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Neuronal Nitric-oxide Synthase Interaction with Calmodulin-Troponin C Chimeras

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Calmodulin (CaM) binding activates neuronal nitric-oxide synthase (nNOS) catalytic functions and also up-regulates electron transfer into its flavin and heme centers. Here, we utilized seven tight binding CaM-troponin C chimeras, which variably activate nNOS NO synthesis to examine the relationship between CaM domain structure, activation of catalytic functions, and control of internal electron transfer at two points within nNOS. Chimeras that were singly substituted with troponin C domains 4, 3, 2, or 1 were increasingly unable to activate NO synthesis, but all caused some activation of cytochrome *c* reduction compared with CaM-free nNOS. The magnitude by which each chimera activated NO synthesis was approximately proportional to the rate of heme iron reduction supported by each chimera, which varied from 0% to ~80% compared with native CaM and remained coupled to NO synthesis in all cases. In contrast, chimera activation of cytochrome *c* reduction was not always associated with accelerated reduction of nNOS flavins, and certain chimeras activated cytochrome *c* reduction without triggering heme iron reduction. We conclude: 1) CaM effects on electron transfer at two points within nNOS can be functionally separated. 2) CaM controls NO synthesis by governing heme iron reduction, but enhances reductase activity by two mechanisms, only one of which is associated with an increased rate of flavin reduction.

Nitric-oxide synthases (NOSs)[1] are bi-domain enzymes comprised of a N-terminal oxygenase domain that binds iron protoporphyrin IX (heme), tetrahydrobiopterin (H_4biopterin), and L-arginine and a C-terminal reductase domain that binds FAD, FMN, NADPH, and the Ca^{2+}-binding protein calmodulin (CaM) (1, 2). All NOS generate nitric oxide (NO) and L-citrulline from L-arginine in a stepwise reaction that requires O_2 and NADPH as co-substrates (reviewed in Ref. 3). During NO synthesis, NADPH-derived electrons pass through the flavins and across domains to the heme iron (4), which enables it to bind O_2 (5) and catalyze the oxidation of L-arginine.

NOS isoforms that are expressed in neurons (nNOS) and endothelium (eNOS) bind CaM reversibly in response to elevated Ca^{2+} levels, and this enables them to participate in biological signal cascades by coupling production of a mediator (NO) to transient rises in intracellular Ca^{2+} (reviewed in Ref. 6). CaM may be unique among the Ca^{2+}-binding proteins in activating NO synthesis (7, 8), and the mechanism is of current interest. Our studies with nNOS show that CaM controls electron transfer at two points in the enzyme (Fig. 1, *upper panel*): it increases the rate at which NADPH-derived electrons transfer into the enzyme flavins (9) and also triggers electron transfer between the reduced flavins and the oxygenase domain heme iron (4). The CaM-dependent increase in flavin reduction is associated with an enhanced rate of electron transfer from nNOS to artificial electron acceptors such as cytochrome *c* or ferricyanide (9–11). Cytochrome *c* and ferricyanide become reduced via direct electron transfer from the nNOS reductase domain and do not require the presence of the nNOS oxygenase domain (10, 12). CaM activation at the second point (heme iron reduction) is associated with initiation of NO synthesis from L-arginine and an increase in NADPH and O_2 utilization (13, 14). Thus, our current model for CaM-nNOS interaction links acquisition of domain-specific catalytic functions to activation of electron transfer at two distinct points within nNOS (Fig. 1).

To further explore this model, we utilized chimeras of CaM and a homologous Ca^{2+} signaling protein, cardiac troponin C (TnC). CaM and TnC show striking structural similarities (Fig. 1, *lower panel*): they are 50% identical at the amino acid level, and both contain 4 EF-hand Ca^{2+}-binding domains, arranged as globular pairs separated by a long central helix (15). Despite this structural similarity, TnC can neither bind to nor activate nNOS (7). Because of the divergence between structure and function, CaM-TnC chimeras are informative probes of the CaM-nNOS interaction (7, 8). For example, several CaM-TnC chimeras bound avidly to nNOS, yet failed to activate NO synthesis by the enzyme, whereas others both bound and activated. This demonstrated that enzyme binding and stimulation of NO synthesis are distinct and separable. The initial studies further established that the domain 1-domain 3 "latch" region of CaM played a critical role in inducing NO production (8).

Here we use seven CaM-TnC chimeras to investigate 1) how CaM promotes electron transfer into the nNOS reductase domain, 2) how CaM promotes electron transfer between the reductase domain and the oxygenase domain heme iron, and 3) how CaM's promotion of electron transfer at each point correlates with its activation of two domain-specific catalytic func-

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[1] The abbreviations used are: NOS, nitric-oxide synthase; NO, nitric oxide; CaM, calmodulin; DTT, dithiothreitol; H_4biopterin, (6*R*,6*S*)-2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine; TnC, cardiac troponin C; BisTris, 2-[bis(2- hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol.

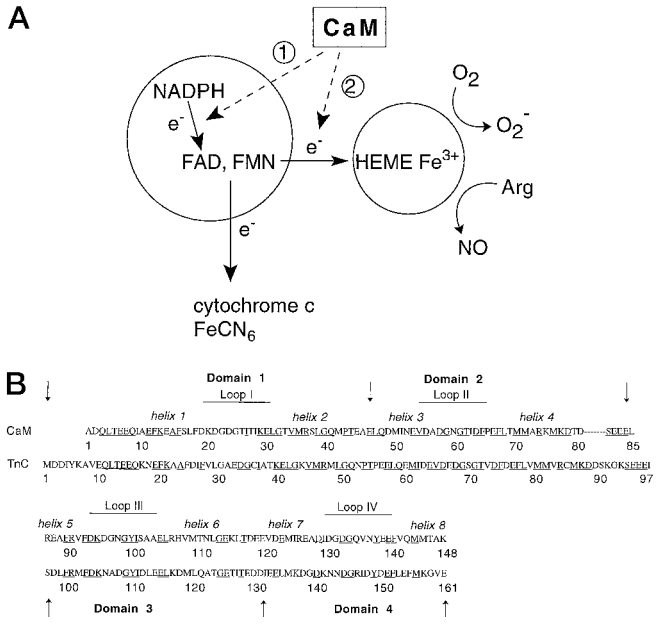

FIG. 1. **CaM control of electron transfer in nNOS and comparative structures of CaM and TnC.** *Upper panel*, the reductase and oxygenase domain of nNOS are drawn as *two circles* in the figure. CaM binding activates at two points in the electron transfer sequence: it increases the rate at which NADPH-derived electrons transfer into the flavins (1) and enables the flavins to pass electrons to the oxygenase domain heme iron (2). Activation at the first point is associated with an increase in reductase domain catalytic activities, such as cytochrome *c* or ferricyanide reduction. Activation at the second point is associated with an increased rate of NADPH oxidation and initiation of NO synthesis from L-arginine or superoxide production in the absence of substrate. Adapted from Ref. 9. *Lower panel*, alignment of CaM and cardiac TnC. Residue numbers and locations of the four EF-hand domains and subdomain loops and helices are as indicated. Common amino acids are *underlined*. *Arrows* indicate the splice points for constructing chimeras. Adapted from Ref. 7.

tions (cytochrome *c* reduction and NO synthesis). We find that CaM-TnC chimeras differentially activate the two nNOS catalytic activities, thus providing the first evidence for CaM mutations that produce abortive or partial activation of an enzyme's function. Also, while promotion of internal electron transfer in nNOS correlated well with a given chimera's ability to activate NO synthesis, it correlated poorly with activation of cytochrome *c* reduction, revealing that CaM can activate reductase domain catalysis by a mechanism other than stimulating electron transfer into the domain.

EXPERIMENTAL PROCEDURES

Reagents—Rat brain nNOS was expressed in a baculovirus-insect cell system and purified as described previously (16). The recombinant nNOS reductase domain (amino acids 724–1429) containing the CaM binding site was expressed in the yeast *Pichia pastoris* and purified as described previously (12). The CaM-TnC chimeras were expressed in *Escherichia coli* and purified as described previously (8). All other reagents and materials were obtained from Sigma or from sources reported previously (12, 14).

Catalytic Measurements—The initial rate of NO synthesis was quantitated at 37 °C using the spectrophotometric oxyhemoglobin assay. Reactions contained nNOS (0.5–2 μg) in 40 mM BisTris propane, pH 7.4, supplemented with 5–10 μM oxyhemoglobin, 0.3 mM DTT, 1 mM L-arginine, 0.1 mM NADPH, 4 μM each of FAD, FMN, and H_4biopterin, 0.83 mM Ca^{2+}, 2 μM CaM or CaM-TnC chimeras, 0.6 mM EDTA, 100 units/ml catalase, 10 units/ml superoxide dismutase, and 0.1 mg/ml bovine serum albumin, to give a final volume of 0.35 ml. In some cases, concentrations above 2 μM were used for certain CaM-TnC chimeras as noted in the text. The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored over time as an absorbance increase at 401 nm and quantitated using a difference extinction coefficient of 38 mM^{-1} cm^{-1} (17). The rate of NADPH oxidation was measured at 340 nm using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. Reactions were run at 37 °C in cuvettes or at 25 °C in microplates and supplemented identically as in the oxyhemoglobin assay except oxyhemoglobin was omitted. Assay volumes were 0.35 ml for cuvettes and 0.15 ml for microwell plates. The NADPH-dependent reduction of cytochrome *c* was measured at 550 nm using an extinction coefficient of 21 mM^{-1} cm^{-1}. Reactions were run in cuvettes or microplates using assay conditions as described for monitoring NADPH oxidation, except that the buffer omitted DTT and H_4biopterin and contained 0.2 mM NADPH, 0.1 mM cytochrome *c*, and 100-fold less nNOS. Reactions were started by adding NADPH.

Optical Spectroscopy and Stopped-flow Measurements—Anaerobic spectra were recorded using septum-sealed quartz cuvettes that could be attached through a ground glass joint to a vaccum and gas train. Enzyme samples, EDTA, and CaM or CaM-TnC chimeras were placed in the cuvette and made anaerobic by repeated cycles of evacuation and equilibration with catalyst-deoxygenated nitrogen. Buffer plus additives were evacuated and gassed with nitrogen in a separate vessel. Solutions were transferred using gas-tight syringes, and cuvettes were maintained under positive pressure during spectral measurements. The kinetics of flavin reduction and CO binding were analyzed as described previously (9, 14) using a stopped-flow apparatus from Hi-Tech Ltd. (model SF-51) equipped for anaerobic work. Measurements were carried out at 10 °C and initiated by rapid mixing a anaerobic solution of 100 μM NADPH (in some cases saturated with CO) with an anaerobic solution containing CaM-free, CaM-bound, or chimera-bound NOS (2 μM) in 40 mM BisTris propane buffer, pH 7.4, containing 10 μM H_4biopterin, 0.3 mM DTT, 2 mM L-arginine, 0.6 mM EDTA, 5 μM CaM or chimeras, and 1 mM Ca^{2+}. Flavin reduction was monitored at 485 nm and CO binding monitored at 444 nm. Signal-to-noise ratios were improved by averaging at least 10 individual experiments. The time course of absorbance change was fit to single or multiple exponential equations by use of a nonlinear least square method provided by the instrument manufacturer (9).

RESULTS AND DISCUSSION

The ability of each CaM-TnC chimera to activate two nNOS reactions that depend on electron transfer to the oxygenase domain heme iron (NO synthesis and coincident NADPH oxidation) or to enhance a reductase domain-specific reaction (cytochrome *c* reduction) is summarized in Fig. 2. Effects on cytochrome *c* reduction were investigated using both full-length nNOS and the recombinant nNOS reductase domain (amino acids 724–1429; Ref. 12) to determine if an attached oxygenase domain would affect response to each chimera. In all cases, the chimeras were present during assay at concentrations previously shown to saturate nNOS (8).

Of seven CaM-TnC chimeras tested, only those containing TnC domain 2, the central helix, or helix 7, activated nNOS NO synthesis to a level that approached native CaM. In contrast, chimeras substituted with TnC domains 4, 3, or 1 lost most or all of their ability to activate this reaction. The relative importance of the nonfunctional Ca^{2+} binding site that is present in domain 1 of CaM 1TnC was probed by utilizing a chimera whose domain 1 Ca^{2+} binding site had been restored (CaM 1TnC BM1). This chimera supported a small but detectable activation of NO synthesis. Thus, while Ca^{2+} binding in domain 1 appears to be essential, structural features not involved in Ca^{2+} binding are also required to fully activate NO synthesis. We conclude that nNOS displays a high degree of structural specificity toward CaM domains 1, 3, and possibly 4 regarding activation of NO synthesis.

Our NO synthesis values were initial rate measurements using the oxyhemoglobin NO capture assay. In general, they confirm results that were obtained using a citrulline formation end point assay (7), with the exception of the CaM 4TnC chimera, which here showed only 13% activation of NO synthesis as compared with 45% activation relative to CaM in the previous report (7). Although the basis for the difference is unclear, our current data imply that CaM domain 4 may be more important in activating NO synthesis than previously thought. Restricting the TnC domain 4 substitution to helix 7 increased the chimera's ability to activate NO synthesis such that it

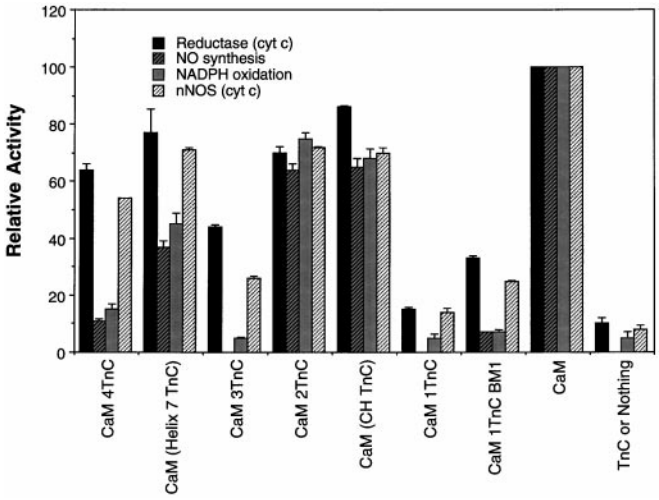

FIG. 2. **Comparative ability of CaM-TnC chimeras to activate three nNOS catalytic functions.** NO synthesis and NADPH oxidation rates were measured in the presence of L-arginine for full-length nNOS (*nNOS*), while cytochrome *c* reductase activities (*cyt c*) were measured for both full-length nNOS and the nNOS reductase domain (reductase). Activities obtained with CaM at 37 °C were all set to 100% and were 60 min^{-1} (*NO synthesis*), 100 min^{-1} (*NADPH oxidation*), 1600 min^{-1} (*nNOS cyt c reduction*), and 2900 min^{-1} (*reductase domain cyt c reduction*). The *bar* values represent the mean ± S.D. for four trials at three measurements per trial.

supported 35% activation relative to CaM. This suggests that elements in domain 4 besides helix 7 also function to activate NO synthesis (8).

As is evident in Fig. 2, increased rates of NADPH oxidation above the basal level were only observed for chimeras that also activated NO synthesis, and in all cases the magnitude of NADPH oxidation and NO synthesis were close to the expected stoichiometry of 1.5 NADPH oxidized per NO formed from L-arginine. Thus, the chimeras mimicked CaM in stimulating coupled NADPH consumption, consistent with their functioning through a common mechanism that involves activating electron transfer to the heme iron (4).

Regarding cytochrome *c* reduction, the full-length nNOS and the nNOS reductase domain responded similarly toward each chimera, as they do toward native CaM (12, 18). Thus, CaM and the chimeras both increase cytochrome *c* reduction by exclusively activating the nNOS reductase domain (10, 12). All chimeras increased nNOS cytochrome *c* reduction to some extent over the CaM-free value, although those containing TnC domains 2, 4, 3, or 1 stimulated progressively less reductase activity relative to CaM. Reconstituting Ca^{2+} binding in TnC domain 1 (*i.e.* CaM 1TnC BM1) enhanced cytochrome *c* reduction only by a factor of two, indicating that elements throughout domain 1 are important for CaM function.

In general, activation of cytochrome *c* reduction was far less sensitive to the single domain substitutions than was NO synthesis. For example, the chimeras containing TnC domains 4, 3, or 1 all activated different degrees of cytochrome *c* reduction but supported either minor or no activation of NO synthesis. The divergence was most striking for CaM 3TnC, which activated 30% maximal reductase activity despite activating no NO synthesis, and for CaM 4TnC, which activated 60% maximal reductase activity *versus* 13% maximal NO synthesis. The divergence was least striking for CaM 1TnC, which activated little cytochrome *c* reduction and no NO synthesis. Thus, CaM domains 3 and 4 are important for activating catalysis by the nNOS oxygenase domain (NO synthesis) but are relatively unimportant for activating reductase domain catalysis (cytochrome *c* reduction). In contrast, CaM domain 1 is critical for activating catalytic functions of both the nNOS oxygenase and reductase domains.

TABLE I

Observed rate constants for NADPH-dependent flavin and heme iron reduction by nNOS-chimera complexes

Experiments were carried out under anaerobic conditions at 10 °C as described under "Experimental Procedures." The nNOS (2 μM) in its CaM-free, CaM-bound, or chimera-bound states was mixed with excess NADPH. Flavin reduction was monitored at 485 nm, while heme iron reduction was measured by CO binding at 444 nm. The data were best fit to single exponential equations in all cases to generate pseudo first-order rate constants. The values are the average obtained with two nNOS preparations.

Protein	k_{obs} (s^{-1})	
	Flavin reduction	Heme iron reduction
nNOS	2.8	0
nNOS/CaM	33	3.6
nNOS/CaM 1TnC	3.6	0
nNOS/CaM 1TnC BM1	2.9	0.56
nNOS/CaM 2TnC	29	1.7
nNOS/CaM (CH TnC)	12.3	2.0
nNOS/CaM 3TnC	4.5	0
nNOS/CaM (helix 7 TnC)	12.8	2.7

Certain CaM-TnC chimeras could stimulate nNOS cytochrome *c* reduction without activating NO synthesis. However, the converse situation (*i.e.* activation of NO synthesis without increasing cytochrome *c* reductase activity) was not observed for any chimera. Together, this provides the first evidence that CaM can control these two catalytic functions independently and argues that CaM activation of reductase domain catalysis is an associated but singularly insufficient step in activating NO synthesis.

CaM is known to increase the rate at which NADPH electrons load into the nNOS flavins (9) and to trigger electron transfer between the flavins and heme iron (4). We had proposed earlier that CaM' s effect on electron transfer at these two points may be the mechanism by which it enhances cytochrome *c* reduction and initiates NO synthesis, respectively (9, 19). We therefore examined each chimera's ability to influence electron transfer into the flavin and heme centers of nNOS.

The psuedo first-order rate constants for flavin and heme iron reduction are listed in Table I, and stopped-flow traces for selected samples are illustrated in Fig. 3, *A* and *B*. NADPH-dependent heme iron reduction was not observed in CaM-free nNOS (data not shown), consistent with previous results (4, 14). In contrast, heme iron reduction in CaM-bound nNOS (as measured by CO binding)[2] was complete within 1 s, indicating rapid reduction occurs under these conditions.[3] The two chimeras that failed to activate NO synthesis (CaM 3TnC and CaM 1TnC) did not support detectable heme iron reduction in the stopped-flow experiment. We also incubated these two particular nNOS-chimera complexes with NADPH for 45 min under an anaerobic CO atmosphere but still observed no CO binding, although both bound CO immediately after addition of dithionite (not shown), which directly reduces the nNOS heme iron (4). This indicates the CaM 3TnC and CaM 1TnC chimeras are completely unable to support electron transfer between the flavin and heme centers of nNOS. Chimeras that supported a slow rate of NO synthesis (for example, 8% relative to control; CaM 1TNC BM1) or a near-normal rate (70%; CaM 2TnC) activated a slow or near-normal rate of heme iron reduction,

[2] CaM does not appear to significantly affect the nNOS heme iron environment or block ligand access (23, 24).

[3] The rate of CO binding observed for CaM-bound nNOS under these conditions matched the rate of CO binding to ferrous nNOS, indicating that the actual rate of heme reduction may be faster than CO binding in this case.

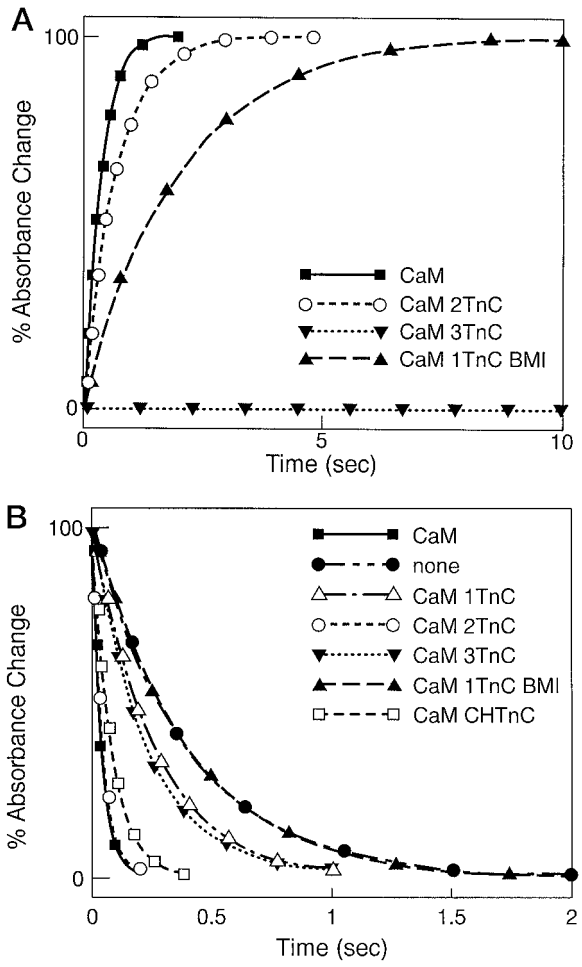

FIG. 3. **Comparative ability of CaM-TnC chimeras to activate heme iron reduction or flavin reduction in nNOS.** The indicated chimera-nNOS complex was mixed with excess NADPH under anaerobic conditions at 10 °C in a stopped-flow spectrophotometer. Heme iron reduction (*A*) was monitored by following formation of the ferrous nNOS-CO complex at 444 nm, while flavin reduction (*B*) was monitored directly at 485 nm. The curves have been smoothed and are representative of at least two separate trials each.

respectively. Regarding nNOS flavin reduction, three of the chimeras tested (CaM 1TnC, CaM 3TnC, and CaM 1TnC BM1) increased the rate of flavin reduction minimally (≤5%) compared with native CaM. On the other hand, CaM 2TnC increased the rate of flavin reduction to a level approaching native CaM, while others (CaM (CH TnC) and CaM (helix 7 TnC)) increased the rate to ~40% that of CaM.

The stopped-flow data suggest a good correlation exists between a chimera's ability to support heme iron reduction and activate NO synthesis. It is remarkable that several chimeras supported heme iron reduction rates that were slower than with CaM and that these slower rates were associated with diminished rates of NO synthesis. Indeed, for certain chimeras (especially CaM 1TnC BM1) the rate of heme iron reduction may be sufficiently slow to become rate-limiting for NO synthesis. This would be a fundamental departure from CaM-supported NO synthesis by nNOS, where heme iron reduction is not rate-limiting (20, 21). Because heme iron reduction is most dependent on structural elements in CaM domains 1 and 3, it is probably controlled by an interaction of nNOS with the "latch domain," which forms from domains 1 and 3 and is important for activating NO synthesis by nNOS (7).

In contrast to the good correlation observed for heme iron reduction and NO synthesis, a chimera's ability to increase the rate of flavin reduction did not always correlate well with stimulation of nNOS catalysis (cytochrome *c* reduction or NO synthesis). For example, the CaM 3TnC chimera caused less than a 5% increase in flavin reduction rate, but still elevated cytochrome *c* reduction 45% relative to CaM without activating heme iron reduction or NO synthesis. A similar situation held for Cam 1TnC BM1. Overall, the data show that activation of reductase domain-specific catalysis (cytochrome *c* reduction) in nNOS is possible without triggering electron transfer to the oxygenase domain, *and without increasing the rate of electron transfer into the nNOS flavins*. This latter conclusion was unexpected based on our model (9) and means that CaM must activate reductase domain catalysis by a mechanism distinct from its increasing the rate of flavin reduction. The mechanism does not likely involve increased affinity toward cytochrome *c*, because cytochrome *c* was present at ten times its K_m concentration in our experiments and its K_m is unaffected by CaM binding (9). Given that nNOS transfers electrons directly to cytochrome *c* (9, 10, 12), we speculate that the activation may involve a CaM-induced conformational change that enables electrons to transfer more easily between the electron-donating flavin and cytochrome *c*. This is consistent with fluorescence data that suggest CaM binding locates the flavins nearer to the surface of the reductase domain (12, 22). Whether this conformational change is involved in triggering electron transfer to the oxygenase domain heme iron is unclear. However, the fact that the CaM 3TnC chimera supported no heme iron reduction while activating significant cytochrome *c* reduction suggests that a distinct additional change must occur before the reductase domain can electronically interact with its natural redox partner (the oxygenase domain).

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