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Single-molecule FRET states, conformational interchange, and conformational selection by dye labels in calmodulin

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Abstract

We investigate the roles of measurement time scale and the nature of the fluorophores in the FRET states measured for calmodulin, a calcium signaling protein known to undergo pronounced conformational changes. The measured FRET distributions depend markedly on the measurement time scale (nanosecond or microsecond). Comparison of FRET distributions measured by donor fluorescence decay with FRET distributions recovered from single-molecule burst measurements binned over time scales of 90 μ s to 1 ms reveals conformational averaging over the intervening time regimes. We find further that, particularly in the presence of saturating Ca^{2+} , the nature of the measured single-molecule FRET distribution depends markedly on the identity of the FRET pair. The results suggest interchange between conformational states on time scales of hundreds of microseconds or less. Interaction with a fluorophore such as the dye Texas Red alters both the nature of the measured FRET distributions and the dynamics of conformational interchange. The results further suggest that the fluorophore may not be merely a benign reporter of protein conformations in FRET studies, but may in fact alter the conformational landscape.

Graphical abstract

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Supporting Information: Analysis of power dependence of FRET distributions, effect of linker length, possibility of donor-acceptor quenching interactions; alternating laser excitation (ALEX) measurements; TCSPC fits and residuals; analysis of the FRET orientational factor. This information is available free of charge via the Internet at <http://pubs.acs.org>.

Introduction

Calmodulin (CaM) is a small (16.7 kD) Ca^{2+} -signaling protein with two globular domains connected by a central linker.¹ Each domain binds two Ca^{2+} ions at Ca^{2+} concentrations from 0.1 to 10 μM (reviewed in ^{1, 2}). Upon Ca^{2+} binding, conformational changes expose hydrophobic regions of the protein^{3, 7} that are then available to bind a wide array of target proteins.⁸⁻¹¹ X-ray diffraction has revealed a remarkably broad range of structures for CaM, from extended^{12, 14} to compact¹⁵ crystal structures of Ca^{2+} -CaM, while solution structures disclose flexible, bent conformations.^{3, 4, 7} The apparent conformational flexibility of CaM^{3, 4} appears to be important for target recognition and binding, but the range of conformations accessible by CaM in its holo (with four Ca^{2+} ions bound), intermediate (Ca^{2+} binding sites partially occupied), and apo (Ca^{2+} -free) states is still unclear.

This laboratory has previously reported single-molecule FRET investigations of the FRET states of fluorescently labeled CaM in solution.^{16, 18} The results revealed multiple FRET states for holo-CaM labeled at sites 34 and 110 with the fluorophores Alexa Fluor 488 and Texas Red. Recent work in our laboratory showed that the nature of FRET states detected in holo-CaM labeled at sites 44 and 117 depend on the identity of the FRET dye pair.¹⁸ In the present paper we report an extensive study of the dependence of FRET states and dynamics of CaM on the identity of the dye pair. We adopted two different methods that recover FRET distributions averaged over different time regimes. Fluorescence decay measurements were used to characterize the FRET distribution over the time scale of the donor fluorescence decay. In each case multiple FRET states were resolved in fluorescence decays. Single-molecule burst measurements^{19, 20} coupled with probability distribution analysis (PDA)^{19, 20} were employed to resolve the underlying FRET distribution over the time scale of 100 μs to 1 ms. Visible circular dichroism spectra of labeled CaM indicate interaction between the fluorophore and the protein.

A model is proposed with multiple conformational states of the protein that interchange on time scales of nanoseconds to microseconds. Interactions of certain fluorophores, notably Texas Red, with the protein may stabilize a compact conformation by conformational selection. Remodeling of the conformational landscape alters both the average FRET efficiency and the time scale of conformational interchange. The mechanism of landscape remodeling by binding of Texas Red in the target binding pocket of CaM may be similar to the mechanism by which CaM binds molecules such as the antipsychotic drug trifluoperazine.

Methods

Alexa Fluor 488 C5 maleimide (AF488), Alexa Fluor 594 C5 maleimide (AF594), and Texas Red C2 maleimide (TRC2) were purchased from Molecular Probes. Atto 594 maleimide (Atto594) was purchased from Atto-Tec GmbH. Texas Red C5 maleimide (TRC5) was purchased from Biotium. The high- Ca^{2+} buffer contained 30 mM HEPES, 0.1 M KCl, 1.0 mM MgCl_2 , 0.1 mM CaCl_2 , at pH 7.4. The low- Ca^{2+} buffer contained 30 mM HEPES, 0.1 M KCl, 1.0 mM MgCl_2 , and either 3 mM EGTA or 1 mM EGTA at pH 7.4.

CaM with mutations T34C, T110C was labeled with maleimide derivatives of donor and acceptor dyes as described previously.^{18, 21} CaM doubly labeled at these sites with AF488 as donor fluorophore and AF594, Atto594, or TRC2 as acceptor fluorophore are here denoted CaM-AF488-AF594, CaM-AF488-Atto594, and CaM-AF488-TRC2, respectively. Single molecule FRET burst measurements were performed as described previously²² on a Nikon TE2000 inverted fluorescence microscope. The sample was excited with a 488 nm Ar ion laser (2201-20SL, JDS Uniphase). The laser excitation power was 25 μ W measured just before the microscope. A 500dcxr microscope dichroic was used to isolate fluorophore emission from scattered light. A 565dclp dichroic was used to separate donor emission from acceptor emission. An ET535/50m and an HQ650/100m-2p band pass filter were placed in front of avalanche photodiode (APD) detectors in the donor and acceptor emission channels, respectively. All filters were purchased from Chroma.

Data were binned into time bins ranging from 90 μ s to 1000 μ s. Single-molecule bursts were selected by identifying bins with counts above a set threshold (typically 5 for 90 μ s bins, 10 for 300 μ s bins, and 10 for 1000 μ s bins). FRET burst data were subjected to probability distribution analysis (PDA)^{19, 20} implemented with classic maximum entropy (cMEM) analysis for recovery of underlying FRET distributions, as described previously.^{18, 22} In many applications of cMEM it has been found advantageous to include smoothing or “blurring” of neighboring elements of the underlying distribution to account for correlation between adjacent cells.²³ Models with various layers of blurring were judged against a model without blurring, and the optimum level of blurring selected based on the Bayesian evidence, as described previously.²²

Fluorescence decays were measured by time-correlated single-photon counting (TCSPC) with a cavity dumped Mira 900 Ti:Sapphire laser (Coherent) and T-format detection as described previously.²⁴ Excitation pulses were coupled into a photonic crystal fiber (NL-PM-750, Thor Labs) to generate a supercontinuum, from which a 10-nm band was selected at 480 nm with a band-pass filter. Fluorescence decays at 520 nm with a bandwidth of 8 nm and polarization at the magic angle were detected by a microchannel plate photomultiplier (Hamamatsu R3809U) with total count rates maintained at less than 1% of the excitation rate. The instrument response function (IRF) had a full width at half maximum of < 50 ps. Fluorescence decays were fit with iterative reconvolution with the IRF by either cMEM as implemented in the program Pulse5 (Maximum Entropy Data Consultants, Ltd) or by nonlinear least-squares fitting to a sum of exponentials. MEM assigns amplitudes to a large number of decay components (here 200) while minimizing the number of features in the amplitude distribution.

Circular dichroism spectra were measured on a JASCO J-810 spectropolarimeter. Background signals were recorded for high or low-Ca²⁺ buffers. Sample CD spectra were recorded at concentrations of 3 to 32 μ M.

Results

Circular dichroism measurements

Figure 1 displays CD spectra in the 550 nm to 650 nm region for CaM-AF488-AF594 and CaM-AF488-TRC2 in high and low- Ca^{2+} buffers. Molar ellipticity was recorded over the visible absorption band of the fluorophores. Negligible CD response was detected for the Texas Red chromophore, sulforhodamine 101 (not shown), consistent with the expected absence of optical activity. Stronger molar ellipticities were detected for both CaM-AF594 and CaM-TRC2. Both the sign and the magnitude of the molar absorptivity depended on both the Ca^{2+} state and the labeling site (C34 or C110). The CD signal in the fluorophore's absorption band for CaM-T34C-AF594 is weak in low- Ca^{2+} buffer but strongly negative in high- Ca^{2+} buffer (Figure 1A). In contrast, the molar ellipticity for CaM-T110C-AF594 is weak in both high and low- Ca^{2+} buffers (Figure 1C). For CaM-T34C-TRC2 a strong positive CD signal is present (Figure 1B) with a Ca^{2+} -dependent band shape, while a strong negative signal was detected for the 110C labeling site (CaM-T110C-TRC2), with greater magnitude at low Ca^{2+} than at high Ca^{2+} (Figure 1D).

Single-molecule burst measurements

Figure 2 illustrates data analysis by cMEM for CaM-AF488-AF594 at high Ca^{2+} with 300 μs bins. Raw data for CaM-AF488-AF594 are graphed as a contour plot of counts in channels 1 and 2. cMEM analysis of the raw data generates the probability $P(E,F)$ of species with apparent FRET efficiency E_{app} and total fluorescence (from both fluorophores) F , as described previously.¹⁸ The apparent FRET efficiency (uncorrected for quantum efficiencies of the donor and acceptor or for detection efficiencies) is given by

$$E_{app} = \frac{F_2}{F} \quad (1)$$

where F is the sum $F_1 + F_2$ of fluorescence counts in channels one and two.

Figure 3 shows cMEM FRET distributions for CaM-34,110 labeled with three different FRET dye pairs under high and low Ca^{2+} conditions. The donor dye in each case was AF488, and the acceptor dye was AF594, Atto 594, or TRC2. FRET distributions were recovered with time bins of 90 μs , 300 μs , and 1000 μs . In all distributions, a population is recovered with an E_{app} of ~ 0.05 as a result of the presence of molecules lacking a functional acceptor. The identity of this population was confirmed by alternating laser excitation (ALEX) measurements (see Supporting Information). This population exhibits only donor fluorescence and probably consists mainly of CaM labeled only with AF488 (at one or both labeling sites) that was not completely removed by HPLC chromatography. Species with a photobleached acceptor fluorophore could also contribute, but this population is expected to be minor due to the low rate of photobleaching (see Supporting Information). The value of E_{app} is greater than zero for these species because of background counts in the acceptor channel and because some donor emission is detected as “crosstalk” in the acceptor channel.

In the low- Ca^{2+} buffer the FRET distributions with AF594 and Atto594 as acceptor fluorophore are nearly identical with an apparent FRET efficiency $E_{app} \approx 0.62$. The low- Ca^{2+} distribution for CaM-AF488-TR has somewhat higher FRET values with a peak around $E_{app} \approx 0.72$ and a small population with $E_{app} > 0.9$. The differences in the Förster radii R_0 for the AF488-TR and AF488-AF594 dye pairs (54 Å and 56 Å, respectively) are too small and in the wrong direction to explain the difference in E_{app} measured for CaM-AF488-TR and CaM-AF488-AF594 or CaM-AF488-Atto594. Hence the differences between the FRET distributions for CaM-AF488-TR compared to CaM-AF488-AF594 and CaM-AF488-Atto594 reveal real differences in the FRET states measured with AF594 or Atto594 vs TRC2 as acceptor fluorophore under low- Ca^{2+} conditions.

In the high- Ca^{2+} buffer, the distributions for CaM-AF488-AF594 and CaM-AF488-Atto594 are again quite similar. The FRET distribution for CaM-AF488-AF594 at high Ca^{2+} shows a population centered at $E_{app} \approx 0.55$. Similarly, for CaM-AF488-Atto594 at high Ca^{2+} , a FRET state was observed with $E_{app} \approx 0.56$ with a small population with $E_{app} > 0.9$. In contrast, for CaM-AF488-TR, FRET states were detected with high amplitudes for $E_{app} > 0.8$, with structures suggesting multiple underlying states. The results for CaM-AF488-TRC2 are consistent with previous measurements from our laboratory.^{16, 17, 25} Again, the differences between the FRET states detected with AF594 or Atto 594 and the FRET states detected with TR cannot be explained by the small differences in the Förster radii.

The FRET distributions shown in Figure 3 were generated with time bins of 90 μs (black line), 300 μs (red), and 1000 μs (blue) for each dye pair. For CaM-AF488-AF594 the recovered distributions are essentially independent of bin width at both low Ca^{2+} and high Ca^{2+} . For CaM-AF488-Atto594 the distributions at low Ca^{2+} are independent of bin width. At high Ca^{2+} a small population with $E_{app} > 0.9$ was found that changes shape with longer bin widths due to averaging. For CaM-AF488-TR at low Ca^{2+} there is little dependence on binning time, although the contribution from the minor population with $E_{app} > 0.9$ may diminish with increasing bin width. For CaM-AF488-TR at high Ca^{2+} the dependence of the FRET distribution on bin width is pronounced. Peaks at $E_{app} \sim 0.8$ and $E_{app} > 0.97$ observable in the FRET distribution for a bin width of 90 μs peaks appear to merge progressively with bin widths of 300 μs and 1000 μs .

FRET states measured in fluorescence decays

FRET efficiencies can also be determined from fluorescence decays of the donor, because energy transfer to the acceptor fluorophore decreases the donor fluorescence lifetime. If it is assumed that other photophysical phenomena are independent of FRET state, then the distribution of fluorescence lifetimes reflects the distribution of FRET efficiencies that exist on the time scale of the fluorescence decay. Thus, time-resolved fluorescence decays provide a window onto the FRET states present on the nanosecond time scale, complementary to single-molecule FRET distributions averaged over tens to hundreds of microseconds. In view of the poor stability inherent in multiexponential fits, fluorescence decays were fit by cMEM, which selects the distribution with the least information from among many combinations of functions that can fit the data with similar χ^2 values. Because the number of

decay components need not be specified, MEM is beneficial for fitting fluorescence decays in situations where the number of subpopulations is not known in advance.²⁶

Lifetime distributions recovered by cMEM are shown in Figure 4. (Fitting parameters are tabulated in the Supporting Information. Fitting by nonlinear least squares regression to three or four exponentials yielded similar lifetimes and relative amplitudes.) For each dye pair tested under both low and high Ca^{2+} conditions, cMEM analysis indicates the presence of at least three and in some cases four FRET populations. (The width of features in the distributions recovered by cMEM could reflect inhomogeneity in these populations but is also a result of measurement noise and fitting uncertainty.) Fits to a single Gaussian distribution were poor, indicating that discrete FRET states more accurately describe the conformations than a broad distribution of conformations. Indeed, if the actual fluorescence decays were best described by single broad lifetime distributions, then one would expect the MEM analysis to recover such distributions because they would have a higher information entropy than the distributions with three or four peaks that were actually recovered.

For CaM-AF488-AF594, the average fluorescence decay time is shorter at low Ca^{2+} compared to high Ca^{2+} , as observed by direct comparison of the raw fluorescence decays (see Supporting Information) and confirmed by cMEM fits (Table S2). This result is consistent with decreased average FRET at high Ca^{2+} as observed in the FRET distributions derived from burst measurements (Fig. 3). The cMEM fluorescence lifetime distribution for CaM-AF488-AF594 consist of three peaks for both low and high Ca^{2+} , suggesting at least three distinct FRET populations. The time constants for AF488 in the CaM-AF488-AF594 construct had nearly the same values at low and high Ca^{2+} , but with altered amplitudes. For example, the amplitude of a component with lifetime around 0.18 ns decreased upon addition of Ca^{2+} while the amplitude of the longest components increased upon addition of Ca^{2+} . The net effect of these changes is an increase in the average fluorescence lifetime at high Ca^{2+} . The cMEM lifetime distributions for CaM-AF488-Atto594 similarly reveal the presence of three or four FRET populations under both low- Ca^{2+} and high- Ca^{2+} conditions. As for CaM-AF488-AF594 the average fluorescence lifetime is shorter in low Ca^{2+} than in high Ca^{2+} , consistent with the higher FRET efficiency detected in burst measurements for CaM-AF488-Atto594 in low Ca^{2+} compared to high Ca^{2+} .

For CaM-AF488-TR there are again four peaks in the cMEM lifetime distribution, both at low Ca^{2+} and at high Ca^{2+} . (The high- Ca^{2+} distribution is replotted here from ref. 27.) Lifetime populations of 0.1 to 0.2 ns and 0.4 to 0.7 ns correlate well with populations with FRET efficiencies of ~ 0.8 and > 0.9 detected in burst measurements for CaM-AF488-TR. Although the lifetimes identified are similar at low and high Ca^{2+} levels, the populations shift so that the shorter lifetime components gain population at high Ca^{2+} . This result is consistent with the shift in the FRET efficiency distribution towards higher FRET at high Ca^{2+} detected in burst measurements of CaM-AF488-TR but in contrast with the dependence of the lifetime distribution on Ca^{2+} for CaM-AF488-AF594 and CaM-AF488-Atto594.

Discussion

We measured FRET distributions for CaM with three sets of donor-acceptor fluorophores and two complementary experimental methods – single-molecule bursts and fluorescence decays – to obtain FRET distributions on two different time scales. In single-molecule burst measurements the FRET efficiency is averaged over the binning time, in this case 90 μ s, 300 μ s, or 1000 μ s, while in fluorescence decay measurements, the averaging time is the fluorescence lifetime of the donor fluorophore. Marked differences were found in the FRET distributions obtained from burst measurements compared to fluorescence decay measurements, demonstrating dynamics occurring on the intervening time scale. Analysis of fluorescence decays revealed the presence of either three or four peaks in the lifetime distribution for CaM for each of the three acceptor fluorophores in both high and low Ca^{2+} buffers (Figure 4), suggesting the presence of at least three distinct FRET populations on the timescale of the fluorescence lifetime. In contrast, burst measurements yielded FRET distributions with one to three populations, depending on both Ca^{2+} occupancy (*holo*-CaM or *apo*-CaM) and the identity of the acceptor fluorophore.

For CaM-AF488-AF594 burst measurements indicate a single FRET distribution for both apo-CaM (Figure 3A) and holo-CaM (Figure 3B). Comparison with the fluorescence lifetime distributions (Figures 3A and 3B) suggests that FRET populations for these constructs interchange over a time regime between the fluorescence lifetime and the binning time, i.e. on a time scale longer than ca. 10 ns but shorter than 100 μ s. CaM-AF488-Atto594 behaves similarly, although a small high-FRET population may be present in the burst FRET distribution for holo-CaM (Figure 2D).

For CaM-AF488-TRC2 somewhat different behavior was observed. At high Ca^{2+} , high-FRET populations appear in burst measurements, suggesting that multiple conformational states persist out to the microsecond time scale for CaM-AF488-TR. Increasing the binning times for analysis of single-molecule bursts for CaM-AF488-TR at high Ca^{2+} from 90 μ s to 300 μ s and then to 1000 μ s results in increasing conformational averaging of a population with $E_{app} > 0.95$ and a population with $E_{app} \approx 0.8$, indicating conformational interchange on the time scale of hundreds of microseconds for this construct. These two populations appear to correspond to the two shorter lifetime conformations found in fluorescence lifetime distributions (Figure 4 F). At low Ca^{2+} there is predominantly a single distribution in the burst FRET distribution (Figure 3E) possibly with a small high-FRET population. Dynamics of CaM-AF488-TRC2 were also detected in previous fluorescence correlation spectroscopy studies with the CaM-AF488-TRC2 construct,²⁸ where FRET fluctuations with a time constant of $\sim 100 \mu$ s were found at high but not at low Ca^{2+} , consistent with the results presented here.

The picture that emerges from these results is one where donor-acceptor labeled CaM explores multiple FRET states. The time scale of conformational interchange lies between the fluorescence lifetime and the 90- μ s binning time for CaM-AF488-AF594, CaM-AF488-Atto594, and CaM-AF488-TR at low Ca^{2+} , but for CaM-AF488-TR at high Ca^{2+} interchange occurs on the hundreds of μ s time scale. The amplitude of the nanosecond FRET states depends on dye pair and on Ca^{2+} in a way that is consistent with the microsecond

results. For example, holo-CaM-AF488-AF594 has lifetime distributions more strongly weighted to longer lifetimes (low FRET) compared to CaM-AF488-TR at high Ca^{2+} , and CaM AF488-TR has lifetime distributions more strongly weighted toward short lifetimes (high FRET) at high Ca^{2+} compared to low Ca^{2+} .

Nature of the FRET states

We address here the question whether the observed FRET states correspond to different conformational states of the protein or merely to different configurations of the donor and acceptor fluorophores. The latter would undermine the reliability of FRET for detecting protein conformational states and their interchange. Multiple FRET states could conceivably result from any of several factors, including (1) multiple orientational states of the fluorophores having different values of the κ^2 factor²⁹; (2) multiple locations of the fluorophores within the range of the linkers connecting them to the protein, resulting in different distances between donor and acceptor; (3) multiple conformations of the protein, having different FRET efficiencies, either because of different distances between donor and acceptor or because of different orientational factors. We consider each of these factors in turn.

Orientational factors of donor and acceptor fluorophores—First, could multiple FRET states result from multiple orientational states of the fluorophores? As reported previously, fluorescence anisotropy decays for several fluorophores linked to CaM at sites 34 or 110^{30, 31} can be fit to two exponential decays, one characteristic of segmental reorientation of the fluorophore relative to the protein and the second characteristic of reorientation of the protein itself. Two models can account for biexponential anisotropy decay of fluorophores linked to proteins. In the first model, fast, segmental reorientation of the fluorophore is restricted to a region of free mobility, typically modeled as a cone with the relative amplitude of the fast decay component characterizing the cone angle. Accessible-volume simulations of the distribution of dye positions^{32, 33} are another variant of this model. If such models can be applied to both donor and acceptor fluorophores with fast orientational averaging over the accessible volume, then all donor-acceptor pairs would be characterized by the same κ^2 orientational factor for a given protein conformation. Hence, in this model, orientational effects would not explain the presence of multiple FRET states. (There would still be the question of the correct value of κ^2 , which would need to be addressed to calculate a donor-acceptor distance from the measured FRET efficiency.)

In the second model there are two populations of fluorophore, one where the fluorophore reorients freely about its tether and a second with the fluorophore stuck to the protein. In this model the relative amplitudes of the fast and slow decay components give the relative populations of free and stuck fluorophores, and there could be four combinations with donor and acceptor each stuck or free. If these four populations have different orientational factors, then up to four FRET states might conceivably be detected for a single protein conformation. For each of these populations it is possible to calculate the most probable κ^2 value and a confidence interval.³⁴ Based on such considerations (see Supporting Information), we can conclude that it is unlikely that the multiple FRET states that we observed by analysis of fluorescence decays can be accounted for by multiple κ^2 populations (stuck and free) with

the same protein conformation. Although, multiple κ^2 populations could give rise to two of the observed peaks if in one of these populations both dyes stick to the protein in orientations leading to a value of κ^2 close to the upper limit of its confidence range, it is unlikely that multiple orientational populations can account for the full range of observed FRET states. (Details are provided in the Supporting Information.) Hence, we can with high probability exclude insufficient orientational averaging as an explanation for the observation of multiple FRET states, although molecular modeling or dynamics simulations may be necessary to reach a definitive conclusion.

Second, we consider whether the length of the linker could explain multiple FRET peaks, that is whether a population with the fluorophore reorienting freely and another with one or both fluorophores stuck to the protein could differ sufficiently in distance between donor and acceptor to explain multiple FRET populations. For five-carbon linkers, the distance might be expected to vary by $\pm \sim 5$ Å. The possible range of FRET efficiencies for fluorophores stuck at the limits of the linker are then readily calculated. Calculation shows again that a fluorophore stuck to the protein at the end of its tether can account for at most for one additional FRET state (see Supporting Information). We note in addition that the FRET efficiency distributions for Texas Red are identical with C2 and C5 linkers (see Supporting Information), indicating that linker length cannot explain multiple FRET states for Texas Red and suggesting that linker length is not a likely explanation for AF594 or Atto594 either.

We conclude that multiple κ^2 populations or multiple populations of the fluorophore relative to its tether might account for two of the FRET populations detected in fluorescence decays, but are highly unlikely to account for more. This analysis therefore strongly suggests that the observed FRET states represent a set of distinct conformational states of the protein.

Effect of dye pair

The results presented here show that the measured FRET distributions depend on the identity of the acceptor fluorophore. A more limited comparison of two dye pairs for a different pair of labeling sites of CaM (44 and 117) led to a similar conclusion.¹⁸ As argued above, it is likely that the observed FRET distributions indicate the presence of multiple conformations of the protein itself. It follows that the nature of the dye pair can affect protein conformations. Since the donor dye was in all cases AF488, differences in measured distributions can be attributed to the identity of the FRET acceptor. In particular, the presence of TR as acceptor appears to stabilize higher FRET conformations. It is possible that dyes such as TR can bind to the protein in its *holo* state, perhaps in a manner analogous to the interaction of certain drugs with CaM,^{35,37} to induce a compact conformation of CaM and thus favor high-FRET conformations of the protein.

The CD results provide further evidence for interaction of the fluorophores with the protein. Induced circular dichroism was observed for both CaM-AF594 (particularly CaM-T34C-AF594 at high Ca^{2+}) and for CaM-TR. The magnitude and the sign of the induced CD were dependent on labeling site (34 or 110), Ca^{2+} level, and fluorophore identity (AF594 or TRC2). Hsu and Woody considered several sources of induced circular dichroism for the heme group in heme proteins³⁸ and concluded that the dominant contribution comes from

coupling with π - π^* transitions in nearby (<12 Å) aromatic amino acids. It is likely that the mechanism for the induced CD signal observed in labeled CaM is similar, consistent with local interaction between the fluorophore and the protein.

The properties of the fluorophores used are summarized in Table 1. The net electric charges on the acceptor dyes differ. AF594 carries a net charge of -2 (at neutral pH), Atto594 possesses a net charge of -1 , whereas Texas Red has a net neutral charge. The dye pairs we investigated can be grouped into two classes based on the FRET distributions generated for CaM under low and high Ca^{2+} conditions. (1) For dye pairs AF488-AF594 and AF488-Atto594, the average FRET efficiency decreased slightly in high Ca^{2+} conditions compared to low Ca^{2+} . High FRET states (FRET efficiency > 0.9) were either not observed at all in burst measurements or had a very low population. (2) For AF488-TRC2, the average apparent FRET efficiency increased in high Ca^{2+} conditions compared to low Ca^{2+} conditions. Previous reports from our laboratory of single-molecule FRET states in CaM were based on the FRET pair AF488-TRC2.^{16, 17, 25, 28}

Dynamics or “dye-namics”?

According to our results, the time scale of conformational interchange depends on the identity of the acceptor fluorophore. Hence, the nature of the dye pair affects not only the *distribution* of FRET states, but also the *dynamics* of their interchange. For CaM-AF488-TRC2 at high Ca^{2+} , high-FRET features generated with different bin widths merge as the bin time is increased on the time scale of hundreds of microseconds, a signature of interchange between FRET states on the time scale of hundreds of microseconds. In contrast, the FRET distributions for CaM-AF488-AF594 and CaM-AF488-Atto594, and for CaM-AF488-TRC2 at low Ca^{2+} depend little on binning time, indicating conformational interchange on a faster time scale. It may be illuminating that the acceptor dyes that are more water soluble, AF594 and Atto594, report only a single FRET population on the time scale of hundreds of microseconds in both high and low Ca^{2+} , whereas the acceptor that is less water soluble, TRC2, reports multiple FRET states at high Ca^{2+} . Interactions between the hydrophobic dye TR and CaM at high Ca^{2+} may be stronger than interactions of AF594 or Atto594 with CaM, resulting in stabilization of states with high FRET efficiency (Figure 5). Such interactions are consistent with the fact that upon Ca^{2+} binding CaM exposes hydrophobic regions rich in methionine residues, and these may present sites for interaction with TR, perhaps in a manner analogous to the compact conformation of CaM bound to small molecules such as the antipsychotic drug trifluoperazine³⁵ with CaM.

Conformational selection

The results we present show the presence of multiple conformational states of CaM on the nanosecond time scale for each of the dye pairs tested. Multiple conformational states thus appear to be intrinsic for CaM, but the FRET distribution depends on both the identity of the dye pair and on the measurement time scale. These results suggest that interactions of dyes with the protein can “remodel” the CaM conformational landscape by stabilizing or destabilizing one or more of these conformations, as illustrated in Figure 5. Subtle changes in the potential landscape can have a significant effect on populations, since these depend exponentially on the potential energy.

The high-FRET state reports a presumed compact conformation of CaM. Such a conformation can form both without¹⁵ and with⁸ binding of a target domain or drug molecules.³⁵ Hence, one possible interpretation of our results is that the compact state for CaM-AF488-AF594 and CaM-AF488-Atto594 forms *without* binding of a fluorophore to the target binding domain of CaM, but TRC2 in CaM-AF488-TRC2 favors formation of a compact state with Texas Red bound to the target binding domain. In this case, CaM-AF488-AF594 and CaM-AF488-Atto594 more accurately report the intrinsic conformations and dynamics of CaM.

An interesting question is whether interaction of a substrate with a protein occurs by conformational selection or whether interaction with the protein induces new protein conformations (as in the induced fit model of protein-substrate interactions). Our analysis of fluorescence decays with three different acceptor fluorophores suggests that similar FRET states are present with each dye pair (Figure 4). The presence of a fluorophore such as Texas Red that interacts more strongly with the protein seems alters the amplitudes of the FRET populations present without extensively shifting the FRET efficiencies. Hence, in this case, interactions with the fluorophore appear to affect the conformational landscape by selection of pre-existing conformations rather than by generating new conformational states (Figure 5).

Conclusions

Single-pair FRET is widely used to probe the conformations and dynamics of proteins.^{39,42} Inherent in such experiments are the assumptions that each FRET state corresponds to a distinct conformational state of the *protein*, and that the dye pair serves as a benign reporter of protein conformations without itself perturbing the protein conformation. Both assumptions are implicit in work reported previously from our lab^{16, 17, 25, 43, 44} as well as from other researchers. In the work reported here, we tested these assumptions by comparing FRET measurements with multiple FRET dye pairs positioned at the same labeling points of the protein.

Using CaM as a test case, we find that a fluorophore such as TR may interact with the protein by stabilizing or destabilizing protein conformational states, thus remodeling the energy landscape of the protein. Conformational remodeling by binding of TR in the target binding pocket of CaM changes both the population of FRET states and their interchange rates. For CaM with AF488 as donor, distinct conformational states were detected on the nanosecond timescale. On the microsecond time scale, conformational averaging reduced the range of measured FRET states for acceptor fluorophores AF594 and Atto 594 and for TR for CaM in its *apo* state, while for TR with CaM in its *holo* state, multiple conformations remained on the timescale of hundreds of microseconds. Thus, it now appears that the FRET states observed in CaM-AF488-TRC2 at high Ca^{2+} are stabilized by the acceptor dye, TR. These results show that the fluorophore can affect the conformational states and interchange dynamics of the protein to which it is linked. These effects appear to occur largely by conformational selection, without extensive changes in the nature of the underlying states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Circular dichroism spectra of acceptor fluorophores. A) CaM-T34C-AF594; B) CaM-T34C-TRC2; C) CaM-T110C-AF594; D) CaM-T110C-TRC2. Red curves show CD spectra in low- Ca^{2+} buffer and black curves in high- Ca^{2+} buffer. The curves in panels C and D have been smoothed by an FFT smoothing algorithm.

Figure 2.

cMEM analysis of burst FRET for CaM-AF488-AF594 at high Ca^{2+} with 300 μs bins. Panel A is a histogram of raw data counts in channels 1 and 2 in 300 μs bins with a threshold of 10 counts. Panel B shows the two-dimensional probability distribution as a function of apparent FRET efficiency and total fluorescence counts as recovered by cMEM analysis. Panel C shows the FRET probability distribution obtained by marginalizing the two-dimensional distribution over fluorescence counts. S1 and S2 are the counts measured in channel 1 (green light) and channel 2 (red light), respectively. E_{app} is the apparent FRET efficiency (uncorrected for cross talk or quantum yields) and F is the total fluorescence counts (donor plus acceptor).

Figure 3.
cMEM FRET distributions for CaM-AF488-AF594 (A and B), CaM-AF488-Atto594 (C and D) and CaM-AF488-TRC2 (E and F). The top row shows results at low Ca^{2+} and the bottom row at high Ca^{2+} . The black lines show analysis with 90 μs bins, the red lines with 300 μs bins, and the blue lines with 1000 μs bins.

Figure 4. cMEM fluorescence lifetime distributions for CaM-AF488-AF594 (A and B), CaM-AF488-Atto594 (C and D) and CaM-AF488-TRC2 (E and F). The top row shows results at low Ca^{2+} and the bottom row at high Ca^{2+} .

Figure 5.

Model for landscape remodeling by dye interactions. In this picture, a medium-FRET state is most stable for CaM-AF488-AF594 (left) while CaM-AF488-TR stabilizes a compact state with high FRET (right) with TR bound in the binding pocket of CaM. Interchange of FRET states of CaM-AF488-AF594 is slower than the fluorescence lifetime but faster than the shortest binning time (90 μs). For CaM-AF488-TR, the high FRET state exchanges on a time scale slow enough that averaging can be detected on the time scale of hundreds of microseconds.

Table 1**Properties of FRET dyes**

Dye	Absorption λ_{max}	Emission λ_{max}	Net Charge	Quantum Yield	$R_0^{(a)}$
Alexa Fluor 488 C5 maleimide	499 nm	520 nm	-2	0.92	
Alexa Fluor 594	592 nm	619 nm	-2	0.66	56 Å
Atto 594 maleimide	601 nm	627 nm	-1	0.85	54 Å
Texas Red maleimide	595 nm	611 nm	0	0.9	54 Å ^(b)

^aThe calculated Förster radii with AF488 as donor for an orientational factor $\kappa^2 = 0.67$.