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Reconstruction of calmodulin single-molecule FRET states, dye interactions, and CaMKII peptide binding by MultiNest and classic maximum entropy

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ABSTRACT

We analyzed single molecule FRET burst measurements using Bayesian nested sampling. The MultiNest algorithm produces accurate FRET efficiency distributions from single-molecule data. FRET efficiency distributions recovered by MultiNest and classic maximum entropy are compared for simulated data and for calmodulin labeled at residues 44 and 117. MultiNest compares favorably with maximum entropy analysis for simulated data, judged by the Bayesian evidence. FRET efficiency distributions recovered for calmodulin labeled with two different FRET dye pairs depended on the dye pair and changed upon Ca^{2+} binding. We also looked at the FRET efficiency distributions of calmodulin bound to the calcium/calmodulin dependent protein kinase II (CaMKII) binding domain. For both dye pairs, the FRET efficiency distribution collapsed to a single peak in the case of calmodulin bound to the CaMKII peptide. These measurements strongly suggest that consideration of dye–protein interactions is crucial in forming an accurate picture of protein conformations from FRET data.

1. Introduction

Single-molecule measurements are an increasingly standard technique in the biophysicist's toolbox. In particular, single-molecule Förster resonance energy transfer (sm-FRET) has been applied to investigate the conformational structure and dynamics of proteins, DNA, and RNA [1–4]. The key to obtaining useful sm-FRET data is a well behaved dye pair. A fluorescent energy donor dye and an energy acceptor must be incorporated into the molecule(s) of interest. These dyes, while attached to biomolecules, should reorient relative to each other on a time scale faster than the FRET efficiency transfer [5]. The dyes should not interact with the parent molecule and, if they become spatially close to one another, they should not interact with each other. The nature of molecular interactions makes these criteria hard to achieve. If dye interactions are well characterized, useful results may nevertheless be derived from the data. Ultimately, the reliability of FRET results should be checked using multiple dye pairs.

Single-molecule burst measurements are a powerful sm-FRET method [6–8]. In these measurements, a dilute solution is placed on a confocal microscope. As single molecules diffuse through a laser focus, fluorescence photons are emitted. The time of arrival of each photon is detected by photodiodes. When a plot of photon arrivals is made, bursts of photons are seen which give the measurement its name; see Fig. 1. Burst measurements allow tens of thousands of single molecules to be probed in a short time. Burst measurements can be made in biologically realistic conditions, with appropriate pH and ionic strength. The measurements do not require immobilization of the molecules by, for example, directly tethering them to a surface or encapsulating them in vesicles or gels. This facilitates investigation of the conformations, kinetics and binding constants with minimal change to the native environment of the biomolecule.

Significant improvements have been made since the first sm-FRET burst measurements. Alternating and pulsed lasers have been used to investigate dye bleaching, fluorescence lifetimes, quantum yield inhomogeneities, anisotropies, and dye-biomolecule stoichiometry [9–13]. Bayesian approaches [14–22] and complementary maximum entropy methods [23–25] have been implemented for single-molecule data analysis.

We studied FRET efficiency distributions obtained from dye-labeled calmodulin, using sm-FRET bursts. Calmodulin is a small 16.7 kDa protein found in all eukaryotic cells. It is an integral part of the cell's calcium signaling pathway. Calmodulin binds up to four Ca^{2+} ions, with high affinities. It activates and deactivates target proteins in accordance with its Ca^{2+} occupancy. Its structure consists of two domains that each contain two EF-hand motifs;

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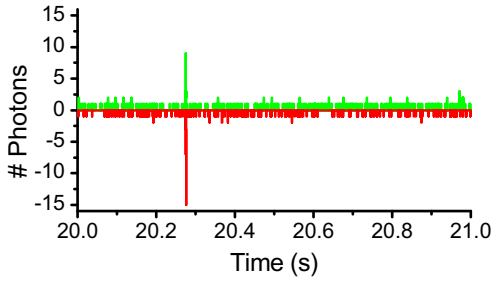

Fig. 1. Photons detected in two detection channels binned into 300 μs time bins. Green corresponds to photons detected in the channel monitoring donor dye emission. Red corresponds to photons detected in the channel monitoring acceptor dye emission. The number of photons detected in the acceptor channel was multiplied by −1 for clarity in the plot. The origin of the time axis is the start of the experiment. Data are for CaM-44-117-AF488-AF594 in high Ca^{2+} buffer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

see Fig. 2 [26,27]. Calmodulin exists in both compact and elongated conformations [27–31].

One of the enzymes that binds calmodulin is calcium/calmodulin dependent protein kinase II (CaMKII) [32]. CaMKII is activated by Ca^{2+}–calmodulin, and phosphorylates its substrates so as to regulate a variety of cellular functions [33]. Isoforms of CaMKII have been found in the heart, brain, lung, pancreas, liver, and skeletal muscle (see [34] and references therein). The structure of calmodulin that is bound to a peptide from the binding domain of CaMKII has been determined [31] (see Fig. 2(B)). The FRET distributions stemming from calmodulin bound to the CaMKII binding domain were investigated in this work.

We attached a fluorescent dye to each domain of calmodulin; see Fig. 2. Upon excitation of the donor dye, FRET can take place between the dyes. Single-molecule burst FRET measurements were made on freely diffusing molecules. The resulting FRET data were then analyzed via a Bayesian nested sampling approach. This analysis has not been performed before and its results were compared to those obtained using the classic maximum entropy method (cMEM) for sm-FRET bursts [22]. Both methods were applied to analyze FRET distributions for calmodulin under conditions of high and low Ca^{2+} and also in the presence and absence of the CaMKII binding-domain peptide. We compared the two methods of analysis and, below, we discuss the role of dye–protein interactions in the FRET distributions.

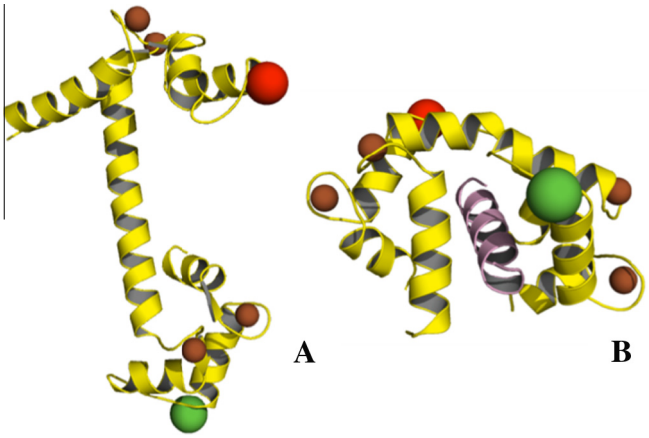

Fig. 2. (A) Ca^{2+}-calmodulin, PDB: 1CLL (B) Ca^{2+}-calmodulin bound to CaMKII peptide fragment (pink), PDB 1CDM. (Both panels) Ca^{2+} ions are brown. Dye labeling site on residue 44 is shown as a red sphere. The dye labeling site on residue 117 is shown as a green sphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Methods

2.1. Sample preparation

Calmodulin was mutated at sites 44 and 117, changing the native threonine residues to cysteines (CaM-44-117). The protein was expressed in *Escherichia coli*. The cells were induced with 0.4 mM IPTG at an O.D. of 0.7. Then they were pelleted by centrifuging and re-suspended in TRIS or MOPS buffer. The cells were lysed by adding 150 μg/ml lysozyme, with incubation at 37 °C, and sonicated three times in an ice bath for 30 s. Cell debris was separated by centrifuging at 19,500 rpm for 30 min at 4 °C. $CaCl_2$ was added to the sample to a final concentration of 5 mM. CaM-44-117 was purified on an Äkta FPLC with a 25 ml bed of Phenylsepharose 6 Fast Flow (high-sub) column (XK 16/20 GE Healthcare). Before sample loading, the column was equilibrated with 50 mM TRIS, 1 mM $CaCl_2$, pH 7.5. The column was then washed with two column volumes of 50 mM TRIS, 1 mM $CaCl_2$, pH 7.5, followed by three column volumes of 50 mM TRIS, 1 mM $CaCl_2$, 500 mMNaCl, pH 7.5, and three more column volumes of 50 mM TRIS, 1 mM $CaCl_2$, pH 7.5. CaM-44-117 was eluted from the column with 10 mM TRIS, 10 mM EDTA, pH 7.5. The elution peaks containing CaM-44-117 were further purified on a Superdex size exclusion column (GE Healthcare) in 10 mM HEPES, 0.1 M KCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4. The protein was aliquoted into 2.1 mg portions and stored at −80 °C.

The cysteines were labeled with fluorescent FRET dye pairs by maleimide reaction chemistry, as described previously [35]. The FRET donor dye was Alexa Fluor 488 C5 maleimide (AF488) (Molecular Probes). The acceptor was either Alexa Flour 594 C5 maleimide (AF594) or Texas Red C2 maleimide (TR) (Molecular Probes). Free dye was separated from dye-labeled protein by means of size exclusion chromatography (G-25 Sephadex fine, GE Healthcare Life Sciences). CaM-44-117 labeled with two donor or two acceptor dyes was separated from the FRET species containing one donor and one acceptor dye by C18 reverse phase HPLC (Jupiter 3u C18 300A 250 × 4.6 mm column, Phenomenex). The HPLC solvent was exchanged with either 10 mM HEPES or 30 mM HEPES, 0.1 M KCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4, and was stored at −80 °C. The resulting samples were CaM-44-117 labeled with AF488 and AF594 (CaM-44-117-AF488-AF594), or CaM-44-117 labeled with AF488 and TR (CaM-44-117-AF488-TR).

Calmodulin samples were diluted to 15–40 pM for sm-FRET burst measurements. The experiments were performed in 30 mM HEPES, 0.1 M KCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4, which will be called high Ca^{2+} buffer, and 30 mM HEPES, 0.1 M KCl, 1.0 mM $MgCl_2$, 3.0 mM EGTA, pH 7.4 (low Ca^{2+} buffer). For measurements of dye-labeled calmodulin in the presence of CaMKII peptide, the peptide was added to the solution up to a final concentration of 1.1 μM. Calcium/calmodulin dependent protein kinase II peptide fragment 290–309 was purchased from Sigma Aldrich. The sample was dissolved in high Ca^{2+} buffer. The peptide was stored at −20 °C prior to use.

2.2. Single molecule microscope

Burst measurements were made with an inverted Nikon TE 2000 fluorescence microscope system, as described previously [36]. Donor dye excitation was performed with a 488 nm argon ion laser (2201-20SL, JDS Uniphase, San Jose, CA) of power 25 μW measured ahead of the microscope. The laser beam was transformed to circular polarization by a consecutive linear polarizer and quarter-wave plate, and was directed into the microscope by a 500DCXR dichroic beam splitter (Chroma). To spectrally separate the donor and acceptor emission bands, we used emission