Minireview

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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 $FADH_2$ 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

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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 2×10^6 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).

$$\underset{\text{on}}{E\text{-FAD} + \text{S}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{on}}{E\text{-FAD}\cdot\text{S}} \xrightarrow{k_2} \underset{\text{off}}{E\text{-FADH}_2 + \text{P}} \quad \text{(Eq. 1)}$$

$$\underset{\text{off}}{E\text{-FADH}_2 + \text{O}_2} \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} \underset{\text{off}}{E\text{-FADH}_2\cdot\text{O}_2} \xrightarrow{k_2'} \underset{\text{on}}{E\text{-FAD} + \text{H}_2\text{O}_2} \quad \text{(Eq. 2)}$$

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.

$$\underset{\text{on}}{E\text{-FAD}\cdot\text{S}} \xrightarrow{k_2} \underset{\text{off}}{E\text{-FADH}_2 + \text{P}} \quad \text{(Eq. 3)}$$

For this scheme, the probability density of the on-time, τ, 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 τ and $\Delta\tau$ 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.

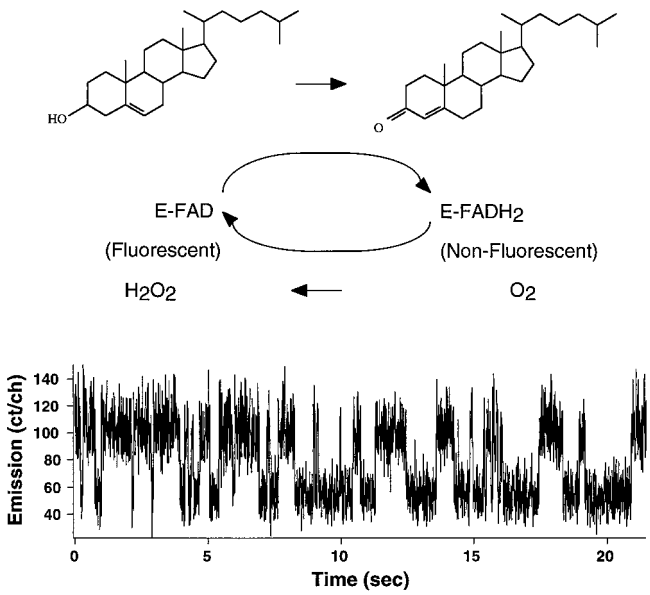

FIG. 1. **Enzymatic cycle of cholesterol oxidase and real-time observation of enzymatic turnovers of a single cholesterol oxidase molecule.** Each on-off cycle in the emission intensity trajectory corresponds to an enzymatic turnover. *ct*, count; *ch*, channel.

A simple example of a Poisson process is the case of a telephone being turned on and off. The Poisson process corresponds to a situation in which all phone calls are independent. This results in an exponential probability density distribution of the lengths of the calls (on-times). In such an analogy, our real-time experiment determines the on-time distribution of a single phone, Yeung's experiment (22) determines the average on-time of a single phone, and an ensemble-averaged measurement gives the average on-time of all the phones in the world.

Fig. 2*A* shows the histogram of on-times derived from a trajectory with a cholesterol derivative of 2 mM concentration, which was fitted with a single exponential. The *inset* of Fig. 2*A* shows the distribution of k_2 among 33 molecules examined, reflecting the large static disorder for the activation step. However, we did not observe static disorder for k_1, k'_1, and k'_2.

The on-time histogram (or probability density) takes a more complex form when k_2 is not rate-limiting. Assuming that $k_{-1} = 0$ in Equation 1, the probability density of the on-times is expected to be the convolution of the two exponential functions (time constants of $1/k_1$ and $1/k_2$) with an exponential rise (the faster one of k_1 and k_2) and an exponential decay (the slower one of k_1 and k_2) (16). Fig. 2*B* shows an on-time histogram for an enzyme molecule with 2 mM cholesterol, with the *solid line* being the convolution of two exponentials. The time lag in the histogram arises from the fact that there is an intermediate, *E*-FAD·S. There is no *E*-$FADH_2$ generated until the intermediate emerges. In the telephone analogy, this corresponds to the caller having to wait for an operator to connect. If there is more than one fluorescent intermediate, the on-times are expected to have a narrower distribution. This is discussed in detail in Ref. 29.

What Is the New Information?

So far, the analyses of on-time distributions illustrate at the single-molecule level the validity of chemical kinetics, in particular, the Michaelis-Menten mechanism (Equation 1). What new information can we obtain from the trajectory analyses? Chemical kinetics holds for Markovian processes, implying that an enzyme molecule undergoing a turnover exhibits no memory of its preceding turnovers. Dynamic disorder is beyond the scope of chemical kinetics. Fig. 2 does not have a good enough signal-to-noise ratio to reveal multiexponential decay because of dynamic disorder. Furthermore, being a scrambled histogram, Fig. 2 is not sensitive to memory effects. What we need is a way to examine how a particular turnover turn is affected by its previous turnovers.

We evaluated the conditional probability, $p(X,Y)$, for a pair of on-times (X and Y) separated by a certain number of turnovers. Fig.

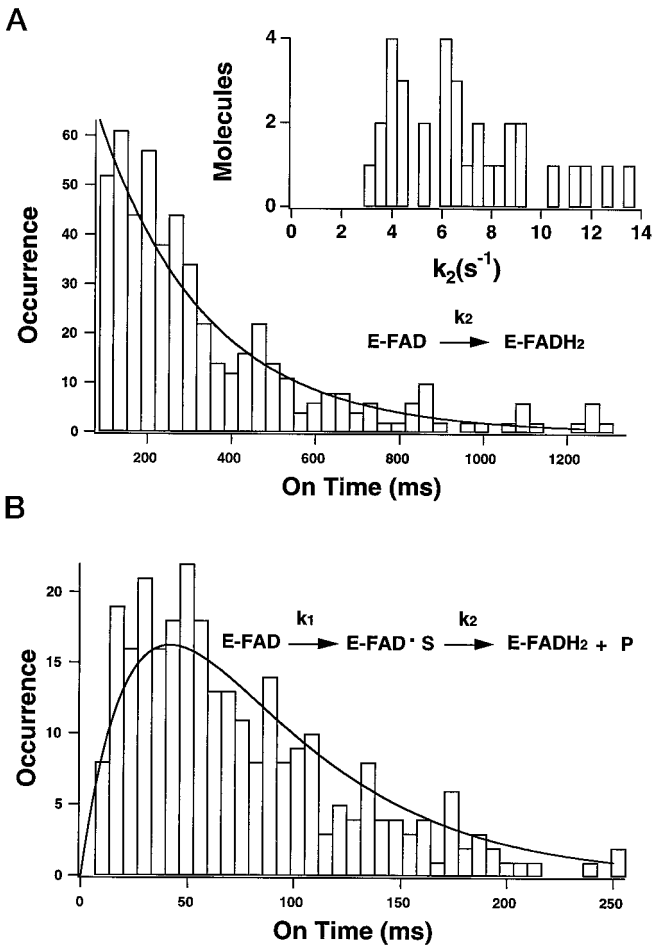

FIG. 2. *A,* histogram of the on-times in a single-molecule turnover trajectory taken with a derivative of cholesterol (see Fig. 3*C*, *inset*) at 2 mM concentration (k_2 being rate-limiting). The *solid line* is a single exponential fit with $k_2 = 3.9 \pm 0.5\ s^{-1}$. The *inset* shows the distribution of k_2 derived from 33 molecules in the same sample. *B,* histogram of the on-times in a single-molecule turnover trajectory taken with cholesterol at 2 mM concentration. The *solid line* is the convolution of two exponentials with time constants $k_1 = 33 \pm 6\ s^{-1}$ and $k_2 = 17 \pm 2\ s^{-1}$.

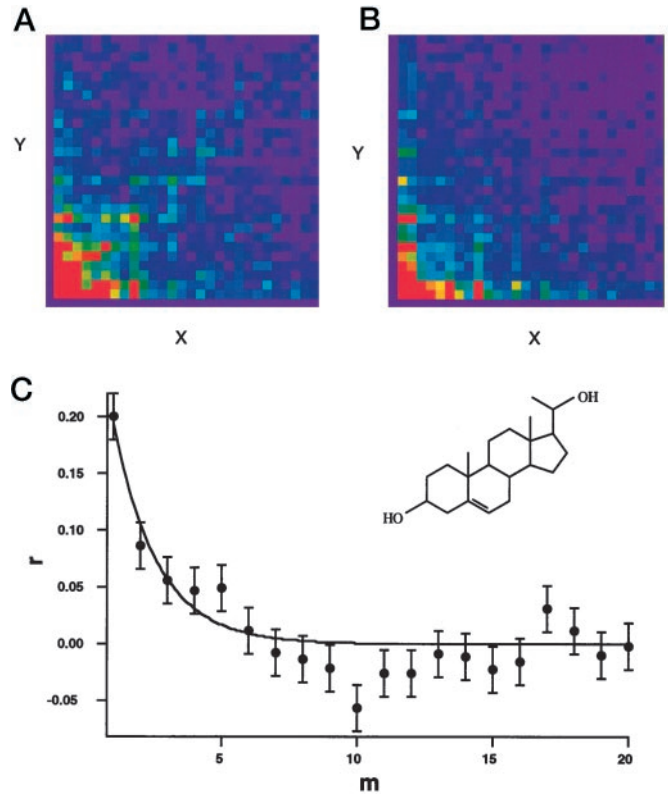

FIG. 3. *A,* two-dimensional histogram of pairs of on-times (X, Y) adjacent to each other. The scales of X and Y are from 0 to 1 s. The diagonal feature indicates a memory effect. *B,* two-dimensional histogram of pairs of on-times separated by 10 turnovers. The on-times that are 10 turnovers apart become independent as the memory is lost. *C,* autocorrelation function of the on-times for a turnover trajectory, $r(m) = <\Delta\tau(0)\Delta\tau(m)>/<\Delta\tau^2>$, where m is the index number of the turnovers and $\Delta\tau(m) = \tau(m) - <\tau>$. The fact that $r(m)$ is not a spike at $m = 0$ indicates dynamic disorder. The time constant of the decay gives the time scale of the k_2 fluctuation. The *inset* shows the structure of the substrate used, which is a derivative of cholesterol.

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. 3*A* and Fig. 3*B* are clearly different. For the separation of 10 turnovers (Fig. 3*B*), 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. 3*A*. For pairs of adjacent on-times (Fig. 3*A*), 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_2). 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, $r(m) = <\Delta\tau(0)\Delta\tau(m)>/<\Delta\tau^2>$, where m is an index number for the turnovers in a trajectory and $\Delta\tau(m) = \tau(m) - <\tau>$ 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_2 and the decay time yielding the time scale of the k_2 fluctuation. Fig. 3*C* shows the $r(m)$ derived from a single-molecule trajectory, with the decay constant being 1.6 ± 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_2 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 and E').

$$E \underset{k_{E'}}{\overset{k_E}{\rightleftharpoons}} E'$$

$$\begin{array}{ccccc} E\text{-FAD} + \text{S} & \xrightarrow{k_1} & E\text{-FAD}\cdot\text{S} & \xrightarrow{k_{21}} & E\text{-FADH}_2 + \text{P} \\ \updownarrow & & \updownarrow & & \updownarrow \\ E'\text{-FAD} + \text{S} & \xrightarrow{k_1} & E'\text{-FAD}\cdot\text{S} & \xrightarrow{k_{22}} & E'\text{-FADH}_2 + \text{P} \end{array}$$

SCHEME 1

The dynamic disorder of k_2 for 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 $k_E/k_{E'} = 1$ and $k_{21}/k_{22} = 5$, matches the observed $r(m)$.[2]

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!

[2] G. Schenter, H. P. Lu, and X. S. Xie, manuscript in preparation.

Open Questions

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 $r(m)$.[2] 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-enzyme molecules can unequivocally reveal detailed mechanistic information hidden in ensemble-averaged measurements. The analyses described here will be generally useful for other enzymatic systems. New single-molecule, *in vitro* enzymatic assays will be developed. Biochemical reactions, such as gene expression (35) and cell signaling, can be observed under physiological conditions. Single-molecule real-time enzymology will allow investigation of molecular and cellular dynamics at a level of great detail.

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