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Calmodulin activates neuronal nitric oxide synthase by enabling transitions between conformational states

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ABSTRACT

We recently showed that inducible nitric oxide synthase conformational intermediates can be resolved via FMN fluorescence lifetimes. Here we show that neuronal NOS activation by calmodulin removes constraints favoring a closed 'input state', increasing occupation of other states and facilitating conformational transitions. The 90 ps FMN input state lifetime distinguishes it from ~4 ns 'open' states in which FMN does not interact strongly with other groups, or 0.9 ns output states in which FMN interacts with ferriheme. Enablement of the conformational cycle is an important paradigm for control in nNOS and related enzymes, and may extend to other control modalities.

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1. Introduction

Nitric oxide production by nitric oxide synthases consumes three NADPH derived electrons per mol of NO in monooxygenase reactions mediated by a heme containing oxygenase domain. The endothelial and neuronal isoforms (eNOS and nNOS) are important signal generators [1–3]. Eukaryotic NOS includes NADPH, FAD and FMN binding domains [4–9].

Calcium–calmodulin (Ca^{2+}/CaM) controls NO synthesis by eNOS and nNOS by activating electron transfer [5]. Ca^{2+} controlled isoforms contain an autoinhibitory element (AI), present as an FMN binding domain insertion, and C-terminal extension that inhibits electron transfer [10–12]. Inducible NOS (iNOS) lacks an AI and binds CaM at all physiological Ca^{2+} concentrations [13].

CaM activation of NOS [14–16] increases NADPH-cytochrome c reductase activity and FMN fluorescence [9,12,15,16]. In our tethered shuttle mechanism the FMN binding domain dissociates from an 'input state', reorienting to reduce the oxygenase domain [7,17–19]. Activation by CaM is the result of increased rates of conformational transitions, which should in general alter the conformational distribution.

We recently showed that conformational states of iNOS and iNOS constructs can be characterized by FMN fluorescence lifetimes [20]. Because of the lack of an AI, the shorter C terminal extension, and differences in the CaM binding site, iNOS activity is insensitive to Ca^{+2}, and the effects of CaM binding on the conformational manifold cannot be studied. Here we show that CaM activation changes the distribution of conformations characterized by the environment of FMN.

2. Materials and methods

Purification of nNOS was previously described [21,22]. Flavin and heme were assessed spectrophotometrically; activity was measured using oxyhemoglobin [22].

Fluorescence decays were recorded using a PicoQuant Fluorotime 100 TCSPC fluorescence lifetime spectrometer [23], or a PTI Picomaster TCSPC spectrometer [20]. FMN was excited at ~440 nm using a pulsed laser diode with 20 MHz repetition; excitation at 378 nm and 473 nm produced similar results. Emission was detected through a polarizer oriented at 54.7° to excitation polarization. Long-pass filters from Chroma Technology [Rockingham, VT] were used to eliminate scattered light.

Abbreviations: NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; CaM, calmodulin; oxyFMN, two-domain NOS construct (FMN and oxygenase domains); FMN, Flavin mono nucleotide ; FAD, Flavin adenine di-nucleotide ; PBS-T, phosphate buffer saline-Tween-20 (see Section 2)

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Fluorescence decays can be represented as sums of exponentials:

$$I(t) = \sum_{ii=1}^{n} \alpha_i \exp(-t/\tau_i) \quad (1)$$

where the α_i represent amplitudes and the τ_i are decay times. The fractional contribution of the ith component to the steady-state intensity is:

$$f_i = \frac{\alpha_i \tau_i}{\sum -j\alpha_j \tau_j} \quad (2)$$

α_i and τ_i were determined as previously described [20].

3. Results and discussion

FMN fluorescence is dominated by a broad emission peak near 525 nm, evoked by pumping isoalloxazine absorbance bands. We recently showed that the weak fluorescence of iNOS is due to FMN quenching by FAD and ferriheme; the interactions are characteristic of obligatory conformational intermediates in the catalytic cycle [20]. FMN is reduced by FAD in a state in which the isoalloxazines are in van der Waals contact, shown in crystal structures of P450 reductase and nNOS reductase complex [24,25]. The isoalloxazine dimer of this state has a very short 90 ps lifetime [20]. A heterogeneous distribution of 'open' states with an average FMN lifetime of ~4.3 ns is the majority state in the two domain oxyFMN construct and a minority state in holoenzyme.A state with an FMN fluorescence lifetime of 0.9 ns, attributed to quenching by interaction with ferriheme, was observed using oxyFMN. Previous studies with P450 and nNOS reported flavin fluorescence lifetimes e.g., [26,27], but insights provided by structural and mechanistic studies allow more productive interpretations.

Because CaM increases cytochrome c reduction, we expect increased interconversion of the input state and nearby states in which FMN is accessible to cytochrome c. Because NO synthesisis undetectable without CaM, we expect interconversion of the input and output states to be negligible in the absence of CaM, and fast enough to support NO synthesis with CaM bound. Rate increases usually change conformational distributions because equilibrium constants are ratios of rate constants. Although we can't directly examine the kinetics of changes in fluorescence decays, it is clear from many previous kinetics papers that calmodulin binding increases the rate of cytochrome c reduction and NO synthesis. This is consistent with an increased rate of release of the FMN binding domain, and not with a decreased rate of input state formation.

Fig. 1 shows fluorescence decays of nNOS holoenzyme excited at 478 nm. FMN fluorescence emission at 525 nm decays as a multiple exponential (filled diamonds). Reasonable fits to were obtained with component lifetimes of 90 ps, 0.9 ns, and 4 ns, with the 90 ps state accounting for 70% of the amplitude. Better fits of the long tail were obtained by replacing the 4 ns component with distributions of states, suggesting that component is heterogeneous. These results are similar to those observed in iNOS [20], except that ~1 ns components could only be observed in iNOS in truncated constructs. Calmodulin addition (square symbols) significantly decreased the 90 ps population, and increased the 0.9 ns and 4 ns states. The effect of endogenous Ca^{2+} in the added CaM (squares) is increased by excess Ca^{2+}(triangles).

The CaM induced shift in the conformational poise of nNOS is summarized in Table 1. The small output state population in nNOS in the absence of CaM is probably real, but CaM activation produces a significant population at the expense of the input state.

Although CaM binding and CaM activation are rapidly reversible with EDTA, fluorescence changes in response to EDTA are slow and incomplete. EDTA addition produced a decay trace after five minutes differing by only a few percent from the CaM activated trace. Steady state FMN fluorescence decays after EDTA addition with half time of 30 min.

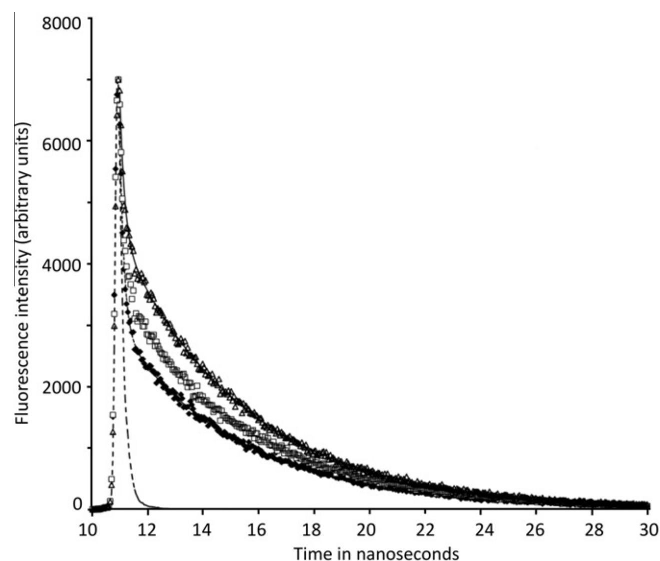

Fig. 1. Effect of calmodulin activation on fluorescence decays of nNOS holoenzyme. FMN was excited with a pulse from a 478 nm laser and the fluorescence decay at 525 nm was observed at 25C. ♦1 μM nNOS in 50 mM MOPS buffer, pH 7.4, 50 mMNaCl with 10% glycerol.2 μM EDTA was added to remove trace calcium. □ after addition of 4 μM calmodulin Δafter the addition of 4 μM calmodulin and 20 μM Ca^{2+}.

The reason for this apparent discrepancy is that CaM primarily affects the rate of interconversion of conformations. An nNOS molecule that releases CaM cannot rapidly relax to other sections of the conformational manifold.

The DG810 mutant of nNOS has a shortened loop along the edge of the FMN binding domain that binds isoalloxazine, resulting in reversal of FMN one electron couples and low NO synthase activity [19]s. CaM binding does not increase DG810 cytochrome c reductase activity or steady state FMN fluorescence. The mutant was originally described as 'already in an activated conformation'.

FMN fluorescence lifetimes of DG810 are shown in Table 1; some fits include a small (~5%) contribution from a 90 ps component. Clearly, DG810 has conformational states similar to those of wild type enzyme. Rather than having a single 'activated' conformation, the mutant enzyme is better described as less constrained than wild type enzyme. Whereas wild type nNOS favors the input state, the mutant is much more capable of assuming open conformations in the absence of CaM, likely because of weakened interactions between the FMN and FAD domain interface [19]. The increased steady-state fluorescence of the mutant in the absence of CaM is due to the higher fluorescence yield of longer-lived states.The Forster equation describing exciton transfer between transition dipoles in s^{-1} is

$$k_{DA} = 8.78 \times 10^{23} J(\lambda)\kappa^2 Q_f t_{D0}^{-1} n^{-4} R^{-6}$$

where J(λ) is the spectral overlap integral, $\kappa^2 = (\mathbf{a}\cdot\mathbf{d}\text{-}3(\mathbf{a}\cdot\mathbf{r})(\mathbf{d}\cdot\mathbf{r}))^2$ is the dipole orientation factor, Q_f is the isolated quantum yield of the donor, $t_{D0}$is the isolated lifetime of the acceptor, n is the refractive index of the surroundings, and R is the effective donor–acceptor distance in Ångstrom units. Because FMN emission overlaps ferriheme α and β bands, FRET readily accounts for quenching of FMN fluorescence in the output state, given the heme-FMN distance of 13–15 Ångstroms suggested by electron paramagnetic resonance [17,20,28].

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