{488}–CALB molecule was positioned under the laser excitation spot ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 520$ nm), a strong emission, followed by an abrupt photobleaching was observed (see Supporting Information). The emission traces of different Alexa^{488}–CALB molecules showed a variation in the time scale of photobleaching of 0 to 15 s, which reflects the different orientations of the fluorophores and the differing degrees of labeling (1 to 11 dye molecules can be attached to the lysine residues on the protein surface).[16] To study the catalysis by the single Alexa^{488}–CALB, we used as the fluorogenic substrate the non-fluorescent ester BCECF, AM (Figure 1 A)[17,18] which upon hydrolysis forms a highly fluorescent product.

Adsorption of the labeled enzymes onto the hydrophobic surface and initial scanning with the confocal microscope revealed spots of fluorescence attributable to the Alexa-labeled single enzymes. Onto this lipase functionalized surface a solution of the substrate was added and the fluorescence emission was monitored. Sequential fluorescence images of the surface revealed an increase in fluorescence emission, originating at the single enzymes and a gradual small increase in the background fluorescence. We attribute this increase in fluorescence emission to the formation and subsequent accumulation of the fluorescent product generated from BCECF, AM. Confirmation that the observed fluorescent product is formed by enzymatic hydrolysis was obtained by studies on the non-fluorescent native CALB (Figure 1 B). In these experiments the enzymes deposited on the surface could not be visualized until the addition of the substrate solution, at which time, points of fluorescence appeared and increased in intensity in a manner similar to that seen for the Alexa^{488}-labeled CALB. In addition these points of fluorescence were observed to blink on and off, a behavior, which we attributed to product formation followed by product diffusion. Each of these blinking (on-off) events is considered to be a single enzymatic turnover cycle (ETOC, see below). Several blank experiments with the enzyme in the absence of substrate and with the substrate in the absence of enzyme confirmed that these blinking phenomena only arise from enzyme-catalyzed hydrolysis (see Supporting Information). The observed enzyme blinking is a direct measure of the enzymatic catalysis and was thus studied in more detail. To be able to monitor the reaction immediately after the addition of substrate, individual Alexa^{488}-labeled CALB molecules were first positioned under the focus point of the laser and then the fluorophores were photobleached prior to the addition of the substrate. Subsequently the substrate solution was added and the fluorescence emission of this specific enzyme was recorded for long periods of time (up to 2 h, Figure 1 C, D).[19] In contrast to previous experiments,[6] where the length of the trajectories was curtailed either because of photobleaching or photooxidation, in our study the length of the trajectories had no limiting factors except the depletion of the substrate.

To analyze the recorded trajectories of the photon counts, we view the ETOC as a two-state process. The two distinct states are defined by the absence (off-state) and the presence (on-state) of fluorescence emission. Accordingly, the ETOC dynamics can be described by the waiting-time probability density function (pdf) of the off-state, $\phi_{off}(t)$, and of the on-state, $\phi_{on}(t)$. Thus, $\phi_{off}(t)dt$ (or $\phi_{on}(t)dt$) is the probability that a given off (or on) event along the trajectory lasts the time between t and $t+dt$. The waiting-time pdfs (which have units of one over time) are the main basic outcome of the trajectory analysis obtained from single-molecule measurements. This result differs from ensemble measurements which provide only a survival probability which is dimensionless.[20] If we denote by $a(t)$ the photon count value at time $t \equiv t_i = i\,\Delta t$ ($\Delta t =$ 1 ms), then, by setting a threshold value, the two states are separated, so that each $a(t)$ can be attributed either to the off-state, $a_{off}(t)$, or the on-state, $a_{on}(t)$ (Figure 1 D).[21,22] (The full local treatment and theoretical analysis will be described elsewhere).[23] As a result of this treatment, the trajectory is now characterized by the binary random process, $\xi(t)$, with values $\xi = 1$ for on events, and $\xi = 0$ for off events. The off and on waiting-time pdfs, $\phi_{off}(t)$ and $\phi_{on}(t)$ can then be constructed by building a histogram from the time durations of the

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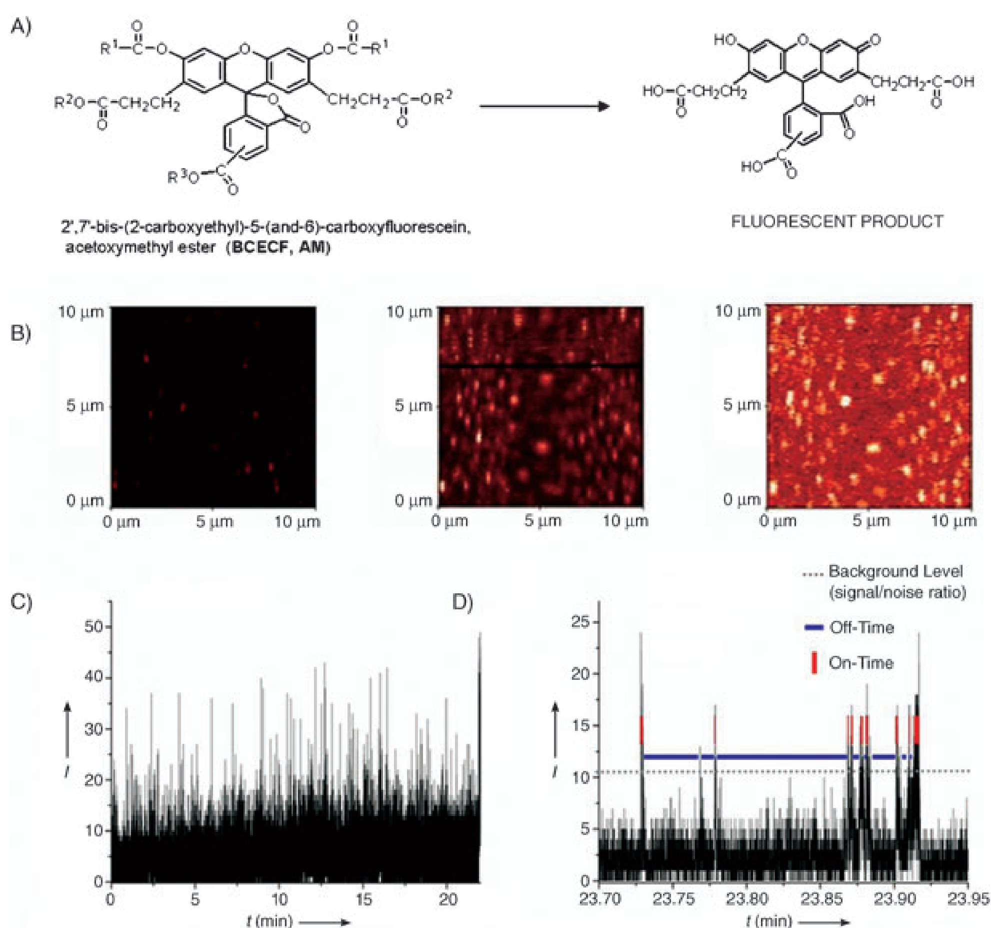

Figure 1. Fluorescence emission studies on the catalytic activity of single CALB molecules. A) Structure of the fluorogenic substrate BCECF, AM. B) Sequential CFM images (10 μm×10 μm, $t=0$ (left), 15 (middle), 30 min (right)) of a native CALB-coated surface during the enzymatic hydrolysis of BCECF, AM. C) Fluorescence emission as a function of time of a single Alexa[488]-labeled CALB molecule during catalysis over 22 min. D) Expansion revealing the signal to noise ratio. By setting a threshold value the photon-count trajectory is transformed into a dichotomous trajectory containing a time series of on and off events.[23]

corresponding events along the trajectory, which allows the analysis of the data accumulated during the single-enzyme-catalyzed reaction.

To investigate the substrate kinetics of an individual enzyme, the behavior of a single lipase molecule was followed at different substrate concentrations. In contrast to other experiments,[6,7] all the experiments described herein were performed using the same single enzyme, since each single enzyme was anticipated to exhibit different activity owing to the non-specific adsorption on the hydrophobic support. To this end, the substrate concentration was gradually increased by sequentially adding a fresh substrate solution onto the same area under the laser excitation spot while the same single CALB molecule was studied. The off-state waiting-time pdf $\phi_{off}(t)$ for three different substrate concentrations [S], [S] = 0.6, 0.9, 1.4 μM, revealed that for all substrate concentrations, the relaxation patterns are non-exponential (Figure 2). The best fit for these curves is found to be a normalized stretched exponential function [Eq. (1)][24] where $\Gamma(-)$ is the gamma function.[25] The exponent parameter α and the parameter τ are independent of [S] within the investigated range, $\alpha=0.15$ and $\tau=1.15$ μs. Note that the pair

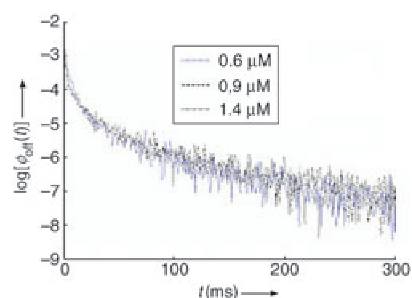

Figure 2. A log-linear scale plot of the off-state waiting-time pdf, $\phi_{off}(t)$, which highlights the non-exponential relaxation pattern. (The normalization used is such that $\phi_{off}(t=0)=1$).

of parameters (α,τ), and not each of these parameters by itself, is the relevant kinetic information obtained on the system, which can be realized by calculating, for example, the average time the process occupies the off state.

$$\phi_{off}(t) = \frac{\alpha/\tau}{\Gamma(1/\alpha)} e^{-(t/\tau)^{\alpha}} \quad (1)$$

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As observed for other systems (such as glassy materials)[26,27] a stretched exponential decaying pattern can arise from the combined contributions of a large number of weighted exponentials owing to an inhomogeneous environment. In the current case of single-enzyme catalysis measurements, the stretched exponential decay pattern of $\phi_{off}(t)$ serves as an indication that an individual enzyme exhibits dynamic disorder. The origin of this dynamic disorder does not stem from different diffusion times of the substrate to the vicinity of the enzyme, since the log linear plots of the off-state waiting-time pdfs at three different substrate concentrations, $[S] = 0.6, 0.9, 1.4\ \mu M$, all have the same values for α and τ (Figure 2). This conclusion is further supported when plotting the number of ETOCs as a function of time (per time interval of 20 min; Figure 3), which was collected from the same single enzyme at different substrate concentrations (201 nM to 1.4 μM). Figure 3 reveals a saturation profile for a single enzyme which is similar to that observed for enzymes in solution. This is the first time that such a kinetic experiment has been performed on the same single enzyme revealing saturation behavior. Thus, the dynamic disorder shown, which leads to a stretched exponential waiting-time pdf, is apparently an inherent property of the enzyme, and can be interpreted as originating from different enzymatic conformations, each of which contributes an exponential to the overall decay of the off-state pdf. This concept of a multiple sub-state enzyme can be compared to the general model [Eq. (2)], which is often applied to enzyme activity.[7,28]

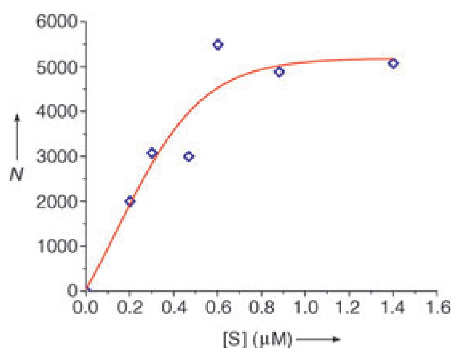
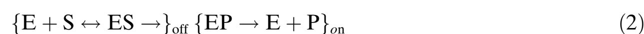


Figure 3. Plot of the number of ETOCs in 20 min (N), as a function of the effective total substrate concentration [S]. The plot reveals a saturation profile, similar to that observed for enzymes in solution, reached at approximately 600 nM.



$$\{E + S \leftrightarrow ES \rightarrow\}_{off}\ \{EP \rightarrow E + P\}_{on} \quad (2)$$

In Equation (2) the symbols E, S, and P stand for the enzyme, substrate, and product molecules, respectively, and the ES stands for the enzyme–substrate complex and EP for the enzyme–product complex. In this model the fluorescent properties of each of the stages are also indicated. More precisely, the symbols represent the species concentrations in bulk experiments and should be replaced by the species probability densities in single-molecule measurements. Given a set of reaction rates and appropriate initial conditions, it is found that the decaying pattern of the off state predicted by the standard scheme in Equation (2) is bi-exponential given a high enough substrate concentration. It is therefore necessary to extend this general model, so that the proposed enzymatic mechanism can account for the functional form of $\phi_{off}(t)$. For this reason we considered a model that involves simultaneously enzymatic activity and conformational changes (Figure 4). The new model describes an off state that consists of a broad spectrum of conformational sub-states, which are coupled. Recently a distribution of reaction rates was extracted from single T4 Lysozyme TOC trajectories (approximately 50), lasting only about 20 s.[10] In our studies, each of the trajectories is considerably long lasting (about 30 min) and contains contributions from the entire distribution, which leads to a non-exponential decay pattern.

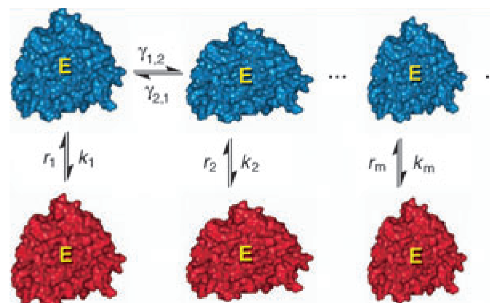


Figure 4. The model for the enzyme (E) conformational–reactivity behavior. The off-stage (blue) consists of a variety of coupled sub-states that interconvert. Each of the off sub-states reacts at a (possible) different reaction rate. Once the enzyme has transformed the substrate into the fluorescent product the on state is seen (red). The product then diffuses away from the enzyme and the process returns to the relevant off sub-state.

The chronologically ordered time duration of the off-state events revealed local trends. Events of similar time duration were observed to follow each other and, noticeable were clusters of "fast" events, with an average of three events per event cluster and an average of 12 ms per event. Considering the average of approximately 5000 events/20 min (5 events/1200 ms) calculated for 1.4 μM BCECF, AM (Figure 3), it is apparent that the enzyme is catalytically less active for long periods of time before it acquires a more reactive conformation.

In summary we have demonstrated that the direct observation of the real-time catalysis and substrate kinetics of a single-enzyme-catalyzed reaction, is possible for a commonly used, robust enzyme by using confocal fluorescence microscopy and a simple method. Furthermore, we have observed a stretched exponential waiting-time pdf in the enzymatic catalytic activity of the single lipase B enzymes from *Candida antarctica* studied that has not been observed earlier. Previous experiments have, however, revealed a non-exponential behavior of the biomolecules in several systems.[9,26] A possible explanation regarding the origin of the non-exponential behavior of the $\phi_{off}(t)$ is that the enzymatic kinetics involves a broad conformational spectrum, of which only certain conformations are catalytically active. Each conformation contributes a specific exponential factor to the

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overall ETOC, an observation, which is often masked by ensemble measurements. The ability to measure the activity of a single enzyme for a long time offers a powerful tool for the investigation of numerous other enzymes and a more complete understanding of their complex behavior.

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