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Dynamic Disorder and Stepwise Deactivation in a Chymotrypsin Catalyzed Hydrolysis Reaction

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Insight into the detailed functioning of enzymes is crucial for understanding their metabolic role and their use as industrial biocatalysts. Not only the activity of a working enzyme but also its transition to a deactivated state is of great interest. Until now, deactivation was generally described as a two-state all-or-nothing process, in which the individual enzyme's activity abruptly disappears.[1,2] In early work on the activity of heat-exposed single galactosidase enzymes, Rotman distinguished two subpopulations: one had retained 100% activity while the other had completely lost its activity.[3] However, in these experiments, no information could be extracted on the deactivation pathway because the deactivation took place in an ex situ incubation process. While NMR, X-ray diffraction, or hydrogen exchange experiments are useful for understanding the complex structural protein dynamics that drive deactivation processes,[2] such techniques lack either the time resolution to study fast dynamic processes or the sensitivity to identify low abundant transient species during spontaneous enzyme deactivation. A recent technique that combines ultrasensitivity with appropriate high time resolution for in situ measurements is single-molecule fluorescence spectroscopy (SMFS). Lately, this powerful technique was successfully applied for proving activity fluctuations of active single enzymes, a phenomenon known as dynamic disorder,[4−9] and for single-turnover counting on various types of catalysts.[10,11] Here, we advantageously employ SMFS to prove the existence of fluctuations in the activity of single α-chymotrypsin enzymes and to show that, during its deactivation, the enzyme is altered stepwise before it eventually deactivates irreversibly.

Our approach to monitor single turnovers relies on the α-chymotrypsin (from bovine pancreas) catalyzed hydrolytic formation of strongly fluorescent rhodamine 110 (**2**) from the profluorescent rhodamine 110 bis(suc-Ala-Ala-Pro-Phe) substrate (**1**) (Figure 1). The fluorescence of individual product molecules **2** released by single α-chymotrypsin monomers, immobilized by entrapment in agarose polymer, was collected through a fluorescence microscope in confocal mode and detected on an avalanche photodiode (see Supporting Information).

In order to determine the optimal reaction conditions for the single-molecule measurements, the ensemble kinetics of the α-chymotrypsin catalyzed hydrolysis of **1** were studied by monitoring the fluorescence increase due to formation of rhodamine 110 (**2**) in a fluorimeter cuvette. Rates were adequately described by the Michaelis−Menten model, proving the absence of substrate inhibition effects or allosteric interactions. The ensemble-averaged kinetic parameters are 3.99 μM for K_M and 3.86 s^{-1} for k_{cat} (see Supporting Information; Figure S1). Consequently, for the single-enzyme measurements, a substrate concentration of around 30 μM was maintained to eliminate artifacts from substrate depletion.

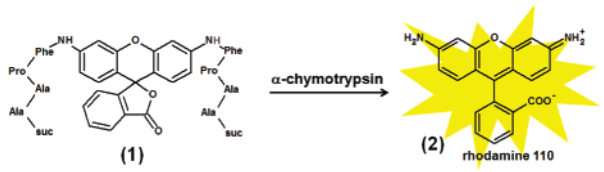

Figure 1. Structure of the profluorescent probe **1** that is converted by chymotrypsin, yielding the strongly fluorescent rhodamine 110 (**2**).

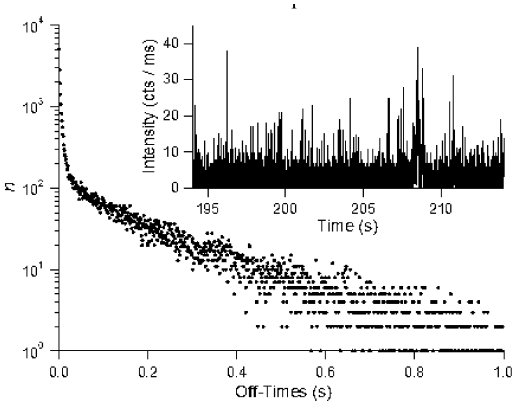

Figure 2. Histogram of the waiting times between two successive turnovers for a typical α-chymotrypsin enzyme during its active period. The nonlinear decay in the semilogarithmic representation indicates dynamic disorder. Inset: Representative time transient for a single α-chymotrypsin enzyme catalyzing the hydrolysis of **1** (30 μM in PBS buffer at pH 7.4). The detected photons are binned in intervals of 1 ms.

Next, activity traces were measured for more than 100 single-enzyme molecules. An example is shown in the inset of Figure 2 (see also Supporting Information; Figure S4). The analysis of the waiting times between successive individual turnovers shows that there is dynamic disorder, even if the activity fluctuations are rather small compared to previous work on other enzymatic systems.[6,7] In the case of an enzyme with a nonfluctuating activity and one first-order rate-limiting step in its catalytic cycle, the waiting times are expected to be distributed according to a monoexponential decay. It is clear, however, that in this case the waiting time distribution is stretched over several orders of magnitude due to dynamic disorder (Figure 2). It has been shown previously that mathematical functions like a stretched exponential are more adequate to fit such distributions.[8]

Autocorrelation analysis on the waiting times reveals the time scale of the activity fluctuations. An elegant method for further analysis is the construction of two-dimensional autocorrelation plots of pairs of waiting times, also called joint probability distributions

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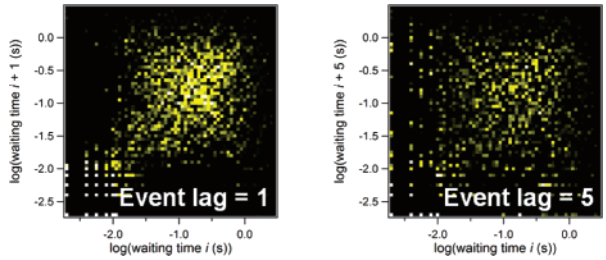

Figure 3. Two-dimensional joint probability graphs for pairs of the logarithm of the waiting times for event lags 1 and 5. The graphs are corrected according to the formula in the text.

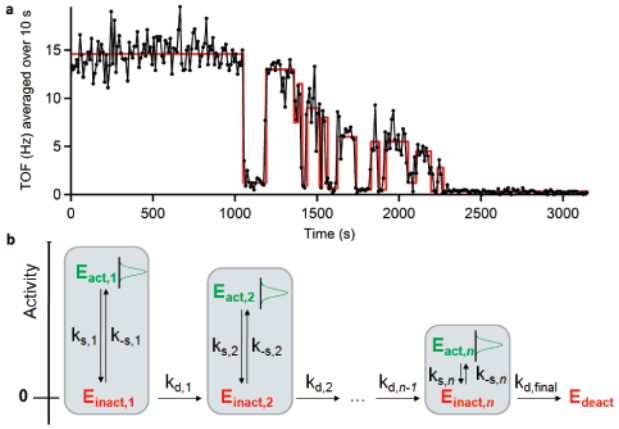

Figure 4. (a) Turnover frequency (TOF, averaged over 10 s) of a deactivating α-chymotrypsin enzyme (see Supporting Information; Figure S6). The red line indicates the different states and serves as a guide to the eye. (b) The extended single-molecule deactivation model for enzymes. A reversible conformational change causes the enzyme to switch between active (green; with dynamic disorder) and inactive (red) states. During this equilibrium, stepwise inactivation occurs before the enzyme deactivates irreversibly.

($p(\tau_j, \tau_{j+i})$ for the correlation between waiting times separated by i turnovers).[4] To identify the correlation effect between pairs of waiting times more clearly, an additional correction can be made, as illustrated by Lerch et al., yielding the difference distribution of waiting times, expressed as $\delta(\tau_j, \tau_{j+i}) = p(\tau_j, \tau_{j+i}) - p(\tau_j) \times p(\tau_{j+i})$.[12] Because of the broad time range of the waiting times distribution, the logarithm of the waiting times was used for constructing the 2D joint probability graphs in Figure 3. For α-chymotrypsin, correlation between waiting times was observed for event lags lower than 15, thus in the time range of a few seconds (see also Supporting Information; Figure S5).

Approximately 95% of the measured enzymes maintained stable activity over the measurement window of ±3 h. The remaining 5% of the enzymes lost its activity according to the trajectory in Figure 4a. This low percentage is the result of the high stability of the immobilized chymotrypsin enzymes. Surprisingly, rather than an abrupt or a continuous gradual deactivation, we observed a transient phase with discrete inactive and active periods, before the enzyme was irreversibly deactivated. After each inactive period, only a fraction of the original activity was recovered. The discrete activity levels in Figure 4a indicate that the consecutive activity decreases are related to events during the inactive periods rather than during the active periods themselves.

The existence of a transient phase in an enzyme's deactivation process has been postulated before[13] and is supported by the observation of genuine temperature optima in bulk kinetic studies of enzymatic activity.[14,15] In Daniel's equilibrium model, an inactive state precedes the deactivated state; while the transition from the active state to the inactive state is reversible, the transition to the deactivated state is irreversible.

$$E_{\text{act}} \underset{k_{-s}}{\overset{k_s}{\rightleftharpoons}} E_{\text{inact}} \xrightarrow{k_d} E_{\text{deact}} \qquad (1)$$

On the basis of our observations, a tentative model for a single enzyme's deactivation is proposed (Figure 4b): from its active state, the enzyme can reversibly switch to an inactive conformation. Only when residing in or during the formation of this inactive state is the enzyme susceptible to important structural changes in such a way that, after switching back to an active state, the averaged activity is lower than before. Even at room temperature, thermal energy seems sufficient to cause transitions between these states. The proposed model in Figure 4b is in agreement with the equilibrium model as described in eq 1 and refines it at a single-molecule level. Extension of these experiments to other enzymatic reactions will reveal whether or not such a stepwise deactivation pathway is common in enzyme chemistry.

Summarizing, the analysis of the chymotrypsin dynamics at the single-enzyme level not only reveals dynamic disorder during the active period but also shows that the deactivation occurs stepwise, rather than by an all-or-nothing event.

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Supporting Information Available: Experimental procedures, data analysis methods, Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

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