Minireview

Single-molecule Enzymology*

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Movies of Molecular Motions and Chemical Reactions of Single Molecules

Viewing a movie of an enzyme molecule made by molecular dynamics simulation, we see incredible details of molecular motions, be they changes of the conformation or actions during a chemical reaction. Molecular dynamics simulations have advanced our understanding of the dynamics of macromolecules in ways that would not be deducible from the static crystal structures (for review see Refs. 1 and 2). Unfortunately, these “virtual movies” do not run long enough compared with the time scale of milliseconds to seconds in which most enzymatic reactions take place. In recent years, rapid advances in the patch clamp technique (for review see Ref. 3), atomic force microscopy (4, 5), optical tweezers (6, 7), and fluorescence microscopy (for review see Refs. 8 and 9) have permitted making single-molecule “movies” in situ at the millisecond to second time scale. Unlike molecular dynamics simulation, these techniques have low time resolutions, but their single-molecule sensitivities allow probing of slow conformational motions, which are otherwise masked in ensemble-averaged experiments. Moreover, chemical reactions can now be observed on a single-molecule basis. For example, enzymatic turnovers of a few motor proteins (10–14), a nuclease (15), and a flavoenzyme (16) have been monitored optically in real time.

Our knowledge of enzyme kinetics has come primarily from experiments conducted on large ensembles of enzyme molecules, in which concentration changes over time are measured. In a single-molecule experiment, the concentration of the molecule being studied becomes meaningless in discussing chemical kinetics. However, this does not negate the fundamental principles of chemical kinetics. As we shall show, chemical kinetics can be cast in terms of single-molecule probabilities. Thinking of chemical kinetics in terms of single molecules is not only pertinent to the ever increasing single-molecule studies but is also insightful and very often more informative.

Such “single-molecule” thinking is also useful in understanding chemistry in living cells. In a living cell, the number of enzyme molecules in a cellular component may not be large. Under this situation, the concentration in a small probe volume is no longer a constant but a fluctuating quantity, as molecules react or diffuse in and out of the probe volume. In fact, the reaction rate (and diffusion rate) can be extracted from the analyses of concentration fluctuations (17, 18). This approach is referred to as fluctuation correlation spectroscopy (for review see Ref. 19) and has recently been conducted with single-molecule sensitivity (20). A typical fluctuation correlation spectroscopy trace, however, is averaged from a large number of molecules diffusing one or a few at a time in and out of a fixed probe volume. There are situations in which we need to focus on the behavior of a single molecule. For example, DNA exists as a “single molecule” inside a bacteria cell. The trajectory of a DNA-enzyme complex can be tracked. In another example, a single receptor protein at a particular spot in a membrane can be interrogated by optical or scanning probe microscopy. Studies in a similar line have been extensively carried out on ion channel proteins with the patch clamp technique (3).

In this minireview, we utilize our recent work on a flavoenzyme (16) to discuss the underlying principles of single-molecule kinetics and the information obtainable from single-molecule studies.

Why Single-molecule Real-time Studies?

What does one gain by doing single-molecule enzymatic studies? The stochastic events of individual molecules are not observable in conventional measurements, and the steady state concentrations of transient intermediates are usually too low to detect. The single-molecule experiments allow direct observations of individual steps or intermediates of biochemical reactions. The trajectories of motor proteins serve as good examples of the visualization of individual steps (10–14). Single-molecule spectroscopy is capable of capturing reaction intermediates.

Perhaps less obviously, single-molecule experiments allow determination of static and dynamic disorder. Seemingly identical copies of biomolecules often have non-identical properties. Static disorder is the stationary heterogeneity of a property within a large ensemble of molecules. Dynamic disorder (21) is the time-dependent fluctuation of the property of an individual molecule. Distributions of molecular properties of an ensemble are usually broad because of both static and dynamic disorder. The distribution is difficult to determine by ensemble-averaged measurements. Furthermore, ensemble-averaged measurements cannot distinguish between static and dynamic disorders.

A recent single-molecule enzymatic assay by Xue and Yeung (22) has revealed static disorder in enzymatic turnover rates of genetically identical and electrophoretically pure enzyme molecules. In a capillary tube containing a solution of highly diluted enzyme molecules (lactate dehydrogenase) and concentrated substrate molecules (lactate and NAD⁺), each enzyme molecule produced a discrete zone of thousands of NADH molecules after 1 h of incubation. The zones were then eluted by capillary electrophoresis and monitored by natural fluorescence of NADH. The enzyme molecules had a broad and asymmetrical distribution of activity, which was otherwise masked by ensemble-averaged measurements. The heterogeneity was found to be static at the hour time scale because the same enzyme molecule produces the same zone intensity after another incubation period. The microscopic origin of the static disorder observed is an interesting subject that deserves future research. Using a similar approach, Craig et al. (23) have studied single alkaline phosphatase molecules and found an even broader, multipeak distribution of activities. The static disorder in this system was attributed to glycosylation and other post-translational modifications, which produce non-identical copies of enzyme in this system.

Such experiments are capable of determining static disorder but not dynamic disorder. Dynamic disorder in enzymatic turnover rates has been observed by real time single-molecule experiments, as is discussed below. With the separation of static and dynamic disorders, relations between the dynamics and functions of enzyme molecules can be better interrogated with single-molecule real-time measurements.

Viewing Single-molecule Enzymatic Reactions by Fluorescence

Consider the example of cholesterol oxidase, a 53-kDa flavoprotein that catalyzes the oxidation of cholesterol by oxygen (Fig. 1). The active site of the enzyme (E) involves a FAD, which is naturally fluorescent in its oxidized form but not in its reduced form. The FAD is reduced by a cholesterol molecule to FADH₂ and is then oxidized by molecular oxygen. As shown in Fig. 1, fluorescence turns on and off as the redox state of the FAD toggles between the
oxidized and reduced states. Each on-off cycle corresponds to an enzymatic turnover. The turnover trajectory contains detailed information about the chemical dynamics.

The single-molecule fluorescence measurements are carried out with an inverted fluorescence microscope, as described elsewhere (16, 24, 30). It is desirable to study immobilized molecules to avoid the complications of the diffusion process. The samples are thin films of agarose gel of 99% water. The single-enzyme molecules are confined in the gel with no noticeable translational diffusion. In contrast, small substrate molecules still diffuse freely. Though confined in the polymer matrix, the enzyme molecules freely rotate within the gel, which was evidenced by a polarization modulation experiment, as previously described (25, 26). This means that the enzyme molecules do not bind to the polymer matrix. Control experiments were done to ensure that the conventional enzymatic assays gave the same results in gel and in solution. Polyacrylamide gel, which has a smaller pore size, has also been used to confine proteins, such as green fluorescence proteins (GFP)\(^1\) (27).

The turnover trajectories contain detailed dynamic information, which is extractable from statistical analyses. Good statistical analyses require long trajectories. The lengths of the trajectories are limited by photobleaching through photochemistry on the excited state (8). We observed a better photostability for the FAD chromophore in protein than for dye molecules, most likely because of the protection by the protein. Trajectories with more than 500 turnovers and \(2 \times 10^6\) detected photons (detection efficiency, 10\%) have been recorded. Similar photostability has been seen for other natural fluorophores, such as those in GFP (27). In the case of GFP, emission of a single molecule blinks off because of photoinduced chemical reactions. We did not observe such photoinduced blinking of cholesterol oxidase. We have also done control experiments to make sure that repetitive excitation/de-excitation does not perturb the enzymatic reactions.

**Chemical Kinetics in Terms of a Single Molecule**

Many two-substrate enzymes, such as cholesterol oxidase, follow the ping-pong mechanism for the two-substrate binding processes, obeying the Michaelis-Menten mechanism (28).

\[
\begin{align*}
E-FAD + S & \stackrel{k_1}{\longrightarrow} E\text{-FAD} \cdot S \longrightarrow E\text{-FADH}_2 + P \\
on & \quad \text{on} \quad \text{on} \\
E\text{-FADH}_2 + O_2 & \stackrel{k_1'}{\longrightarrow} E\text{-FADH}_2 \cdot O_2 \rightarrow E\text{-FAD} + H_2O_2 \\
\text{off} & \quad \text{off} \quad \text{on} \quad \text{on}
\end{align*}
\]

(Eq. 1)

The most obvious feature of the turnover trajectory in Fig. 1 is its stochastic nature. The emission on-times and off-times correspond to the "waiting time" for the reductive and oxidative reactions, respectively. The simplest analysis of the trajectory is the distribution of the on- or off-times. We limit our discussion below to the on-time distributions although similar analyses can be done for off-times as well.

First, take a simple case in which \(k_2\) is rate-limiting. This situation can be created with a slowly reacting substrate (derivative of cholesterol) and a high concentration of the substrate. The FAD reduction reaction follows a simple kinetic scheme.

\[
E\text{-FAD} \cdot S \stackrel{k_2}{\longrightarrow} E\text{-FADH}_2 + P \quad \text{(Eq. 3)}
\]

For this scheme, the probability density of the on-time, \(\tau\), for a single-molecule turnover trajectory is an exponential function, \(p_{on}(\tau) = k_2 \exp(-k_2 \tau)\), with the average of the on-times being \(1/k_2\), the time constant of the exponential. The exponential function follows from the fact that Equation 3 is a Poisson process. One caveat is that this does not mean that zero on-time has the highest probability; \(p_{on}(\tau)\) is the probability density, whereas the probability for the on-time to be between \(\tau\) and \(\tau + \Delta\tau\) is given by \(p_{on}(\tau)\Delta\tau\), with the integrated area under \(p_{on}(\tau)\) being unity.

\[^1\] The abbreviation used is: GFP, green fluorescence proteins.
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3, A and B, shows the two-dimensional histograms of a pair of on-times adjacent to each other and those separated by 10 turnovers, respectively. In the absence of dynamic disorder, \( p(X,Y) \) should be independent of the separation of turnovers. However, Fig. 3A and Fig. 3B are clearly different. For the separation of 10 turnovers (Fig. 3B), the loss of memory leads to \( p(X,Y) = p(X)p(Y) \), where \( p(X) \) and \( p(Y) \) are the same as in Fig. 3A. For pairs of adjacent on-times (Fig. 3A), there is a diagonal feature, indicating that a short on-time tends to be followed by another short on-time, and a long on-time tends to be followed by another long on-time. This means that an enzymatic turnover is not independent of its previous turnovers. The memory effect arises from a slowly varying rate \( (k_E) \). Coming back to the telephone analogy, this corresponds to the average lengths of phone calls varying over the course of a day.

Although the two-dimensional conditional probability plot provides a clear visual illustration, it needs to be constructed of a large number of turnovers of many molecules. A more practical and quantitative way to evaluate the dynamic disorder is the autocorrelation function of the on-times, \( r(m) = \frac{\langle \Delta r(0) \Delta r(m) \rangle}{\langle \Delta r^2 \rangle} \), where \( m \) is an index number for the turnovers in a trajectory and \( \Delta r(m) = r(m) - <r> \) and where the bracket denotes the average along the trajectory. The physical meaning of \( r(m) \) is as follows. In the absence of dynamic disorder, \( r(0) = 1 \) and \( r(m) = 0 \) \((m > 0)\). In the presence of dynamic disorder, \( r(m) \) decays, with the initial \((m = 1)\) amplitude reflecting the variance of \( k_E \) and the decay time yielding the time scale of the \( k_E \) fluctuation. Fig. 3C shows the \( r(m) \) derived from a single-molecule trajectory, with the decay constant being \( 1.6 \pm 0.5 \) turnovers.

We attribute the dynamic disorder behavior to a slow fluctuation of protein conformation, which was independently observed by spontaneous spectral fluctuation of FAD (16, 30) on the same time scale of \( k_E \) fluctuation. Slow conformational fluctuations on a similar time scale have been observed on other systems with single-molecule experiments (4, 31–34). The simplest model we proposed involved (at least) two slowly converting conformational states \((E \text{ and } E')\).

The dynamic disorder of the Michaelis-Menten mechanism (Equation 2) can be accounted for by the more complicated kinetics scheme (Scheme 1) with time-independent rates \( k_{21} \) and \( k_{22} \). A simulation of \( r(m) \) based on this kinetic scheme, assuming that \( k_5/k_W = 1 \) and \( k_{31}/k_{32} = 5 \), matches the observed \( r(m) \).

In summary, although the Michaelis-Menten mechanism provides a good description for the averaged behaviors of many molecules and for the averaged behaviors of many turnovers of a single molecule, it does not provide an accurate picture of the real-time behavior of a single molecule. On a single molecule basis, the rate for the activation step is fluctuating, and this is a not a small effect!

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The influence of conformational dynamics on protein functions has been a subject of extensive studies (36–43). Our observations have revealed the slow conformational motions that influence the enzymatic functions. However, we still need to understand the microscopic origin of the slow conformational change of cholesterol oxidase, which has a relatively large activation barrier between the two conformational states. Although the two-state model suffices to account for the fluctuation of $k_2$ and the spectral mean in our initial study, there can be more than two conformational states, or even a continuous distribution of conformational states, each with a distinctly different $k_2$. Our observations confirm that conformational transitions take place among metastable states in the energy landscape (44). Simulations based on the two-state model and the continuous-state (45, 46) model give a similar r(t). Further experiments are needed to probe the distribution of conformational states, the distribution of rates, and their correlation.

The physiological relevance of the phenomenon is of particular interest. Similar slow conformational changes have been inferred from other monomeric enzyme systems, which were believed to be associated with physiological enzymatic regulation (47–49). Interestingly, an ensemble-averaged enzymatic measurement of cholesterol oxides has revealed a sigmoid dependence of the enzymatic rate on the substrate concentration (50). Our simulation showed that the proposed mechanism in Scheme 1 might account for this sigmoidal behavior. The implication of this finding on the behavior of the monomeric enzyme is intriguing.

Not assumed in Scheme 1 is the possibility of conformational changes induced by substrate binding and/or the redox reactions, which can either lead to new conformational states or shift the equilibrium between the existing conformational states (47–49). Conformational changes associated with the "induced fit" have been illustrated (51). At this point, our single-molecule experiments have not yet detected the induced conformational changes. A study of cross-correlations between simultaneous spectral and turnover trajectories is under way to investigate this possibility.

In the Future

Thinking of chemical kinetics in terms of single molecules is becoming a necessity as new tools of microscopy allow investigations of the dynamics of individual molecules. We have shown that statistical analyses of turnover trajectories of single-protein molecules can unequivocally reveal detailed mechanistic information hidden in ensemble-averaged measurements. The analyses described here will be generally useful for other enzymatic systems. The analyses of turnover trajectories is under way to investigate this possibility.

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