Single-Molecule Fluorescence Spectroscopy: New Probes of Protein Function and Dynamics

Single-molecule fluorescence methods provide new tools for the study of biological systems. Single-pair fluorescence resonance energy transfer has provided detailed information about dynamics and structure of the Ca²⁺-signaling protein calmodulin. Single-molecule polarization modulation spectroscopy has probed the mechanism by which calmodulin activates the plasma membrane Ca²⁺ pump.

Ca²⁺-signaling protein calmodulin (CaM) functions as a molecular switch in the transduction of Ca²⁺ signals. The proper regulation of the Ca²⁺ signal is critical to the temporal and spatial propagation of intracellular responses, which in turn govern important cellular functions such as induction of gene expression, release of neurotransmitters, ion transport, smooth muscle contraction, neuronal plasticity, learning, and memory formation (3).

Single-pair fluorescence resonance energy transfer (FRET) measures the efficiency of energy transfer from an excited fluorophore (the “donor,” D) to another fluorophore (the “acceptor,” A) (24). Because the efficiency of transfer depends on distance, FRET has been called a “spectroscopic ruler” (24) and can be used to measure distances on the scale of tens of Ångstroms. On the single-molecule level, single-pair FRET (spFRET) has emerged as a powerful method for tracking dynamics and mechanisms in biomolecules (7, 10) and has been exploited to detect conformational dynamics of proteins such as syntaxin 1 (15) and T4 lysozyme (4) as well as unfolding intermediates of cold-shock protein (21), chymotrypsin inhibitor 2 (8), and adenylate kinase (20).

We applied spFRET to detect the binding of single CaM molecules to target peptides such as the CaM-binding domain of CaM-dependent protein kinase II (CaMII). To track a single CaM molecule over long time periods, proteins can be immobilized in agarose gel, which provides an aqueous environment allowing the protein to function without impairment by glass surfaces. CaM itself is not immobilized in an agarose gel, possibly because of its small size, so...
we constructed a fusion protein of CaM with maltose-binding protein (MBP-CaM), which is immobilized in agarose (1, 2). We constructed a mutant T104C, T110C-CaM and labeled the two Cys residues with donor and acceptor FRET fluorophores, Alexa Fluor 488 and Texas red (1, 2). We demonstrated FRET in fluorescently labeled single-molecule MBP-CaM constructs (denoted MBP-CaM-DA) and found that binding of a target peptide to MBP-CaM-DA strongly quenches fluorescence of the dye pair Alexa Fluor 488-Texas red attached at sites 34 and 110 (1). Fluorescence quenching in these constructs provides the potential for ultrasensitive assays of CaM binding to proteins and drugs. We determined a $K_d$ of $103 \pm 35$ pM by counting fluorescent (unquenched) single molecules of MBP-CaM-DA immobilized in an agarose gel as a function of CaMKII concentration (1). This novel method of detecting target binding has the potential to determine binding affinities down to femtomolar levels.

**Fluorescence correlation spectroscopy and spFRET**

In contrast to methods that probe translationally restricted molecules, the study of biomolecules in solution eliminates the need for immobilization. In fluorescence correlation spectroscopy (FCS) (5, 12, 13), molecules are detected as they diffuse freely through the focal region of the fluorescence microscope. Fluctuations in fluorescence signals are analyzed by autocorrelation and cross-correlation of signals from one or more fluorescent species. FCS and spFRET form a powerful combination that is able to reveal dynamics and conformational substates of freely diffusing biomolecules (6).

In our laboratory, we used spFRET-FCS to detect intramolecular dynamics and conformational distributions of fluorescently labeled CaM molecules (CaM-DA) by FRET (Ref. 22 and unpublished observations) (see FIGURE 2). spFRET is able to uncover subpopulations in heterogeneous systems. Distance distributions of CaM-DA reveal the presence of multiple conformational substates (22) (see FIGURE 3). The dominant conformation is centered at a distance between residues 34 and 110 of ~34 Å in Ca$^{2+}$-CaM and ~38 Å for apoCaM. A second conformation was observed at ~50–60 Å and a third, more compact conformation at ~28 Å. Such multiple conformations cannot readily be detected by ensemble methods, which measure an average structure, whether in solution or in a crystal. Thus single-molecule methods provide a unique capability to detect the conformations present under physiological conditions. Furthermore, from ensemble measurements it is not possible to distinguish whether a change in the average conformation of CaM as conditions (such as pH) are altered results from a) a change in the structure of all molecules in the distribution or 2) a change in the relative populations of conformational substates while the structure of the conformational substates themselves remain unchanged. Single-molecule measurements can resolve this question (unpublished observations).

Single-molecule spectroscopy is also able to provide information about conformational dynamics of proteins. Dynamics can be characterized in terms of the ratio of donor and acceptor channel counts yields the FRET efficiency $E$ and the corresponding distance $R$ between donor and acceptor fluorophores. The factor $c$ corrects for differences in quantum yield and detection efficiency between donor and acceptor channels; $b$ corrects for cross-talk of donor emission into the acceptor channel. $R_0$ is the distance of 50% energy transfer from donor to acceptor. The histogram of distances between residues 34 and 110 for Ca$^{2+}$-CaM shows the presence of three distinct conformations. The dominant conformation (32–40 Å) does not correspond to any of the crystal structures of CaM. The longer and shorter conformations are consistent with extended (pdb 1cll) and compact (pdb 1prw) structures.

**FIGURE 2. Combined single-pair fluorescence resonance energy transfer correlation spectroscopy in fluorescence correlation spectroscopy (FCS), as single fluorescently labeled calmodulin molecules (CaM-DA) pass through the focal region, fluorescence bursts are generated in donor and acceptor channels. The ratio of donor ($I_d$) and acceptor ($I_a$) counts yields the fluorescence resonance energy transfer (FRET) efficiency $E$ and the corresponding distance $R$ between donor and acceptor fluorophores.**
spFRET-FCS by cross-correlation analysis of signals from donor and acceptor fluorophores. spFRET in the presence of dynamic motion between lobes of CaM leads to anticorrelation between D and A signals, contributing a rising component to the cross-correlation function. We detected such a component on the submillisecond time scale in the cross-correlation function of CaM-DA, revealing fast conformational fluctuations of CaM (22).

**Single-molecule polarization modulation**

The polarization of light used to excite a single molecule provides a means to probe the orientation dynamics of single molecules (see FIGURE 3) (11, 27). We used single-molecule polarization modulation spectroscopy to probe the interactions of CaM with one of its target enzymes, plasma-membrane Ca\(^{2+}\)-ATPase (PMCA) (17, 19). Single-molecule spectroscopy opens a new approach to experiments on proteins such as PMCA that are available in low abundance. To apply single-molecule polarization methods to CaM, we constructed T34C-CaM and labeled it with the fluorophore tetramethylrhodamine (TMR). We detected Ca\(^{2+}\)-dependent binding of single CaM-TMR molecules to PMCA immobilized in agarose gels.

**FIGURE 4** shows the modulation depth distributions measured for PMCA-CaM complexes at two different Ca\(^{2+}\) concentrations. Two distinct populations were detected. We argued that the more orientationally mobile population corresponds to PMCA-CaM complexes with a dissociated autoinhibitory domain of the Ca\(^{2+}\) pump (19). This population thus corresponds to active PMCA. However, a less mobile population emerges at a reduced Ca\(^{2+}\) concentration in the absence of ATP. The distribution of orientation mobilities shown in **FIGURE 4** is not consistent with the usual two-state model of PMCA function (9), which dictates that when CaM is bound to PMCA, the autoinhibitory domain is necessarily dissociated from the nucleotide-binding region of the pump. The presence of a low-mobility population at reduced Ca\(^{2+}\) levels suggests the existence of an intermediate state in the activation of the pump (19) in which CaM is bound (or partially bound by one of its two globular domains) but the autoinhibitory domain is still associated with the active site. This model is illustrated at the top of **FIGURE 4**. The presence of this subpopulation would have been difficult to detect by conventional ensemble methods, but it was revealed by mapping out the distribution of orientation mobilities of individual complexes.

More recent single-molecule experiments probed the effect of oxidative damage to CaM on the distribution of orientation mobilities of PMCA-CaM complexes (see **FIGURE 4**) (17). Single-molecule results show that oxidative damage of CaM produces a significantly higher population of PMCA-CaM complexes with low orientation mobility even in the presence of ATP, suggesting that oxidative modification of CaM results in a loss of ability of CaM to induce dissociation of the autoinhibitory domain (17).

**Caveats**

Although single-molecule fluorescence spectroscopy can provide the experimenter with an extraordinarily sensitive probe of biomolecular systems, it is accompanied by potential pitfalls that must be avoided. First, single-molecule fluorescence detection in most cases requires attachment of extrinsic fluorophores. Thus activity assays and other controls are required to show that fluorescence labeling does not perturb biomolecular function. A related concern is the nature of the interaction of the fluorescent dye with the biomolecule to which it is attached. This issue arises in FRET experiments, for example, because the FRET efficiency depends not only on the distance between donor and acceptor fluorophores but also on their relative orientation (via the infamous $k^2$ factor (25)). It is usual-
without releasing the autoinhibitory domain. CaM bound and the autoinhibitory domain dissociated (right). The top interaction of the autoinhibitory domain of PMCA with the catalytic site (low-modulation depth distribution) or associated autoinhibitory domain (low-mobility, high-modulation depth distribution). The proposed model for C population (middle) shows the proposed intermediate state in which CaM is bound to PMCA without releasing the autoinhibitory domain.

**Conclusions**

Emerging single-molecule spectroscopic techniques have demonstrated the ability to map distributions of biomolecules, uncovering properties that are hidden in the ensemble averaging of conventional methods. Single-molecule methods also offer the potential for ultrasensitive assays with enhanced throughput. spFRET applied to the CaM-dependent activation of PMCA revealed conformational substates of CaM, demonstrating the presence of multiple conformations of CaM in solution rather than a single, well-defined structure and suggesting that multiple conformations endow CaM with the ability to interact with a diverse range of target enzymes. Single-molecule polarization modulation spectroscopy applied to the CaM-dependent activation of PMCA revealed a previously undetected intermediate state, which we proposed corresponds to PMCA with a nondissociated autoinhibitory domain. This state may play a regulatory role in fine-tuning the activity of the enzyme. Application of single-molecule methods thus allows new questions to be answered about the conformational states, dynamics, and function of biological systems, and one can expect exciting new developments as single-molecule methods are applied to single cells.

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Present address for M. W. Allen: Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803.

**References**


