Probing Single-Molecule Protein Conformational Dynamics

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ABSTRACT
Protein conformational fluctuations and dynamics, often complex and associated with inhomogeneities, play a crucial role in biomolecular functions. It is extremely difficult to characterize such inhomogeneous dynamics in an ensemble-averaged measurement, especially when the proteins are involved in multiple-step, multiple-conformation complex chemical interactions and transformations, such as in enzymatic reactions, protein−protein interactions, and ion-channel membrane protein processes. Alternatively, single-molecule spectroscopy is a powerful approach to probing and analyzing protein conformational dynamics in real time.

Introduction

Protein molecules typically engage in complex and dynamic interactions with other protein species or local components of their environments. Protein conformations are highly dynamic rather than static, and subtle conformational changes play a crucial role in protein function. It has been widely recognized that knowledge about static structure alone is not sufficient to understand real-time protein functions[1−15] and that the conformational changes and ordered-disordered protein structure transitions[6,7,16,17] play important roles in determining protein functions. Nevertheless, static structure analyses from ensemble-averaged measurements at equilibrium have been the norm in understanding of protein structure and functions. Recently, however, single-molecule spectroscopy has been demonstrated as a powerful complementary approach in characterizing dynamic conformations and understanding correlated protein functions in real time. Single-molecule studies allow characterization of temporal, spatial, and energetic properties of protein conformations, which involve complex interactions among protein molecules, enzyme−substrate interactions and enzymatic reactions, and ion channel processes in membranes. Single-molecule studies can thus result in a detailed, mechanistic understanding of some of the most important biological processes, including receptor sensing, cell redox respirations, cell signaling, and enzymatic reactions. Several elegant articles[1−5] have provided extensive reviews of single-molecule spectroscopy and its applications.

Single-molecule fluorescence photon-stamping time trajectory detection[6,9,18−21] and statistical analysis have revealed static and dynamic inhomogeneities in the dynamics of a number of protein systems.[1−4,6−9,11−14] Static inhomogeneity describes inhomogeneous molecular dynamics from molecule to molecule, whereas dynamic inhomogeneity originates in the temporal fluctuations of the kinetic rates for individual molecules, which is beyond the scope of conventional kinetics.[1,4,6−9,13,14,20−23] It is nearly impossible for an ensemble-averaged measurement to selectively identify and characterize both static and dynamic inhomogeneities.

The following Account focuses on three areas of our recent work on single-molecule conformational dynamics: (1) the enzyme−substrate complex formation in T4 lysozyme hydrolization enzymatic reactions,[8−10] (2) gramicidin-dimer ion channels at lipid bilayers,[11,12] and (3) protein−protein interactions in a cell signaling system.[6,18]

Enzyme Conformational Dynamics in Catalytic Reactions

Enzymatic reactions typically involve multiple kinetic steps, complex molecular interactions, inhomogeneous conformational changes, and confined local environments. These complexities, associated with intrinsic static and dynamic inhomogeneities, often make an ensemble-averaged measurement alone insufficient for characterizing enzymatic reaction dynamics. To obtain a molecular-level understanding of the enzymatic reaction dynamics, conformational dynamics, intermediate enzyme−substrate complexes, and energy landscape of specific conformational changes along reaction coordinates,[8] our approaches have combined single-molecule spectroscopy, molecular dynamics (MD) simulation, and theoretical modeling.

One object of study has been the T4 lysozyme hydrolysis reaction of a polysaccharide.[8−10] The wild-type T4 lysozyme, having two domains connected by an α-helix, adopts different conformations as the two domains undergo a hinge-bending motion.[24] Previously, there have been no real-time observations of how much an active site opens to initiate the formation of an enzyme−substrate complex under physiological conditions, although the literature shows that the scale of the motion can be 4 to 8 Å.[24−26] Furthermore, except for an assessment of the overall reaction rates, other features of the process, e.g., the multistep reaction dynamics, the enzyme−substrate intermediate states, and the correlated conformational dynamics, have also been unknown because there were no adequately selective and temporal characterization methods for such complex reaction systems. By covalently attaching a fluorescence resonance energy transfer (FRET)[8,27] donor−acceptor TMR/Texas Red pair site-specifically to noninterfering sites, i.e., two cysteine residues on the enzyme (Figure 1A, inset), we were able to measure the hinge-bending motions of the enzyme by recording the time trajectories of the single-pair donor−

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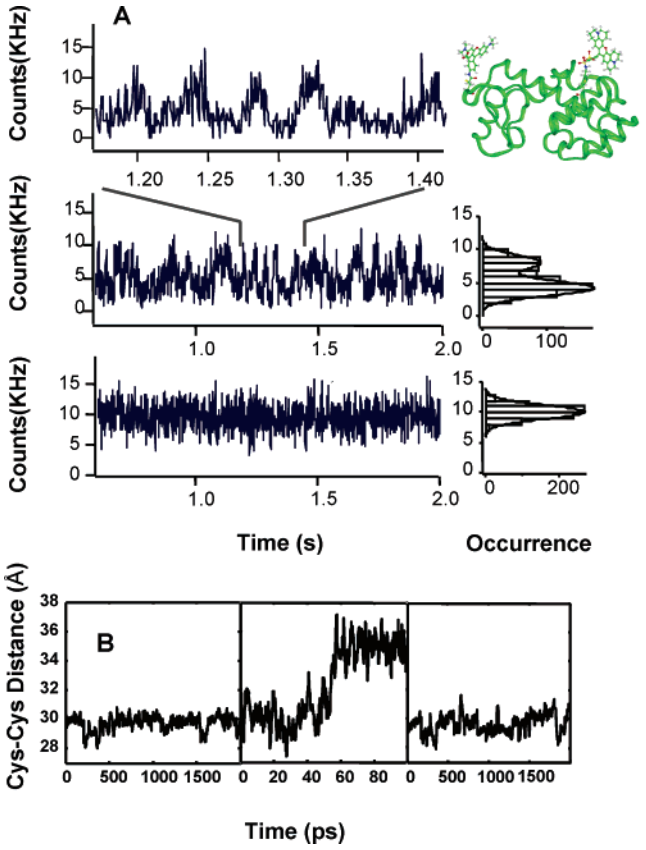

FIGURE 1. (A) Probing of a single T4 lysozyme enzymatic reaction turnover trajectory. The upper panel shows an expanded portion of a trajectory from donor fluorescence of a donor−acceptor-labeled single T4 lysozyme under an enzymatic reaction. The lower panel shows a trajectory of a donor-alone-labeled enzyme. The fluorescence intensity distributions derived from the two trajectories are shown in the insets of the middle and lower panels. The solid lines are fit using bimodal and Gaussian functions, respectively. (Inset, top-right) Crystal structure of wild-type T4 lysozyme with donor−acceptor labeling at Cys-54 and Cys-97. (B) Molecular dynamics simulation of T4 lysozyme hinge-bending motion. Three conformation trajectories of the distance between two −SH of Cys-54 and Cys-97 of T4 lysozyme in solution at room temperature are presented: free enzyme without substrate (E) (left panel), nonspecific binding of polysaccharide (E + S → ES) (middle panel), and active enzyme-polysaccharide complex (ES*) (right panel). Adapted with permission from ref 8. Copyright 2003 American Chemical Society.

acceptor emission intensities. We characterized single-molecule FRET and anisotropy dynamics over a wide range of time-scales, both the nanosecond fast dynamics and the sub-millisecond or longer slow dynamics.[8,9] In our experiments, the dye-labeled T4 lysozyme proteins were covalently tethered on a hydrocarbon-modified glass surface through a bifunction-linker molecule under buffer solution.[8,9]

Single-molecule FRET fluorescence trajectories contain detailed information about the conformational motions associated with the enzymatic turnovers. Using MD simulation, we have calculated that the distance change between the two dye-tethered cysteine residues is from 30.5 to 35 Å.[8] Considering the lengths of the two covalent linking groups of the donor and acceptor dipoles, the actual donor−acceptor dipole−dipole distance change is estimated to be ∼5.5 Å. At the Förster distance R_0 of 50 ± 5 Å, the distance change of 5.5 Å can cause a change of 2∼3 times in the intensity of donor fluorescence, although the change in the acceptor fluorescence intensity is less than 10%.[8] The upper panel in Figure 1A shows an expanded portion of a trajectory (middle panel) recorded from donor fluorescence of a single-pair donor−acceptor labeled protein with substrate present. By comparison, the lower panel shows a portion of a donor-fluorescence trajectory recorded from a donor-only labeled T4 lysozyme, not sensitive to the enzyme open-close hinge-bending motions, under the same conditions. The large-amplitude, lower-frequency wiggling of the donor fluorescence intensity in the upper panel, with a bimodal fluorescence intensity distribution (inset), reflects the open and closed conformational states of a T4 lysozyme.[8]

We have attributed each wiggle to a hinge-bending motion, i.e., an open-closed cycle, involved in either an enzymatic reaction turnover or nonproductive binding and releasing of the substrate, based on a series of ensemble-averaged and single-molecule control experiments.[8] The enzyme and polysaccharide chains engage in a nonspecific interaction through diffusion to form a nonactivated complex state, ES. Then, the active-site cleft binds to the polysaccharide chain to form specific hydrogen bonds in the activated complex state, ES*. The subsequent release of product and the enzyme searching for the next reactive site in the substrate complete the enzymatic turnover cycle. The donor fluorescence intensity increases as the active site opens due to substrate inserting and decreases as the active site closes to form an active enzyme−substrate complex. This attribution is consistent with the results of the ensemble-averaged FRET measurements made with and without a substrate.[8] Further evidence that we are measuring the hinge-bending motion comes from evaluating autocorrelation functions calculated from the single-molecule donor and acceptor trajectories when changing the laser excitation intensity, the pH, and the substrate concentration.[8]

Single-molecule FRET is sensitive to conformational change dynamics but not necessarily accurate in determining exact donor−acceptor distance changes of the conformations due to spectral fluctuation[28] and dye photophysics.[29] To evaluate quantitatively the amplitude of the protein-domain open-closed motions in forming an activated enzyme−substrate complex, we applied MD simulation to explore the conformations of the intermediate states of the enzymatic reaction.[8] MD simulation is intrinsically a single-molecule computational experiment involving picosecond (ps) to nanosecond (ns) short-time trajectories as "snapshots," as opposed to the millisecond time-resolved single-molecule experimental trajectories. Our MD simulation (Figure 1B) showed that when the substrate enters the active site, forming ES, the two domains of the enzyme exhibit a breathing-type hinge-bending motion along the α-helix, causing a 4.5 ± 0.2 Å distance increase ($\langle X_N(t)\rangle/2$) between the two cysteines.[8]

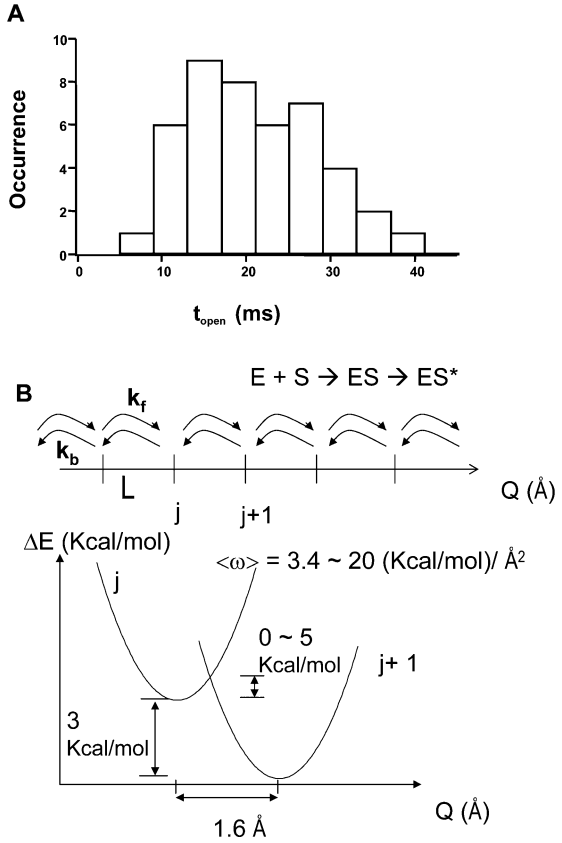

FIGURE 2. (A) Active-complex, ES*, formation time (t_{open}) distribution deduced from single T4 lysozyme fluorescence trajectories under enzymatic reaction. The t_{open} is the duration time of each wiggling of the intensity trajectories above a threshold. (B) Energy potential surface of T4 lysozyme-substrate complex formation process. The conformational change dynamics involving multiple intermediate states is analyzed based on a one-dimensional random walk model coupled with the parameters obtained from our single-molecule experimental spectroscopy and MD simulation. Since the total energy change between E and ES* states is about −18 kcal/mol, assuming six intermediate states would result in an average energy difference of 3 kcal/mol for each associated conformational state along the α-helix coordinate during the hinge-bending motion. We postulate that the activation energy associated with the k_f of the forward step is about 0∼5 kcal/mol, considering the slow forward rate and the entropy decrease in the complex formation process. It is reasonable to assume the energy potential surface along the conformational change nuclear coordinate to be parabolic for each intermediate state. Therefore, the averaged force constant of the potential surface for each intermediate state is calculated to be 3.4∼20 (kcal/mol)/Å². Adapted with permission from ref 8. Copyright 2003 American Chemical Society.

Experimentally, the formation time, t_{open}, of ES and ES* is probed in real time by recording single-molecule FRET trajectories.[8] Figure 2A shows a distribution of the open-time (t_{open}) deduced from single-molecule trajectories. The first moment of the distribution, $\langle t_{open}\rangle = 19.5 \pm 2$ ms, corresponds to the mean formation time of the ES* state. The standard deviation of the distribution, $\sqrt{\langle \Delta t_{open}{}^2\rangle} = 8.3 \pm 2$ ms, reflects the distribution bandwidth.

We model the hinge-bending motion associated with interactions between the enzyme and substrate as a classical particle one-dimensional multiple-step random walk in the presence of a force field (Figure 2B).[8,30] Based on this model analysis, we were able to obtain the diffusion coefficient, $D = 3.8 \times 10^{-14}$ cm²/s, of the T4 lysozyme substrate-enzyme complex formation conformational motions from measurable parameters, both the single-molecule spectroscopy data and MD simulation results:[8]

$$D = \frac{(\sqrt{\langle \Delta t_{open}{}^2\rangle}\langle X_N(t)\rangle)^2}{2\langle t_{open}\rangle^3}$$

We also obtained the mean drifting velocity, $\langle v\rangle = \langle X_N(t)\rangle/\langle t_{open}\rangle$, of the conformational change, 4.6×10^{-6} cm/s.

We further characterized the energy landscape of the hinge-bending conformational change dynamics by estimating the minimum number of the intermediate conformational states involved in the complex formation and by calculating the averaged rates of forming these conformational states. From the random walk model (Figure 2B upper panel),[8] we have $D/\langle v\rangle = L[(k_f + k_b)/(2(k_f - k_b))]$, and $L = 2D/\langle v\rangle = 1.6$ Å, when $k_b \rightarrow 0$; therefore, the minimum number of intermediate conformation states is $m = \langle X_N(t)\rangle/L = 5.6$. The average rate for each step is $m/\langle t_{open}\rangle = 280$ s^{-1}. This result ($m > 2$ at the limit of $k_b \rightarrow 0$) suggests that there are definitely more than two conformational intermediate states in addition to ES and ES*.[8] Our model analysis, based on both experimental results and MD simulation, also identified the friction coefficient, the energy consumed by friction along the reaction coordinates, and the averaged force constant of the potential surface for each intermediate state (Figure 2B).[8]

The information about the existence of the multiple intermediate conformation states involving the enzymatic active complex formation, an experimental characterization of the energy landscape (Figure 2B), and dynamics of the complex formation process cannot be obtained solely by either an ensemble-averaged experiment, a single-molecule experiment, or a computational MD simulation approach. For studies of protein conformational dynamics, a combined approach, using both single-molecule spectroscopy and MD simulations, is essential.

Using the methodology described above, which has allowed a better understanding of hydrolysis enzymatic reaction in dissolving cell walls, we are now studying the spatially inhomogeneous and temporally intermittent single-molecule enzymatic reactions in living cell surfaces. Using single-molecule injection,[10] we directly place a single-molecule T4 lysozyme on a cell wall surface and watch the highly effective enzyme proteins in action.

Probing Multiple-State Conformational Dynamics of Single Ion-Channel Membrane Proteins

Ion channels are proteins that control ion flux across the cell membranes. Subtle conformational changes of ion-channel proteins are often critical in the functioning of ion channels. However, the conformational dynamics for

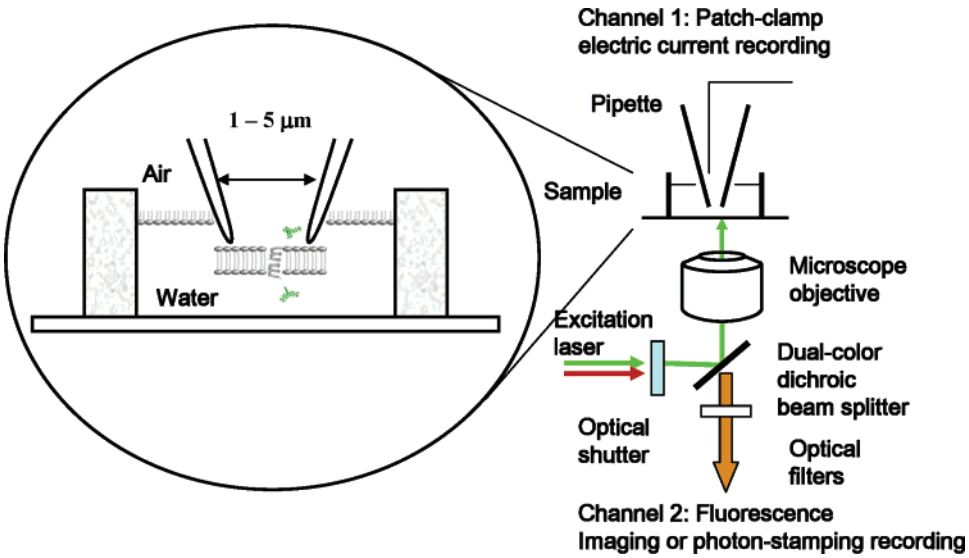

FIGURE 3. Experimental apparatus and measurement configuration of PCFM. Lipid bilayers were formed at the tip of a patch pipet by apposition of two monolayers of mixed lipids (4:1 dPhPE:DPhPC). Dye-labeled gramicidin monomers are introduced from both sides of the bilayer, and single-channel electric currents are recorded by using typical patch-clamp instrumentation. The wide-field or confocal excitation was focused on the pipet tip, and the fluorescence was imaged on a CCD or a pair of photostamping modules. Adapted with permission from ref 11. Copyright 2003 American Chemical Society.

most ion-channel proteins are still largely unknown due to lack of direct measurements in real time. The patch-clamp recording technique,[31] which records ion currents of cations or anions such as sodium ions and protons flowing across biological membranes through ion channel proteins, has had a major impact on the understanding of ion-channel processes. However, the conformational dynamics of the ion channel proteins has been primarily extrapolated by model analyses of the electric current trajectories that are not particularly sensitive to subtle conformational intermediate states, including electrically undetectable "silent" conformational states.

In 1999, Yanagida and co-workers[32] reported single-channel fluorescence images acquired before or after a single-channel current measurement on the same sample lipid bilayer, utilizing a micro-pinhole patch technique. During that time and since then, there have been significant efforts and advancements.[11,12,33−41] These research efforts have begun to provide a new paradigm for studying ion-channel conformational dynamics and mechanisms and for obtaining information not obtainable by the conventional patch current recording measurements, a primary experimental approach for decades.

The following discussion is focused on the new scientific information obtained in our single-molecule studies on the conformation-gated dynamics of the gramicidin ion channels in lipid bilayers by using a new approach,[11,12,41] patch-clamp fluorescence microscopy (PCFM) (Figure 3), which combines simultaneous single-channel electric current patch recording with single-molecule fluorescence imaging of co-localization and FRET, nanosecond lifetime and anisotropy, and fluorescence self-quenching.

Linear gramicidin, produced naturally from the bacterium *Bacillus brevis*, is probably one of the mostly studied ion channels.[42−44] According to the literature, there are two conformational states involved in the channel open-closed activity. Its conductive and open state is a dimer of two 15-amino acid peptides forming β-helices, which meet head-to-head at the N-termini in the interior of a lipid bilayer; a dissociated dimer constitutes a closed state. However, even for this "simple" ion channel, the nature of the structural changes and the conformational dynamics that regulate the open and closed states are still being debated.[44] Our experimental results, not obtainable by using ensemble-averaged analyses or traditional single-channel patch electric recording, revealed that multiple intermediate conformational states are involved in the channel's open-closed activities. Such results advanced our knowledge of ion channel dynamics and significantly modified the conventional two-state model of gramicidin.

Using PCFM, we were able to record simultaneously the single-channel electric current and spectroscopic images and thus identify and characterize the single ion-channel protein conformational dynamics. Asymmetric incorporation of Cy5-gramicidin C and TMR-gramicidin C[11,12] led to the formation of single channels tagged with pairs of FRET donors and acceptors. Co-localization and FRET within a single gramicidin heterodimer were probed by dual-color excitation and donor−acceptor two-channel fluorescence imaging (Figure 4A) and by single-molecule photon time-stamping.[6,9,11,12,18−21] Dye molecule spin or wobbling motions were at a nanosecond time-scale[8,12,18] and averaged out in the FRET imaging measurements at a millisecond time-scale.[11,12] The mean spFRET efficiency of 0.59 ± 0.05 and the fwhm of 0.21 (Figure 4B) correspond to a mean donor−acceptor distance of ~56 Å and a range of ~52 Å $\leq d \leq$ ~63 Å. Dihedral angular distortion of the two monomers due to hydrogen bonding destabilization[11,12] may also contributed to the broad distribution. Nevertheless, FRET results suggest the existence of the multiple intermediate conformational states, beyond the

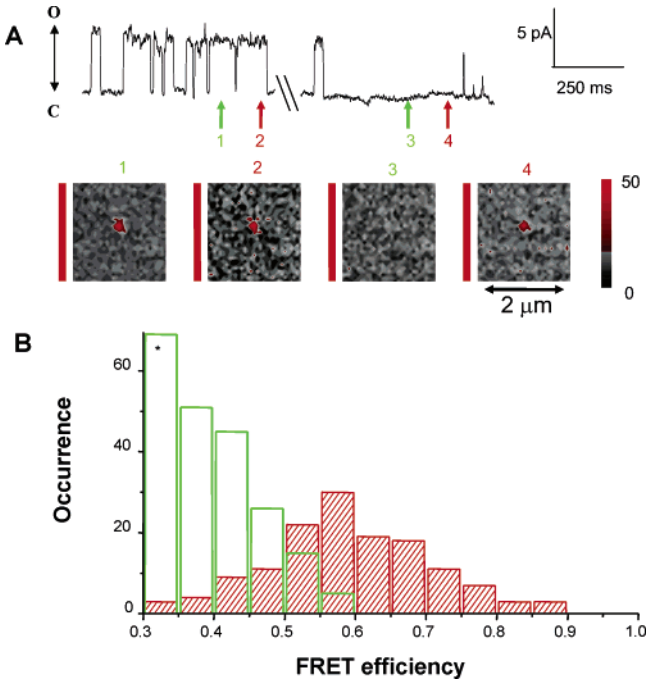

FIGURE 4. PCFM measurement of single TMR/Cy5-gramicidin heterodimer ion channels. The "green" and "red" arrows mark the timing of the toggled excitation at 514 and 632 nm, respectively. (A) Single-channel electric current trajectory (upper panel) with four arrows indicating the exposure of four consecutively acquired FRET imaging frames. Frames 1 and 3 were taken at 514-nm excitation, and Frames 2 and 4 were taken at 632-nm excitation. Frame 1 indicates that FRET occurred between the TMR/Cy5 spFRET pair when the gramicidin channel was at an open state. Frame 2 verifies that a single Cy5 molecule was present. Frame 3 indicates that no FRET occurred when the channel was at a closed state. Frame 4 verifies that the Cy5 was still present. (B) FRET efficiency distribution of "channel open" (shaded bars) and "channel closed" (open bars). It is experimentally difficult to differentiate FRET below the efficiency of 0.3 or no FRET (indicated by *). Adapted with permission from ref 11. Copyright 2003 American Chemical Society.

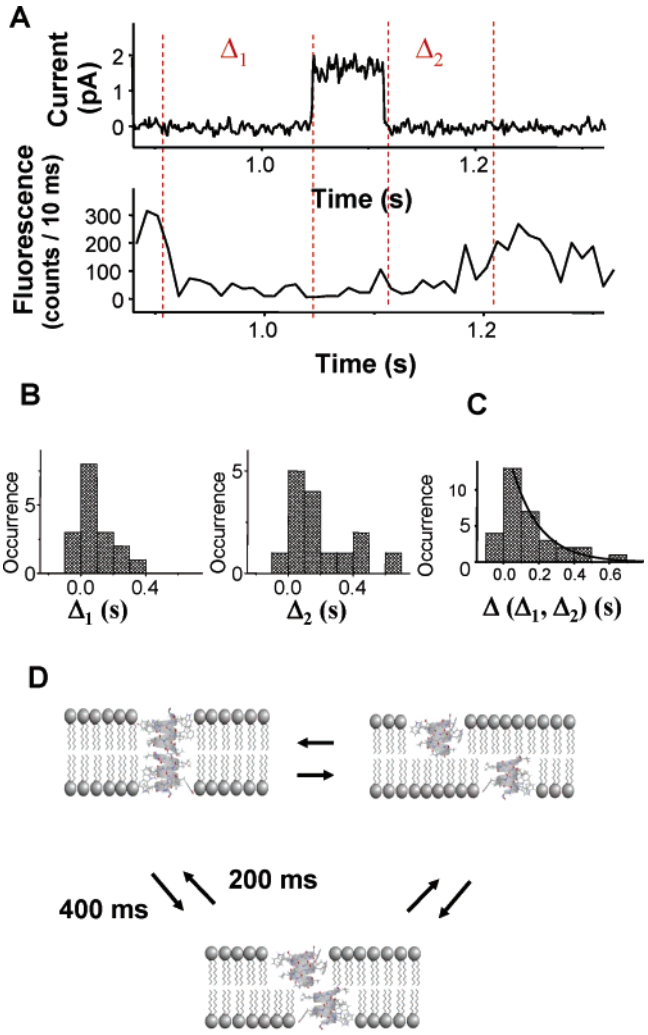

FIGURE 5. Identification and distribution of the conformation-associated silent time. The fluorescence self-quenching of a TMR-dye pair on a gramicidin dimer causes an intensity decrease. (A) Identification of the silent times, the time lags between the channel electric current changes, and fluorescence intensity changes. (B) The distribution of the silent times. (C) The collective distribution of the silent times. (D) Proposed multi-state model of gramicidin dynamics indicating a third "multi-state" or a set of intermediate conformation states of nonconducting or partially conducting channels with larger separation between the fluorophore probes (middle).

conformational fluctuation within a conformational state or between two conformational states of open-closed states.[11,12] The broad distributions of FRET efficiency, correlated with both open and closed states, suggest that the channels could be in closed states even when the two gramicidin monomers are still in an intermediate non-dissociated state.

Figure 5A shows a single-channel electric current trajectory simultaneously recorded with a synchronized fluorescence-quenching time trajectory. The fluorescence intensity and the electric current trajectories show a distinctive anti-correlated pattern when the channel is in an open state due to the close proximity of the pair of fluorophores across the lipid bilayer.[12] A further analysis (Figure 5A−C) shows that there are statistically significant time mismatches between the two trajectories in the channel-opening and -closing events. We attributed the time mismatches to conformation-associated activity induction times or the silent times, Δ_1 and Δ_2, between the channel conformational changes and the channel opening and closing events, respectively (Figure 5A).[12] The distributions of the stochastic Δ_1 and Δ_2 are essentially Poisson and can be fitted with single-exponential decays (Figure 5B and 5C). The mean time of conformational change among the intermediate states and open-closed states was determined to be 200 ± 50 ms for Δ_1 and 400 ± 50 ms for Δ_2. The multiple intermediate states are involved in gramicidin channel dynamics, in addition to the channel open and closed states, and their interconversion time is at a subsecond time-scale.

Based on our experimental results,[11,12] we proposed a new model of multi-state gramicidin channel dynamics (Figure 5D). The open (upper left), closed (upper right), and intermediate (middle) states can presumably be the outcomes of intra- and intermolecular conformational changes. There is a subset of the intermediate states. We postulate that the intermediate conformers result predominantly from the six intermolecular H-bond fluctua-

tions that disrupt the pore structure of the gramicidin dimer.[11] Lately, this postulated mechanism has been supported by MD simulations.[45] Subtle conformational changes at the N-terminal linkage of the dimer may significantly disrupt the channel cavity structure and the electrostatic field within the channel, thereby perturbing the channel conductance. It is also noticeable that the gramicidin conformational dynamics are coupled with the peptide-lipid interactions.[11,12]

The unique real-time imaging and spectroscopy of the dynamics of individual gramicidin ion channels enabled us to reveal a hitherto undetected correlation of multiple conformational states of the channel consistent with transitions among closed, intermediate, and open states. The methodology we developed should be applicable for studying ion-channel protein conformational dynamics and mechanisms in more complex membrane protein systems, including the ion channel proteins that involve strong protein−lipid interacting modulations. It is now possible to explore the function-regulating and site-specific conformational changes of single ion-channel membrane proteins in living cells and under physiological conditions.[41]

Protein−Protein Interaction Dynamics in Cell Signaling

Proteins rarely act alone but often involve complex protein−protein interactions. Most of the important biological processes, including cell signaling, gene expression, enzymatic metabolism, and bacterial redox respiration, have crucial contributions from protein−protein interactions. Most often, protein−protein interactions are intrinsically single-molecule processes in living cells. For example, cell-signaling processes can be initiated through a few copies of interacting protein complexes, which determine pathway branching and are amplified along the signaling pathway.

New information obtained from our single-molecule spectroscopy studies[6,7] shows that protein−protein interactions in cell signaling[6] and DNA damage recognition[7] may involve highly flexible or disordered tertiary structures and slow conformational fluctuations at time-scales of sub-milliseconds to seconds. The large-amplitude conformational fluctuations underlie significant static and dynamic inhomogeneities in the interactions of protein complexes.[6,7] To a certain extent, recent NMR static and ensemble-averaged structural analyses of protein complexes have also indicated that binding domains often undergo dramatic conformational changes, from disordered to ordered tertiary structures, upon induced-fit binding complex formation.[16,17] It is reasonable to suggest that structural transitions of flexible conformational domains, which can be identified and characterized in detail by measuring single-molecule conformational fluctuation dynamics, are likely common in biomolecular recognition processes.[6,7]

We have applied single-molecule spectroscopy to study the interactions of an intracellular signaling protein Cdc42, a monomeric GTPase protein, with its downstream effector protein WASP (Wiskott-Aldrich Syndrome Protein).[6] In this work, a WASP fragment, 13-kDa CBD (Cdc42 binding domain of WASP) that binds only the activated Cdc42, was used and labeled with a solvatochromic dye (I−SO)[6] to probe the hydrophobic interactions at the protein−protein interface that are significant to Cdc42/WASP signaling recognition. Cdc42 acts as a molecular switch in signaling pathways to regulate various cellular responses.[46] Cdc42 GTPase can be activated by binding to GTP, and the activated Cdc42 then binds and activates a series of effector proteins via direct and consecutive protein−protein interactions.[47,48] Our single-molecule spectroscopy study revealed both static and dynamic inhomogeneities on the conformational fluctuations of the protein complex involving bound and loosely bound states of the protein−protein interaction complex.[6]

Collecting fluorescence emission images and photon-stamping time trajectories (Figure 6A) from individual Cdc42-CBD complexes immobilized in an agarose gel (0.5%) thin film, we observed a more than 3-fold increase in fluorescence intensity fluctuations at a time-scale ranging over several orders of magnitude, from milliseconds to sub-seconds. To identify the origination of the fluorescence fluctuations, we conducted a series of control experiments and reached the following conclusions:[6] (1) The measured conformational fluctuations were spontaneous, as the decay rate constants of the autocorrelation functions were independent of the laser excitation intensity at a level less than 250 W/cm^2 under our experimental conditions. (2) Fluorescence fluctuation did not originate from dye interactions in the CBD biosensor because dye-labeled CBD alone did not give high enough fluorescence intensity to be detected at the single-molecule level (Figure 6B). Only the Cdc42-CBD complex was "visible" to our single-molecule measurements. (3) Fluorescence fluctuation was not a result of interaction between the dye-labeled CBD and the agarose gel because the fluorescence quantum yield of dye-labeled CBD remained as low in the agarose gel as in the buffer solution. (4) A triplet-state formation of the dye molecule in the Cdc42-CBD complex was unlikely to produce such fluorescence fluctuation because the fluctuation is spontaneous rather than photoinduced; besides, the triplet state dynamics would have been at a much faster time-scale[28,49] under ambient conditions than the 10-to-100-ms time-scale observed in our fluorescence intensity fluctuations. The single-molecule control experiments[6] and the ensemble-averaged measurements[6,48] both support attributing dye fluorescence fluctuations to protein−protein interactions and to conformational fluctuations at the protein−protein interface being probed.[6]

We attributed the dynamics of the conformational fluctuations to both bound (B) and loosely bound (LB) states of the protein complex.[6] The LB states were presumably a subset of conformations that deviated from the bound equilibrium states, probed by the dye, without disrupting the sub-nanometer long-range interactions, so the overall protein complex is still associated. Compared

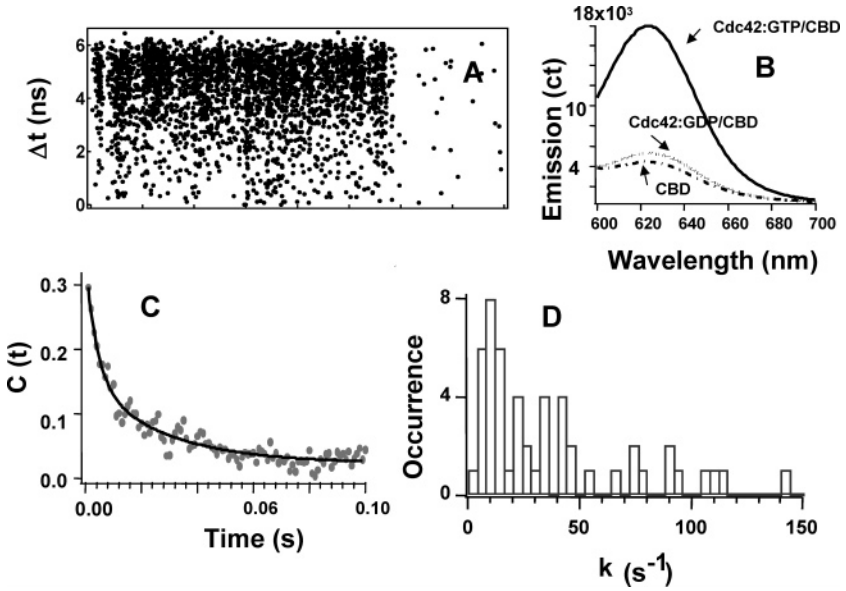

FIGURE 6. Single-molecule spectroscopy and imaging study of Cdc42-CBD binding dynamics. (A) Single-molecule photon time-stamping trajectory. Each dot corresponds to a photon stamped with an arrival time (t) and a delay time (Δt). The delay time is the duration from the time of excitation of the single molecule to the emission by the same molecule, which is a one-event measurement of the fluorescence lifetime of this molecule. (B) Ensemble fluorescence assays of Cdc42 with CBD biosensor using fluorescence spectral measurements. Fluorescence spectra were obtained with excitation at 568 nm. The solid curve represents the active GTP-activated Cdc42 forming complexes with dye-labeled CBD; the dotted curve represents the GDP-loaded Cdc42 with dye-labeled CBD; and the dash−dotted curve represents the dye-labeled CBD alone. A non-hydrolyzable GTP analogue, GTP-γ-S, was used to lock Cdc42 in the active conformation. Thus, the GTP binding and unbinding process was eliminated from the single-molecule experiments that measured only the activated Cdc42 interacting with CBD. The effects of GTP-γ-S are the same as that of GTP, and the biological relevance and validity of using GTP-γ-S has been well established in the literature.[48] **(C)** The autocorrelation function, C(t), calculated from an intensity trajectory of a single Cdc42-CBD complex, with biexponential decays of 250 ± 60 s^{-1} and 45 ± 10 s^{-1}. **(D)** The occurrence histogram of the single-complex conformational fluctuation rates. Adapted with permission from ref 6. Copyright 2004 American Chemical Society.

with the B state, the LB state gives significantly lower fluorescence intensity, as the distorted protein−protein interaction interface becomes more solvent-accessible and hydrophilic. The spectroscopic characterization of the B and LB states showed them to be analogues of CDC42−CBD and CBD-alone states measured in a control ensemble-averaged experiment (Figure 6B).[6] Our MD simulation demonstrated that the solvent-accessible surface area of the dye molecule undergoes a decrease from ~377 Å^2 to ~225 Å^2 upon Cdc42-CBD binding.[6]

One of the most intriguing observations is that by using autocorrelation function analyses conformational fluctuation rates were found to be highly inhomogeneous (Figure 6C and 6D). Variations of more than 2 orders of magnitude occurred in the conformational fluctuation rates among individual protein complexes under the same solution condition. Although it is still difficult to identify exactly how many conformational states contribute to the inhomogeneous distribution, we postulated that there are at least two subgroups of states associated with conformational fluctuations at ~10 s^{-1} and ~40 s^{-1} (Figure 6D). About 25% of the autocorrelation functions of the single-complex fluorescence intensity trajectories demonstrated biexponential decays, indicating non-Poisson kinetics.[6] The non-Poisson behavior suggests that the Cdc42-CBD interactions have both static and dynamic inhomogeneities.

The real-time observations of the large-amplitude and millisecond conformational fluctuations of the protein−protein interactions in a cell signaling system can only be achieved by single-molecule spectroscopy, as the stochastic conformational fluctuations are typically averaged out in an ensemble-averaged measurement. The millisecond slow dynamic fluctuation of a highly flexible domain likely plays a crucial role in the protein−protein recognition complex, showing that the induced-fit protein−protein interactions are highly dynamic rather than static.[6,7] Is the slow conformational fluctuation essential for cell-signaling protein−protein interactions? A definitive answer has yet to be obtained and may be obtained by combined biological mutation, single-molecule spectroscopy, MD simulation, theoretical modeling, and structural analyses by NMR and X-ray crystallography.

Concluding Remarks

Single-molecule spectroscopy is particularly powerful for identifying and characterizing spatially and temporally inhomogeneous protein conformational dynamics. For the last two decades, we have witnessed a rapid development of this field, and its future will definitely involve further advanced single-molecule approaches: single-molecule combined experimental multiple-parameter probing with high chemical selectivity and wide temporal and spatial

resolutions; computational molecular dynamics simulation analysis, based on static protein structures and correlated experimental single-molecule trajectory analyses; and theoretical modeling with experimentally accessible physical parameters. Already, single-molecule spectroscopy has substantially changed the landscape of the biological and biophysical sciences. We now have an open window for observing complex and inhomogeneous protein conformational fluctuations and motions in real time under physiological conditions. Ultimately, the single-molecule conformational dynamics of protein−protein and protein-DNA interactions, enzymatic reactions, and ion-channel protein activities will be directly studied in living cells.

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