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# Fluorescence quenching studies of structure and dynamics in calmodulin–eNOS complexes

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ABSTRACT

**Activation of endothelial nitric oxide synthase (eNOS) by calmodulin (CaM) facilitates formation of a sequence of conformational states that is not well understood. Fluorescence decays of fluorescently labeled CaM bound to eNOS reveal four distinct conformational states and single-molecule fluorescence trajectories show multiple fluorescence states with transitions between states occurring on time scales of milliseconds to seconds. A model is proposed relating fluorescence quenching states to enzyme conformations. Specifically, we propose that the most highly quenched state corresponds to CaM docked to an oxygenase domain of the enzyme. In single-molecule trajectories, this state occurs with time lags consistent with the oxygenase activity of the enzyme.**


## 1. Introduction

The nitric oxide synthases are functional homodimers that, upon activation by CaM, catalyze the release of NO gas [1]. An N-terminal oxygenase domain comprises the enzyme reaction center with its associated heme and tetrahydrobiopterin cofactors. The C-terminal reductase domain contains NADPH/FAD and FMN binding modules. The oxygenase and reductase domains are separated by a CaM-binding sequence immediately adjacent to the FMN module. Transfer of electrons from NADPH through FAD and FMN to the oxygenase heme requires a series of conformations that sequentially position each electron donor and acceptor pair in close proximity. (See references [2,3] for reviews.)

An understanding of the mechanism of NO generation by CaM–NOS therefore requires knowledge of CaM–eNOS conformations and their interchange dynamics. The position of the CaM binding domain adjacent to the FMN module suggests the possibility to detect changes in its position by monitoring changes in fluorescence of a bound, labeled CaM. Indeed, previous work showed quenching of fluorescence-labeled CaM bound to eNOS, due principally to Förster resonance energy transfer (FRET) from the excited dyes to the reaction-center hemes [4].

We have carried out fluorescence lifetime and single-molecule fluorescence measurements of eNOS complexed with fluorescently labeled CaM. The results demonstrate distinct fluorescence quenching states, which we hypothesize correspond to conformational states of the enzyme. Single-molecule trajectories reveal transitions on time scales from tens of milliseconds to several seconds, consistent with the solution kinetic data for the enzyme. A highly quenched state with long average time duration in single-molecule trajectories strongly supports the presence of a conformation with CaM docked to the oxygenase domain of eNOS, as suggested by recent EM and hydrogen–deuterium exchange investigations [5–7]. Single-molecule trajectories allow us to relate

*Abbreviations:* AF488, Alexa fluor 488; AF594, Alexa fluor 594; BSA, bovine serum albumin; CaM, calmodulin; eNOS, endothelial nitric oxide synthase; EGTA, ethylene glycol tetraacetic acid; EM, electron microscopy; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FRET, Förster resonance energy transfer; iNOS, inducible nitric oxide synthase; NADPH, nicotinamide adenine dinucleotide phosphate; nNOS, neuronal nitric oxide synthase; TCSPC, time-correlated single photon counting; TRIS, tris(hydroxymethyl)aminomethane

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conformational states (in particular the docked, highly quenched state) with conformational interchange kinetics. The results suggest that the formation and dissociation rates of the docked state are rate limiting for the activity of the enzyme.

## 2. Materials and methods

A previously characterized 6-His tagged S1179D mutant of bovine eNOS was purified as described previously [8,9]. It was used because it was the subject of a cryo-electron microscopy (cryo-EM) reconstruction of CaM–eNOS complexes [5], and because the phosphomimetic substitution for S1179 enhances the probability of detecting active conformations of the enzyme [8]. T34C-CaM was generated and purified as previously reported [10,11] and labeled at Cys-34 with maleimide derivatives of Alexa Fluor 488 (AF488) or Alexa Fluor 594 (AF594) (Molecular Probes) as described previously [10] to generate labeled CaM (denoted CaM-AF488 and CaM-AF594).

Fluorescence lifetime experiments were performed by time-correlated-single-photon-counting (TCSPC) as described previously [12]. The output of a cavity-dumped Ti:Sapphire laser (Coherent Mira) emitting 150 fs pulses at 800 nm with a 2.28 MHz repetition rate was focused into a photonic crystal fiber (Thorlab NL-PM-750) to generate white light, which was directed through 10 nm band-pass filters for excitation of the AF488 or AF594 labels. Fluorescence emission was monitored at 517 nm for AF488 and 617 nm for AF594 with a bandwidth of 7.6 nm and polarization at the magic angle. Excitation levels were low enough that emission counts were detected for less than 1% of excitation pulses.

Fluorescence decays were recorded after adding varying amounts of high-$Ca^{2+}$ buffer (50 mM TRIS at pH 7.4, 100 mM KCl, 2 mM EGTA, 10 mM $CaCl_2$, and 0.1 mg/mL BSA) to a solution of 200 nM CaM and 800 nM eNOS in nominally $Ca^{2+}$-free buffer (50 mM TRIS at pH 7.4, 100 mM KCl, 2 mM EGTA, and 0.1 mg/mL BSA). The concentrations of free $Ca^{2+}$ in the reaction mixtures were determined by comparison with identically prepared $Ca^{2+}$ buffer solutions containing the $Ca^{2+}$-sensitive dye quin-2 (Molecular Probes). The $Ca^{2+}$ dependence of quin-2 fluorescence was calibrated against a $Ca^{2+}$ calibration buffers (Molecular Probes).

Fluorescence decays were analyzed with in-house software by iterative non-linear least-squares fitting to exponentials convoluted with the instrument function and by maximum-entropy analysis. The maximum entropy algorithm (Pulse5, Maximum Entropy Data Consultants, Ltd.) fits fluorescence decays by assigning amplitudes to 200 logarithmically spaced decay components while minimizing amplitude variations [13]. This method minimizes the number of features in the amplitude distribution and is thus appropriate for cases where the number of decay components is not known in advance.

Complexes between fluorescence labeled CaM and eNOS were prepared by incubating 800 nM S1179D-eNOS with 200 nM fluorescently labeled CaM in a buffer containing 50 mM TRIS at pH 7.4, 100 mM KCl, 1 mM $CaCl_2$, and 0.1 mg/mL BSA. After incubation for 30 min this mixture was diluted to produce a final CaM concentration of <1 nM. CaM–eNOS complexes were immobilized on $Cu^{2+}$ coated coverslips (MicroSurfaces, Inc.) via 6-His tags on eNOS. Trajectories were collected by positioning complexes over the focal region of an inverted fluorescence microscope (Nikon TE300). Fluorescence was excited with an Ar ion laser at 488 nm (JDS Uniphase) or a He–Ne laser at 594 nm (Melles Griot 25-LYP). Excitation powers were 500 nW or less. Trajectories were obtained with 20-ms time steps.

Trajectories were selected for analysis and truncated immediately preceding photo-bleaching events. Trajectories that appeared to originate from aggregates, displayed no transitions, or appeared to derive from mobile molecules were rejected for analysis. Time correlation functions, $C(t)$, were calculated for each individual trajectory, $I(t)$, according to the relation:

$$C(t) = \frac{\langle \Delta I(t) \Delta I(0) \rangle}{\langle \Delta I^2 \rangle} \quad (1)$$

where $\Delta I = I - \langle I \rangle$ and $\langle \cdots \rangle$ denotes the time average. The $t = 0$ values of the correlation functions $C(0)$ were excluded from fitting because they include a contribution arising from uncorrelated noise. The correlation functions were then rescaled so that the fitting functions decayed from a starting value of $C(0) = 1$.

## 3. Results

### 3.1. Fluorescence lifetimes for labeled CaM–eNOS complexes

The maximum-entropy lifetime distributions derived for free CaM-AF488 and for labeled CaM–eNOS complexes in high-$Ca^{2+}$ buffer are shown in Fig. 1. CaM-AF488 by itself exhibits a single fluorescence lifetime of 4.0–4.1 ns. Regardless of fluorescence label, four lifetime peaks were derived from the data for CaM–eNOS complexes by maximum-entropy fitting, suggesting the presence of four different populations of CaM in the presence of eNOS. The slight differences between the lifetime distributions for CaM-AF488 and CaM-AF594 can be attributed to different Förster radii for energy transfer to heme or flavin (see Supporting Information), as well as inherent uncertainties in recovered distributions.

The maximum-entropy lifetime distributions for CaM-AF488 in the presence of S1179D-eNOS are presented in Fig. 2 for free $Ca^{2+}$ concentrations ranging from nominally zero to a value of 6 μM, which is known to be sufficient to saturate a CaM–eNOS complex [8,9]. Chelation of $Ca^{2+}$ by addition of 2 mM EGTA resulted in a single lifetime of 4 ns, consistent with the value obtained for CaM-AF488 in the absence of the synthase. Increases in the free $Ca^{2+}$ concentration caused the appearance of additional lifetime components with amplitudes that increased with increasing $Ca^{2+}$ concentration.

The normalized changes in amplitude of each lifetime component are plotted versus the free $Ca^{2+}$ concentration in Fig. 3. The fractional changes in amplitude $F$ were fit to a Hill equation for $Ca^{2+}$ binding [9]:

$$F([Ca^{2+}]_{free}) = \frac{[Ca^{2+}]^n_{free}}{[Ca^{2+}]^n_{free} + K^n} \quad (2)$$

The values obtained for the dissociation constant $K$ and the Hill coefficient $n$ (Table 1) are similar to those derived by Tran et al. from data for CaM binding and enzyme activation at different free $Ca^{2+}$ concentrations [8]. Similar values were obtained from fits to the amplitudes from non-linear least-squares fits of the fluorescence decays to a sum of four exponential decays.

### 3.2. Single-molecule trajectories

Fig. 4 shows representative single-molecule fluorescence trajectories obtained for CaM–S1179D-eNOS complexes. Each trajectory shown represents a different set of 200 or more trajectories collected under the conditions indicated. The single-molecule trajectories reveal distinct levels of fluorescence that interconvert on multiple time-scales. The corresponding histograms of fluorescence counts in each trajectory, also shown in Fig. 4, suggest two, three, or, occasionally, four distinct levels of fluorescence.