Activation of Constitutive Nitric Oxide Synthases by Oxidized Calmodulin Mutants†

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ABSTRACT: Several calmodulin (CaM) mutants were engineered in an effort to identify the functional implications of the oxidation of individual methionines in CaM on the activity of the constitutive isoforms of nitric oxide synthase (NOS). Site-directed mutagenesis was used to substitute the majority of methionines with leucines. Substitution of all nine methionine residues in CaM with leucines had minimal effects on the binding affinity or maximal enzyme activation for either the neuronal (nNOS) or endothelial (eNOS) isoform. Selective substitution permitted determination of the functional consequences of the site-specific oxidation of Met^{144} and Met^{145} on the regulation of electron transfer within nNOS and eNOS. Site-specific oxidation of Met^{144} and Met^{145} resulted in changes in the CaM concentration necessary for half-maximal activation of nNOS and eNOS, suggesting that these side chains are involved in stabilizing the productive association between CaM and NOS. However, the site-specific oxidation of Met^{144} and Met^{145} had essentially no effect on the maximal extent of eNOS activation in the presence of saturating concentrations of CaM. In contrast, the site-specific oxidation of Met^{144} (but not Met^{145}) resulted in a reduction in the level of nNOS activation that was associated with decreased rates of electron transfer within the reductase domain. Thus, nNOS and eNOS exhibit different functional sensitivities to conditions of oxidative stress that are expected to oxidize CaM. This may underlie some aspects of the observed differences in the sensitivities of proteins in vasculature and neuronal tissues to nitration that are linked to NOS activation and the associated generation of peroxynitrite.

Calcium (Ca^{2+})[1] is an important signaling ion that functions to initiate a range of cellular processes, including muscle contraction, neurotransmission, memory, cell fertilization, cell proliferation, cell defense, and cell death (*1*). In non-muscle cells, calmodulin (CaM) represents the major Ca^{2+} sensor that functions to rapidly upregulate intracellular metabolism through the coordinated activation of approximately 30 intracellular proteins (*2*). The linkage between Ca^{2+} signaling, CaM activation, and cellular metabolism suggests that Ca^{2+} activation will result in an improved generation of reactive oxygen and nitrogen species, which function to mediate a range of adaptive and pathological cellular responses (*3*). For example, following Ca^{2+} activation, CaM binds to the constitutive forms of nitric oxide synthase (NOS, EC 1.14.13.39) and induces global conformational changes that enhance the rate of nitric oxide (•NO) production in both neuronal NOS (nNOS) and endothelial NOS (eNOS) by promoting electron transfer through the flavins (*4*, *5*). •NO itself is an important signaling molecule, which at sub-micromolar concentrations functions to facilitate synaptic plasticity in neurons and relaxation of the vasculature (*6*−*8*). However, at higher concentrations, •NO reacts with superoxide to form peroxynitrite, and has been implicated as a causative agent in a number of different cellular pathologies resulting from the nitration and functional inactivation of cellular proteins (*9*−*11*). Peroxynitrite acts as a feedback inhibitor of •NO generation by NOS through oxidation of methionines in CaM (*12*).

Oxidation of methionines in CaM occurs during biological aging, where essentially all CaM isolated from senescent brain is oxidized (*13*). CaM is particularly sensitive to oxidation under conditions of oxidative stress as a result of the large number of surface-exposed methionines, which are involved in target protein recognition and activation (*2*). In contrast, methionines tend to be buried in the vast majority of proteins and are relatively insensitive to oxidative modification (*14*). Thus, coupled with the functional importance of CaM to intracellular signaling and the functional role of methionines in binding sites that mediate target protein binding and activation, these results suggest that the

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[1] Abbreviations: Ca^{2+}, calcium; CaM, calmodulin; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H_4B, (6*R*)-5,6,7,8-tetrahydrobiopterin; IPTG, isopropyl β-D-1-thiogalactopyranoside; NADPH, nicotinamide adenine dinucleotide phosphate; •NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; SOD, superoxide dismutase.

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oxidation of CaM associated with normal biological aging may function to downregulate the activity of NOS (*13*). Unfortunately, interpretation of previous measurements is complicated by the formation of a heterogeneous mixture of oxidized CaM that contains nine methionines that are oxidized to differing extents. Since methionines in CaM play different roles in the binding and activation of target proteins, it has not been possible to differentiate alterations in binding affinity from differences in the ability of oxidized CaM to maximally stimulate enzymatic activity.

We have constructed CaM mutants that permit the site-specific oxidation of methionines to investigate the regulation of NOS activity by oxidation of specific methionines in CaM. This approach has allowed an assessment of the activation of the constitutive forms of NOS by homogeneously oxidized CaM samples. The choice of CaM mutants was based on earlier observations suggesting that the site-specific oxidation of Met^{144} and Met^{145} resulted in the inability of CaM to activate the plasma membrane Ca-ATPase (*15*). Furthermore, mutation of Met^{144} to valine results in a 75% decrease in the ability of CaM to activate nNOS (*16*), suggesting a critical role for this methionine in the productive association between CaM and nNOS that leads to enzyme activation. CaM mutants were engineered to permit the selective oxidation of Met^{144} and Met^{145} in CaM to assess their effects on the CaM-dependent activation of nNOS and eNOS. The site-specific substitution of individual methionines with leucines permitted the oxidation of the remaining methionines. Leucines were chosen because their volume is similar to that of methionines and, unlike valines, because of their propensity to stabilize α-helical structures (*14*).

The constitutive forms of NOS (i.e., nNOS and eNOS) are dimeric enzymes in which each monomer contains a carboxy-terminal reductase domain where NADPH, FAD, and FMN bind. The amino-terminal oxygenase domain contains binding sites for protoporphyrin IX and tetrahydrobiopterin (H_4B) as well as the substrates L-arginine and molecular oxygen (*17*). The CaM binding domain separates the oxygenase and reductase domains of NOS. The binding of CaM to nNOS and eNOS facilitates electron transfer (1) from NADPH to the flavins within the reductase domain and (2) from the flavins to the heme in the oxygenase domain or to exogenous electron acceptors such as cytochrome *c* (*17*−*19*). To assess enzymatic function, three distinct activities associated with NOS were measured involving NADPH oxidation, cytochrome *c* reduction, and •NO generation as assessed by the hemoglobin capture assay. These assays measure the extent of electron transfer involving the reductase domain by measuring the levels of NADPH oxidation, cytochrome *c* reduction (which is generally attributed to electron transfer from FMN), and interdomain electron transfer (FMN to heme) that is associated with •NO generation.

EXPERIMENTAL PROCEDURES

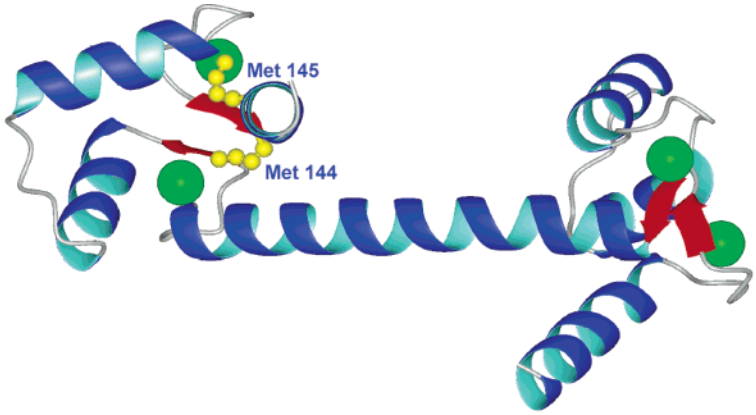
Construction and Expression of the Wild Type and CaM Mutants. Four CaM mutants in which variable numbers of methionine residues were mutated to leucines were constructed. To accomplish this, the coding region for chicken CaM [accession number MCCH (PIR database) or P02593 (SWISS-PROT database)] was excised from the plasmid pCaMPL provided by S. George (Duke University, Durham, NC) and subcloned into the mutagenesis and expression vector pALTER-Ex1, as previously described (*20*). The CaM-L7 mutant has the first seven methionine residues in CaM (methionines 36, 51, 71, 72, 76, 109, and 124) mutated to leucines (Figure 1). CaM-L8M145 has the first seven methionine residues mutated to leucines and the eighth methionine residue, Met^{144}, also changed to a leucine. CaM-L8M144 is similar to CaM-L8M145 except that Met^{145} is mutated to a leucine while Met^{144} is unchanged. The fourth mutant, CaM-L9, was constructed by mutating all the methionine residues in CaM to leucines. The mutated genes for CaM-L7, CaM-L8M144, CaM-L8M145, and CaM-L9 were produced by sequentially mutating all nine methionine residues to leucine using the PCR-based mutagenesis method outlined previously (*21*, *22*). The intact mutant CaM genes were subcloned into pET-15b and individually transformed into the *Escherichia coli* BL21(DE3) cell strain for protein overexpression. The bacteria were grown in minimal media, and protein production was induced with IPTG. Both wild-type and mutant CaM were purified by chromatography on phenyl-Sepharose CL-4B, essentially as previously described (*23*). Wild-type CaM, oxidized wild-type CaM, and the unoxidized and oxidized CaM mutants were dialyzed overnight in 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, and 0.5 mM dithiothreitol (DTT). Buffer was exchanged once during this time. CaM concentrations were determined using an extinction coefficient at 277 nm of 3029 M^{-1} cm^{-1}.

Table 1: Masses of Expressed and Oxidized CaM[a]

	mass (Da)[b]			
	expressed protein[d]		oxidized[e]	
CaM[c]	observed	theoretical	observed	theoretical[f]
wild-type	16 707	16 706	16 850	16 851
CaM-L7	16 579	16 580	16 612	16 611
CaM-L8M144	16 562	16 562	16 577	16 578
CaM-L8M145	16 561	16 562	16 578	16 578
CaM-L9	16 544	16 544	16 544	16 544

[a] Whole protein masses were measured in the positive ion mode using a Q-Tof 2 quadrupole, time-of-flight hybrid instrument (Micromass Ltd., Manchester, U.K.). The analysis of intact CaM was done by desalting 20 μg of protein on a trapping column (1.5 cm × 1 mm inside diameter) hand-packed with Zorbax SB-C18, 5 μm (Agilent Technologies, Wilmington, DE.), washing with 1% acetic acid, and then eluting directly into the source with 90% MeOH and 0.5% formic acid. Instrument parameters were found that offered the best compromise of sensitivity and minimizing the water loss peak, and were 60 V on the cone and 12 V on the collision cell with no Ar. [b] Masses of deconvoluted ESI-MS spectra were determined with an accuracy of ±1 Da. [c] Wild-type CaM and CaM mutants were expressed following the site-directed substitution of seven, eight, and nine leucines for methionines, as defined in Figure 1. [d] CaM samples prior to oxidation. [e] CaM samples following addition of 100 mM H_2O_2. [f] Calculated masses assuming the selective oxidation of methionines.

Oxidation of CaM. Quantitative oxidation of all methionine residues to methionine sulfoxides in wild-type CaM and the four mutants was assessed by mass spectrometry (Figure 2 and Table 1) and involved incubation of 60 μM CaM (1.0 mg/mL) with 50 mM hydrogen peroxide in 50 mM HO-MOPIPES (pH 5.0), 0.1 M KCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ for approximately 24 h at 25 °C, essentially as previously described (*15*). No other amino acids were oxidized. The nomenclature that refers to the treatment with



Wild-type	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTVM^{36}*R*SLGQNPTEAELQDM^{51}INEVDADGNGTIDFPEFLTM^{71}M^{72}A*RK*M^{76}
L7	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTVL^{36}*R*SLGQNPTEAELQDL^{51}INEVDADGNGTIDFPEFLTL^{71}L^{72}A*RK*L^{76}
L8-M144	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTVL^{36}*R*SLGQNPTEAELQDL^{51}INEVDADGNGTIDFPEFLTL^{71}L^{72}A*RK*L^{76}
L8-M145	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTVL^{36}*R*SLGQNPTEAELQDL^{51}INEVDADGNGTIDFPEFLTL^{71}L^{72}A*RK*L^{76}
L9	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTVL^{36}*R*SLGQNPTEAELQDL^{51}INEVDADGNGTIDFPEFLTL^{71}L^{72}A*RK*L^{76}

Wild-type	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HVM^{109}TNLGE*K*LTDEEVDEM^{124}I*R*EADIDGDGQVNYEEFVQM^{144}M^{145}TA*K*
L7	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HVL^{109}TNLGE*K*LTDEEVDEL^{124}I*R*EADIDGDGQVNYEEFVQM^{144}M^{145}TA*K*
L8-M144	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HVL^{109}TNLGE*K*LTDEEVDEL^{124}I*R*EADIDGDGQVNYEEFVQM^{144}L^{145}TA*K*
L8-M145	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HVL^{109}TNLGE*K*LTDEEVDEL^{124}I*R*EADIDGDGQVNYEEFVQL^{144}M^{145}TA*K*
L9	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HVL^{109}TNLGE*K*LTDEEVDEL^{124}I*R*EADIDGDGQVNYEEFVQL^{144}L^{145}TA*K*

FIGURE 1: Wild-type calmodulin structure and primary sequence of wild-type and mutant calmodulins. Ribbon diagram depicting the backbone fold of Ca^{2+}-saturated CaM (top) and primary sequence of wild-type and mutant CaM constructs (bottom). Secondary structural elements such as helices (blue), sheets (red), loop structures (gray), Met^{144} and Met^{145} (yellow), and bound calcium (green spheres) are indicated in the ribbon diagram. Positions of methionines and their associated site-directed substitutions are highlighted in red in the sequence.

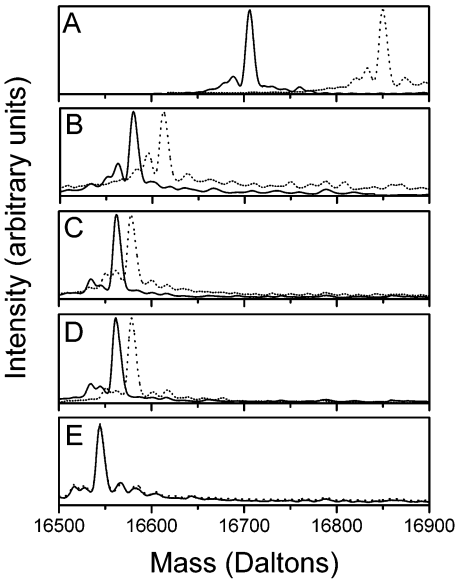


FIGURE 2: Electrospray ionization mass spectra of wild-type and mutant calmodulin. Spectra corresponding to wild-type CaM (A), CaM-L7 (B), CaM-L8M144 (C), CaM-L8M145 (D), and CaM-L9 (E) before (—) and after (···) oxidation by hydrogen peroxide. Spectra were obtained following deconvolution of multiply charged ions. Experimentally, 20 μg of CaM in 0.1 mM EGTA and 10 mM $(NH_4)_2CO_3$ (pH 8.6) was trapped, desalted, and then directly infused (on-line) into a Q-Tof 2 mass spectrometer.

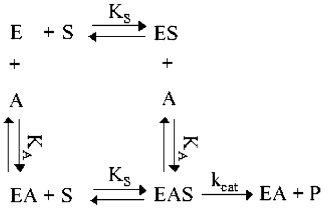
hydrogen peroxide is CaM_{ox}, CaM-$L7_{ox}$, CaM-$L8M145_{ox}$, CaM-$L8M144_{ox}$, and CaM-$L9_{ox}$.

Protein Expression and Purification. Rat neuronal and bovine endothelial NOS were expressed in *E. coli* and purified as previously described (*24*).

Nitric Oxide Synthase Activity. The hemoglobin capture assay (*25*, *26*) was used to determine the rate of •NO generation. Because of the slow binding of wild-type CaM to NOS (*27*), proteins were preincubated together for 5 min at 25 °C before initiation of the reaction by the addition of L-arginine and NADPH. Quadruplicate reactions were monitored on a 96-well plate reader (Spectramax 190, Molecular Devices, Sunnyvale, CA) at 25 °C. Nitric oxide-mediated oxidation of oxyhemoglobin was monitored at 401 nm (ϵ = 0.1036 OD unit/nmol) (*25*). The concentration of CaM was varied over a range of 5−3000 nM with at least six different CaM concentrations. Reaction mixtures contained, in a final volume of 100 μL, 50 mM Tris-HCl (pH 7.5), 500 μM NADPH, 5.0 μM H_4B, 200 μM $CaCl_2$, 20 μM DTT, 1.0 μM FAD, 1.0 μM FMN, 100 units/mL superoxide dismutase (SOD), 50 units/mL catalase, 0.2 mg/mL bovine serum albumin, 25 μM L-arginine, and nNOS [15, 30, 45, 60, 75, or 90 nM; V_{max} = 355 ± 31 nM min^{-1} mg^{-1}; K_M(L-Arg) = 1.8 ± 0.3 μM] or eNOS [25, 50, 75, 100, or 150 nM; V_{max} = 31.1 ± 1.4 nM min^{-1} mg^{-1}; K_M(L-Arg) = 3.1 ± 0.9 μM].

The percent of •NO production by nNOS and eNOS incubated with the mutant CaM proteins (1 μM) was determined relative to the activity obtained with wild-type CaM (1 μM) and is an average of reactions performed in quadruplicate at varying NOS concentrations. Initial veloci-

Scheme 1



ties were corrected for a change in signal in blank wells containing no CaM.

Cytochrome c Reductase Activity. The NADPH-dependent reduction of cytochrome *c* was monitored at 550 nm (ϵ = 0.0488 OD unit/nmol) as described previously (*28*). Quadruplicate reactions were initiated by the addition of NADPH and monitored on a 96-well plate reader (Spectramax 190, Molecular Devices) at 25 °C. The concentration of CaM that was used was 3 μM, which gave maximal •NO synthesis activity. Reaction mixtures contained, in a final volume of 100 μL, 50 mM Tris-HCl (pH 7.5), 200 μM NADPH, 1.0 mM $CaCl_2$, 1.0 μM FAD, 1.0 μM FMN, 10 units/mL SOD, 10 units/mL catalase, 100 μM horse heart cytochrome *c*, 0.1 mg/mL bovine serum albumin, and 5.5 nM nNOS or 50 nM eNOS.

NADPH Oxidase Activity. The consumption of NADPH by NOS was monitored at 340 nm (ϵ = −0.0152 OD unit/nmol) as previously described (*29*). Quadruplicate reactions were initiated by the addition of L-arginine and monitored on a 96-well plate reader (Spectramax 190, Molecular Devices) at 25 °C. The concentration of CaM that was used was 3 μM, which gave maximal •NO synthesis activity. Reaction mixtures contained, in a final volume of 100 μL, 50 mM Tris-HCl (pH 7.5), 500 μM NADPH, 5.0 μM H_4B, 1.0 mM $CaCl_2$, 300 μM DTT, 1.0 μM FAD, 1.0 μM FMN, 100 units/mL SOD, 50 units/mL catalase, 0.2 mg/mL bovine serum albumin, 5 mM L-arginine, and 68 nM nNOS or 100 nM eNOS.

Calculation of Kinetic Constants. Calmodulin binding to cNOS was assumed to be noncompetitive with L-arginine (*27*). Scheme 1 depicts noncompetitive binding between the substrate (S, L-arginine), activator (A, CaM), and the enzyme (E, cNOS).

Assuming the rapid establishment of equilibrium among all species, the activator concentration that results in half-maximal activity of an enzyme−substrate−activator complex conforming to Scheme 1, with depletion of free activator due to binding with enzyme, is given by eq 1:

$$AC_{50} = 0.5E_o + K_A \quad (1)$$

where AC_{50} is the activator concentration that results in half-maximal activity at a given enzyme concentration (nanomolar), E_o is the total enzyme concentration (nanomolar), and K_A is the activation constant (nanomolar) given by eq 2:

$$K_A = \frac{[E][A]}{[EA]} = \frac{[ES][A]}{[EAS]} \quad (2)$$

AC_{50} values were determined at several enzyme concentrations (Figure 3A) by fitting fractional initial velocity data to eq 3 by nonlinear regression (Statistics Toolbox, MATLAB version 5.3, Mathworks, Boston, MA):

$$v_f^{wt} = \frac{v}{v_o^{wt}} = \frac{v_{fmax}^{wt}}{1 + \left(\frac{AC_{50}}{A_o}\right)^n} \quad (3)$$

where A_o is the total activator concentration (nanomolar), v_o^{wt} is the initial velocity as the concentration of the wild-type activator approaches infinity (nanomolar per second), v is the initial velocity at an activator concentration of A_o (nanomolar per second), v_f^{wt} is the fractional initial velocity obtained by dividing v by v_o^{wt} (dimensionless), v_{fmax}^{wt} is the value of v_f^{wt} as A_o approaches infinity, and n is the logistic sensitivity (Hill coefficient). The use of eq 1 to calculate K_A is analogous to methods employed for the estimation of dissociation constants of tight binding inhibitors (*30*, *31*). Previous studies involving CaM have calculated an AC_{50} value at a single enzyme concentration (*12*, *32*). Although not essential to studies where relative changes in binding and maximal activity between wild-type and mutant activators are of primary interest, our approach eliminates the possibility that the calculated K_A values are merely titrating to one half of the total active enzyme concentration. The estimated activation constant for wild-type CaM binding to nNOS, K_{CaM}^{wt}, was calculated from the ordinal intercept of the plot of AC_{50} versus NOS concentration (Figure 3B). Similar plots were obtained for eNOS with wild-type CaM and for nNOS and eNOS with the unoxidized and oxidized CaM mutants. The K_{CaM} values obtained in this way for all CaM proteins with nNOS and eNOS are given in Tables 2 and 3, respectively. Two-tailed *t* tests performed at a 95% confidence level (α = 0.05) and corrected for multiple comparisons were used to determine statistical significance.

RESULTS

Oxidation of Calmodulin. Studies have shown a requirement for Met^{144} or Met^{145} in CaM for the stimulation of nNOS. The oxidation of the C-terminal methionines or the site-specific substitution of Met^{144} to valine in CaM resulted in a weakened ability to activate the target enzyme (*12*, *16*). To clarify the role of these methionines and investigate the effects of their site-specific oxidation, we engineered CaM mutants in which variable numbers of the nine methionines in wild-type CaM were replaced with leucines (Figure 1). Leucine was chosen since this amino acid substitution has a minimal impact on the tertiary structure of CaM (*33*). These CaM mutants were further modified by oxidation of the remaining methionine residues.

ESI-MS was used as a complementary tool to assess the homogeneity of CaM samples and to rule out additional sites of modification. A single major peak is observed in the ESI-MS spectra for each CaM mutant both before and after oxidation, which corresponds to the expected mass for each mutant protein prior to and following, respectively, the quantitative oxidation of all methionines (Figure 2 and Table 1). No significant spectral intensity is observed above the major peak associated with the oxidation of all methionines in the ESI-MS spectrum, indicating that no other amino acids are oxidized. Despite the presence of lower-mass peaks that correspond to ionization-induced dehydration artifacts (*34*),

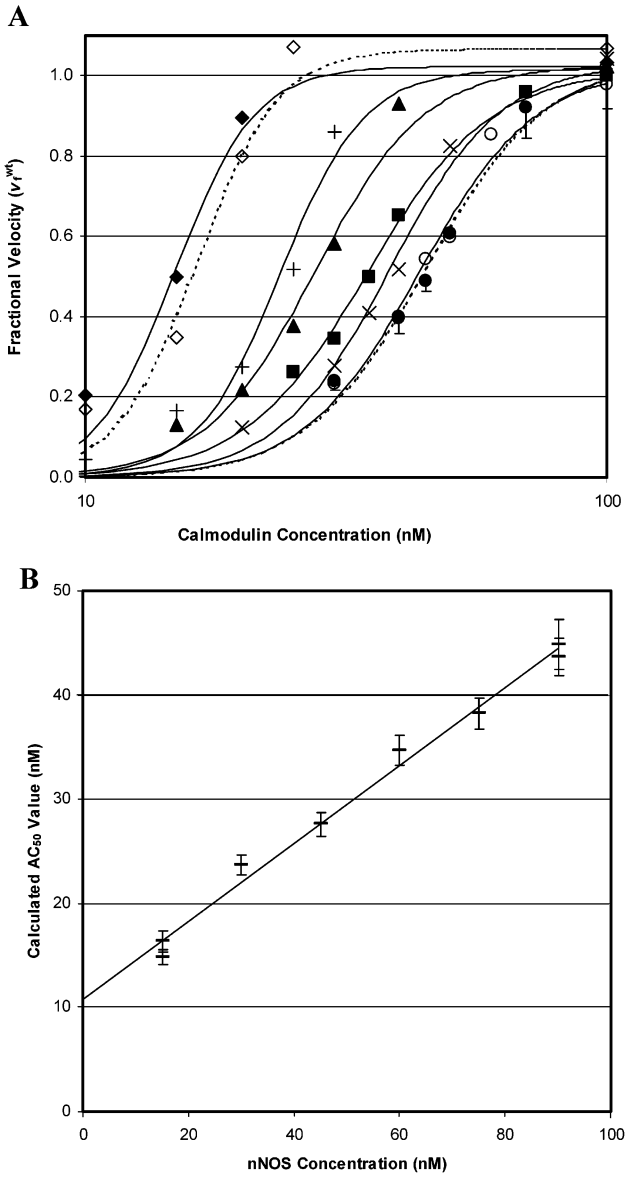


FIGURE 3: Calculation of the activation constant of wild-type calmodulin binding to nNOS. (A) AC_{50} curves of wild-type CaM at nNOS concentrations of 15 nM (◆, 15 nM), 15 nM (◇, 16 nM), 30 nM (+, 24 nM), 45 nM (▲, 28 nM), 60 nM (■, 35 nM), 75 nM (×, 38 nM), 90 nM (○, 45 nM), and 90 nM (●, 44 nM) were determined. AC_{50} values for each curve are given in parentheses. (B) AC_{50} vs nNOS concentration. The activation constant for wild-type CaM binding to nNOS was calculated from the ordinal intercept of panel B as determined by linear regression of the data ($R^2 = 0.99$) and was equal to 11 ± 1 nM. Wild-type CaM and nNOS were preincubated for 5 min at 25 °C. Reactions were initiated by the addition of L-arginine and NADPH to final concentrations of 25 and 500 μM, respectively. Error bars in panels A and B denote the standard deviation and 95% confidence intervals, respectively, and are representative of collected data.

the overall shapes of the mass spectra for the expressed and oxidized CaM proteins are nearly identical, indicating homogeneous oxidation that reflects the homogeneity of the wild-type sample.

Nitric Oxide Synthesis. The hemoglobin capture assay was used to determine the rate of CaM-activated •NO production by nNOS and eNOS. Wild-type CaM fully activated both of the NOS enzymes with maximal rates of activation of 355 ± 31 nM min^{-1} mg^{-1} for nNOS and 31.1 ± 1.4 nM min^{-1} mg^{-1} for eNOS.

Estimates of the activity constants for CaM-dependent activation of the cNOS enzymes were obtained using calculations based on Scheme 1. A fit of the fractional initial velocity versus wild-type CaM concentration to eq 3 was performed at several concentrations of nNOS (Figure 3A), and the resulting plot of the calculated AC_{50} values versus the nNOS concentrations (Figure 3B) is shown. The excellent agreement between the replicated experiments at nNOS concentrations of 15 and 90 nM, the narrow 95% confidence intervals of the regressed AC_{50} values, and the high R^2 value of the plot (0.99) demonstrate that the proposed calculation methodology is suitable for use with the oxyhemoglobin assay. The estimated activation constant for wild-type CaM binding to nNOS, K_{CaM}^{wt}, was calculated from the ordinal intercept of Figure 3B and was equal to 11 ± 1 nM. Although monitoring enzyme activation as opposed to simple binding, our results are consistent with the reported K_d of 5 nM obtained using ^{125}I-labeled CaM to monitor binding to nNOS (*27*). This method of analysis gave an activation constant for eNOS using wild-type CaM of 9 ± 2 nM. The same method of analysis was used for all mutant CaM proteins, and the results are given in Tables 2 and 3.

Role of Select Methionines in the Activation of nNOS. The oxidation of wild-type CaM resulted in no •NO synthesis by nNOS. Mutation of the first seven methionine residues in CaM to leucine produced the CaM-L7 mutant that retained Met^{144} and Met^{145}. This mutant CaM did not fully activate nNOS [~25% decrease in the •NO production rate relative to those from wild-type CaM studies (Table 2)] and had a 2-fold higher K_{CaM} than wild-type CaM. Oxidation of CaM-L7 (CaM-$L7_{ox}$) did not significantly affect the maximal rate of •NO synthesis, while the K_{CaM} value more than doubled (Table 2, 22 ± 2 nM for CaM-L7 vs 56 ± 6 nM for CaM-$L7_{ox}$). Thus, the oxidation of the remaining methionine residues (Met^{144} and Met^{145}) significantly affected the binding of CaM to nNOS.

The role of Met^{144} was specifically investigated by replacing eight of the methionine residues (methionines 36, 51, 71, 72, 76, 109, 124, and 145) of CaM with leucines to produce the CaM-L8M144 mutant. The level of •NO synthesis by nNOS incubated with CaM-L8M144 was significantly higher than the levels found in the presence of wild-type CaM, but required an ~3-fold increase in K_{CaM}. The oxidation of CaM-L8M144 led to a significant decrease in the rate of •NO synthesis by nNOS to levels found for nNOS in the presence of either CaM-L7, CaM-L8M145, or their corresponding oxidized forms (Figure 4). The Met^{144} residue is required for full activation of the nNOS enzyme, but oxidation of this residue increases the affinity of CaM for nNOS as evidenced by a significant decrease in the K_{CaM} value.

Mutation of the first eight methionine residues of CaM to leucine resulted in a protein termed CaM-L8M145 that retained Met^{145} and exhibited an ~30% decrease in the maximal level of •NO synthesis and an ~2-fold increase in K_{CaM}. The rate of •NO synthesis exhibited a significant increase upon oxidation of the CaM-L8M145 mutant (CaM-$L8M145_{ox}$), and a small increase in K_{CaM} was again found for the oxidized form (Table 2). The disruption of the interaction between Met^{145} of CaM and nNOS increases the relative maximal rate of •NO production as found by

Table 2: CaM-Dependent Activation of nNOS

	NADPH oxidation[a]		Cyt *c* reduction[a]		•NO production[b]		affinity (K_{CaM})[c]	
CaM	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (nM)	Met oxidation (nM)
wild-type	100 ± 6	8 ± 1	100 ± 2	29 ± 1	100 ± 2	0	11 ± 1	–
CaM-L7	68 ± 3	66 ± 2	40 ± 2	47 ± 2	74 ± 3	81 ± 3	22 ± 2	56 ± 6
CaM-L8M144	97 ± 1	81 ± 1	49 ± 1	41 ± 2	121 ± 3	76 ± 3	42 ± 1	31 ± 2
CaM-L8M145	60 ± 1	68 ± 1	44 ± 1	43 ± 1	66 ± 4	84 ± 6	21 ± 2	29 ± 2
CaM-L9	89 ± 3	86 ± 6	49 ± 2	51 ± 1	114 ± 3	113 ± 7	30 ± 2	42 ± 2

[a] Assays were performed in quadruplicate. [b] •NO production values shown are the mean of regressed v^{wt}_{fmax} values. The number of replicates for each CaM mutant ranged from four to eight. Each v^{wt}_{fmax} value was determined from a fit of fractional initial velocity vs CaM concentration to eq 3 by nonlinear regression. Each set of fractional initial velocity determinations was performed at seven CaM concentrations in quadruplicate. The estimated standard errors of the regressed v^{wt}_{fmax} values showed good agreement with the calculated standard deviations of the replicate populations. [c] K_{CaM} values were calculated from the ordinal intercept of AC_{50} vs enzyme concentration as determined by linear regression. The number of data points used in the linear regression for each CaM mutant ranged from four to eight. R^2 values were ≥0.98, except for that of CaM-$L7_{ox}$ which equaled 0.95. AC_{50} values were determined from a fit of the fractional initial velocity vs CaM concentration to eq 3 by nonlinear regression as noted above. The ± symbol denotes the standard error of the regressed K_{CaM} value.

Table 3: CaM-Dependent Activation of eNOS

	NADPH oxidation[a]		Cyt *c* reduction[a]		•NO production[b]		affinity (K_{CaM})[c]	
CaM	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (%)	Met oxidation (%)	Met to Leu mutations (nM)	Met oxidation (nM)
wild-type	100 ± 5	9 ± 1	100 ± 2	5 ± 1	100 ± 4	0	9 ± 2	–
CaM-L7	80 ± 5	84 ± 3	86 ± 2	87 ± 2	73 ± 11	72 ± 5	26 ± 1	41 ± 4
CaM-L8M144	69 ± 3	72 ± 4	87 ± 1	82 ± 1	71 ± 8	70 ± 6	45 ± 3	24 ± 1
CaM-L8M145	75 ± 4	70 ± 3	82 ± 1	81 ± 3	74 ± 7	67 ± 3	18 ± 1	30 ± 1
CaM-L9	76 ± 4	83 ± 3	84 ± 1	81 ± 2	72 ± 3	69 ± 11	30 ± 1	42 ± 1

[a] See footnote a of Table 2. [b] See footnote b of Table 2. [c] See footnote c of Table 2.

comparing CaM-$L8M145_{ox}$ versus CaM-L8M145, as well as comparing CaM-$L7_{ox}$, CaM-L8M144, and CaM-L9 versus CaM-L7. This indicates that Met^{145} may act as a negative tether to control nNOS activity.

Mutation of all nine methionines in CaM to leucine produced the CaM-L9 mutant. This mutation enhanced CaM-dependent nNOS activity as seen for the other CaM mutants that disrupt Met^{145} to nNOS interactions. Oxidation of CaM-L9 (CaM-$L9_{ox}$) did not significantly affect the maximal rate of •NO synthesis by nNOS. These results are consistent with the C-terminal domain of CaM having a role in binding to the nNOS enzyme. While the Met^{144} residue of CaM appears to be required for full activation of the enzyme, its replacement with leucine in CaM-L9 gave full activity even when the protein was oxidized.

Role of Select Methionines in the Activation of eNOS. The oxidation of all nine methionine residues in wild-type CaM resulted in no •NO synthesis by eNOS. None of the CaM mutants, either oxidized or not, could activate eNOS to a level comparable to that found with wild-type CaM (Table 3 and Figure 4B). In fact, the CaM mutants only stimulated eNOS to ~70% of the maximal activation level of •NO synthesis observed with wild-type CaM. These studies clearly show that eNOS does not interact with CaM in exactly the same manner as nNOS.

As for the case of nNOS, the eNOS experiments using CaM-L8M144 showed a significant decrease in the K_{CaM} upon oxidation, suggesting the Met^{144} residue affected CaM binding to eNOS (Figure 4). All CaM mutants, except for CaM-L8M144, exhibited an elevation of their K_{CaM} values upon oxidation. The replacement of the methionine residues resulted in a more than 2-fold increase in K_{CaM} that was further increased upon oxidation. Thus, some or all of the seven other methionine residues in CaM are required for the proper binding and full activation of eNOS.

Role of Methionines in the Transfer of Electrons within the Reductase Domain. The rate of reduction of cytochrome *c* was found to be greater for nNOS [16 290 ± 330 nmol of cytochrome *c* reduced min^{-1} (mg of nNOS)$^{-1}$] than for eNOS [518 ± 11 nmol of cytochrome *c* reduced min^{-1} (mg of eNOS)$^{-1}$] in the presence of wild-type CaM. This is consistent with their previously reported rates for cytochrome *c* reduction (*35*). The level of cytochrome *c* reduction by nNOS decreased by more than 70% when the enzyme was incubated with the oxidized wild-type form of CaM. The reduction of cytochrome *c* by nNOS activated by the other mutated forms of CaM, independent of oxidation state, showed a decrease of more than 50% relative to that of wild-type CaM (Table 2). These results are consistent with the reported 45% decrease in the rate of cytochrome *c* reduction by nNOS when oxidized CaM was present (*12*).

The eNOS enzyme incubated with the oxidized form of CaM exhibited very little activity (Table 3). The unoxidized or oxidized mutant CaM proteins incubated with eNOS gave higher relative rates of cytochrome *c* reduction of ~84% of that obtained using wild-type CaM. While there was no apparent association between the mutation or oxidation of selected methionine residues and the rate of cytochrome *c* reduction by eNOS, there was a clear difference when their effects on the •NO generation by the two NOS isoforms were compared.

NADPH Oxidation. The rates of NADPH oxidation by nNOS and eNOS were stimulated by CaM binding [258 ± 7 nmol of NADPH oxidized min^{-1} (mg of nNOS)$^{-1}$ and 57 ± 3 nmol of NADPH oxidized min^{-1} (mg of eNOS)$^{-1}$, respectively]. The various forms of CaM used in the

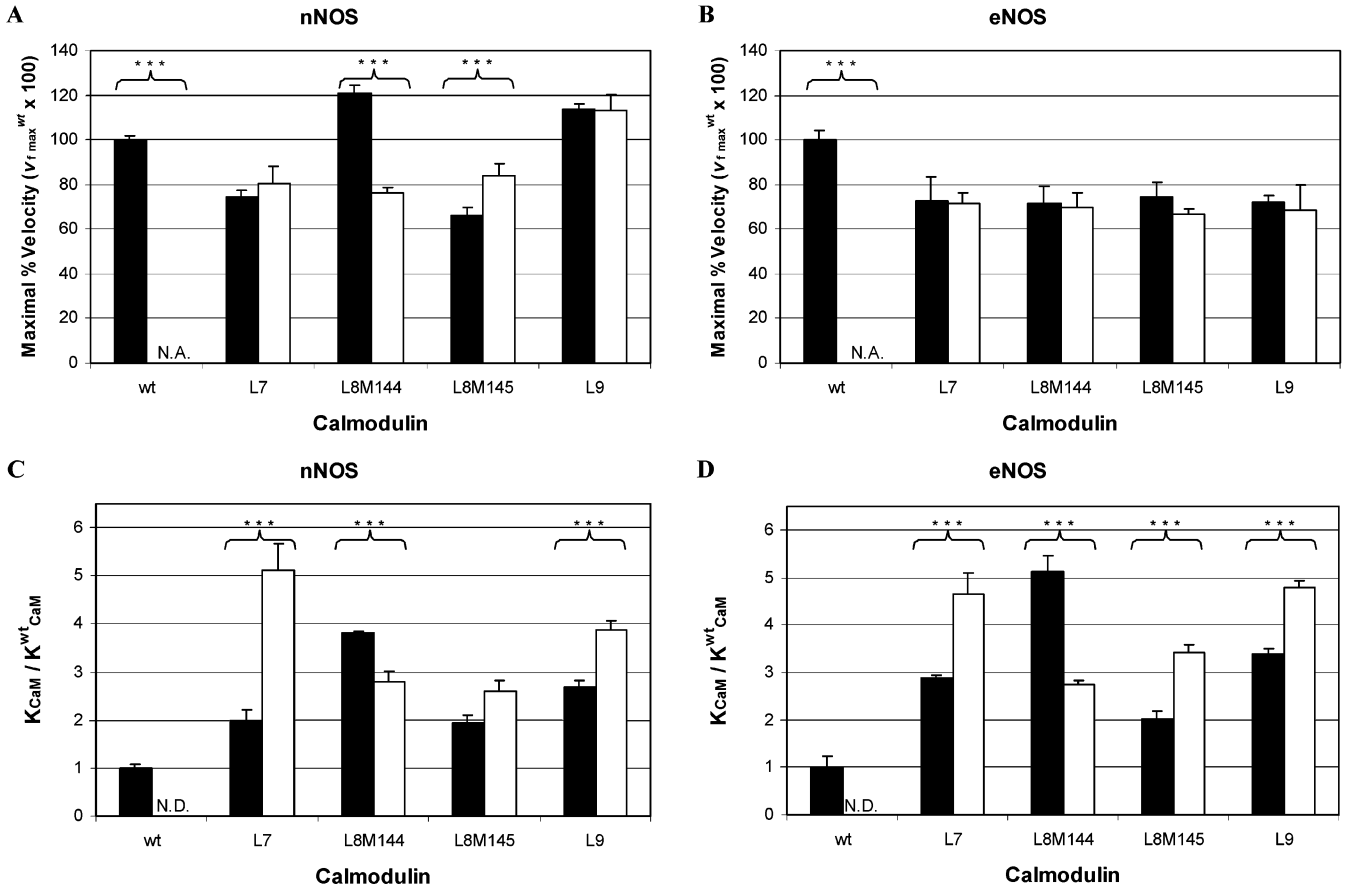


FIGURE 4: Percent initial velocity and the ratio of K_{CaM} to K^{wt}_{CaM} for nNOS and eNOS in the presence of unoxidized and oxidized wild-type and mutant calmodulins. (A and B) Maximal % velocity, v^{wt}_{fmax}, of nNOS and eNOS in the presence of wild-type and mutant CaM proteins, respectively. For both nNOS and eNOS the percent initial velocity was significantly different from that of wild-type CaM for all mutant CaM proteins. (C and D) Ratio of K_{CaM} to K^{wt}_{CaM} for nNOS and eNOS incubated with wild-type and mutant CaM proteins. For both nNOS and eNOS, the ratio of K_{CaM} to K^{wt}_{CaM} was significantly different from that of wild-type CaM for all mutant CaM proteins. For all panels, significant differences between unoxidized (black bars) and oxidized (white bars) CaM proteins are indicated (***). Statistical hypothesis tests were performed at a 95% confidence level ($\alpha = 0.05$). Error bars denote the standard deviation. N.A. means not active and N.D. not determined.

investigation showed coupled NADPH consumption as previously reported for CaM−troponin c chimeras (*32*). The rates of NADPH oxidation for the different mutant and oxidized CaM proteins correlated well with the rates of •NO synthesis for both isoforms. The oxidation of all nine methionines reduced the level of NADPH oxidation by more than 90%.

DISCUSSION

Summary of Results. The oxidation of Met^{144} and Met^{145} in CaM to their corresponding methionine sulfoxides generally results in an ~2-fold increase in the CaM concentration necessary for half-maximal activation of both eNOS and nNOS. The observed changes in the CaM concentration associated with half-maximal enzyme activation are suggested to reflect changes in the binding affinity between CaM and the cNOS enzymes. This latter interpretation follows from the fact that these CaM samples are homogeneously oxidized, and cNOS binds with a stoichiometry of one CaM per enzyme. The calculated binding affinities are consistent with those previously measured for both nNOS and eNOS (*27*, *36*). The mutation of Met^{145} to a leucine led to significant increases in maximal enzyme activity in nNOS, but not eNOS. While oxidation of either Met^{144} or Met^{145} in CaM has little effect on the maximal extent of enzyme activation for eNOS, there is a greater than 30% reduction in the activation of nNOS following the selective oxidation of Met^{144}. These results clearly demonstrate differences in the roles of Met^{144} and Met^{145} in the regulation of eNOS and nNOS and, for the first time, demonstrate the potential for the selective oxidation of these sensitive sites to regulate CaM-dependent enzymes.

Role of CaM in the Regulation of NOS. CaM-mediated Ca^{2+} signaling regulates a wide variety of proteins. Examination of the CaM binding sequences within these proteins has revealed some similar structural characteristics, such as an amphiphilic α-helix, but both the length and content of these binding regions vary, suggesting differences in the binding of CaM/Ca^{2+} to target proteins (*37*−*39*). Ca^{2+}/CaM differentially regulates the NOS isoforms, with CaM-bound inducible NOS (iNOS) fully active at low basal levels of Ca^{2+}, while the Ca^{2+}-dependent nNOS and eNOS isoforms are active only in the presence of elevated Ca^{2+} concentrations. The CaM-dependent activation of NOS has been investigated using CaM mutant and chimeric proteins. Replacement of individual EF-hands of CaM with the corresponding hands of cardiac troponin c has shown that the latch domain, consisting of helix 2 in site 1 and helix 6

in site 3, is critical for NOS activation (*40*). The first, third, and fourth EF-hands of CaM are important in regulating the nNOS's heme domain (*41*). Interchanging CaM EF domains has shown that replacement of the first EF domain with the third EF domain can significantly reduce the level of nNOS activation (*42*). Further studies by Persechini et al. (*43*) on nNOS activation by engineered CaM proteins with duplicated or exchanged EF-hand pairs indicated that the EF-hand pairs contain distinct but overlapping sets of determinants for binding and activation of enzymes. CaM proteins with mutations in each EF-hand were used to show that some of the EF-hand Ca^{2+}-binding sites affect the oxygen-dependent functions in a manner different from those of the reductase domain-specific functions (*19*). The active site substrates L-arginine and tetrahydrobiopterin do not affect CaM binding characteristics (*27*).

NMR studies of CaM interactions with peptides comprising the CaM binding domain of nNOS demonstrated that the two methionine-rich hydrophobic regions of CaM are directly involved in binding to the target sequence (*44*). Studies on the binding of CaM to NOS peptides have determined that the high-affinity Ca^{2+}-binding carboxy terminus of CaM binds first, followed by the binding of the low-affinity Ca^{2+}-binding amino terminus of CaM (*44*, *45*). Mutagenesis studies have revealed the importance of the first Ca^{2+} binding lobe of CaM in the promotion of electron transfer through the reductase domain to the heme domain of NOS (*19*). The fourth Ca^{2+} binding lobe in CaM is involved in the binding of CaM to NOS, given that mutations in this lobe not only decreased the activity of NOS but also required a greater concentration of the CaM mutants for activity (*19*, *47*).

Role of CaM Methionines in the Regulation of NOS. It is noteworthy that most investigations have focused solely on the nNOS isoform. We have extended these investigations by including both cNOS enzymes. Consistent with the aforementioned EF-hand studies, replacement of the first seven methionine residues in CaM with leucine resulted in an ~20% decrease in the maximal activity of both isoforms. Furthermore, while the activities of both nNOS and eNOS were completely lost when assayed using the fully oxidized form of wild-type CaM, the oxidized form of CaM-L7 retained more than 70% of the activity. A single methionine to leucine mutation at residue 36 in CaM reduced CaM-dependent cyclic nucleotide phosphodiesterase activity by more than 60% (*48*). Furthermore, the crystallographic structure of CaM bound to a 20-residue peptide comprising the eNOS CaM-binding region shows that Met^{76} in the central helix of CaM participates in the binding of the eNOS CaM domain (*49*). Thus, some of the first seven methionine residues are clearly implicated in CaM binding and activation in both the eNOS and nNOS enzymes.

Investigations with CaM proteins with point mutations have shown that the amino acid at position 144 plays a critical role in nNOS activation but may have little effect on the activation other target enzymes (*16*). This investigation also shows the importance of the Met^{144} residue of CaM for the activation of nNOS. The specific oxidation of Met^{144} (CaM-L8M144$_{ox}$) resulted in a greater than 30% decrease in the activity of nNOS (Figure 4A). In contrast, the oxidation of this residue has no effect on the activation of eNOS. The Met^{144} residue of CaM also affects binding as its oxidation results in a significant decrease in the K_{CaM} value for both eNOS and nNOS. Free energy simulations predict that the oxidation of Met^{144} should enhance target peptide binding (*50*). The crystal structure of CaM bound to the eNOS peptide shows residues Phe^{496}, Lys^{497}, and Ala^{500} of eNOS in contact with Met^{144} of CaM, indicating a NOS-specific requirement for a bulky hydrophobic residue at position 144 in CaM (*49*). All three residues are conserved in nNOS, and the decrease in K_{CaM} for both cNOS isoforms following oxidation of Met^{144} may be due to an electrostatic attraction between Lys^{497} and the methionine sulfoxide. There is no apparent reason for the differences in maximal activity between the two isoforms based on the structural studies of eNOS except for the possibility of another electrostatic effect in nNOS since Glu^{498} in eNOS aligns with a lysine residue in nNOS.

The removal of Met^{145} (CaM-L9, CaM-L9$_{ox}$, and CaM-L8M144) led to significant increases in activation of nNOS relative to studies using wild-type CaM or CaM-L7 (Figure 4A). Even the oxidation of the Met^{145} residue in CaM-L8M145 resulted in a significant increase in nNOS activity. In contrast, there is no apparent trend in the maximum velocities of eNOS incubated with CaM carrying a mutation at Met^{145} (Figure 4B). The crystal structure of the CaM-bound eNOS peptide shows considerable interaction between Met^{145} of CaM and Ala^{500}, Asn^{501}, and Lys^{504} in the eNOS peptide (*49*). The three residues are nearly conserved in eNOS and nNOS, except that nNOS has a glutamate in place of Asn^{501}. This sequence variance may account for the isoform-specific differences that we observed in our investigation. The results for the activation of the nNOS enzyme suggest that Met^{145} may not be the optimal residue at this position in CaM for activating this particular enzyme. This is not surprising considering the significant variance in the primary sequence of the binding sites found in more than 30 CaM-binding proteins.

The activation of eNOS by CaM may be more sensitive to modified methionines within CaM as replacing all the methionines of CaM with leucines did not restore the activity of eNOS to the level found with wild-type CaM (Figure 4B). The sensitivity of eNOS to the modified methionine residues of CaM may be part of a regulatory process. Studies have shown that in the absence of H_4B, superoxide is produced by eNOS from the dissociation of the ferrous dioxygen complex in the oxygenase domain (*52*, *53*). Addition of H_4B fully couples L-arginine oxidation to NADPH consumption to produce $^{\bullet}NO$ and L-citrulline (*52*, *53*). Therefore, an alteration in the regulation of $^{\bullet}NO$ synthesis and superoxide production by eNOS could lead to a reaction between these two radicals, which would produce peroxynitrite, a potent oxidizing agent. Peroxynitrite is capable of oxidizing the methionine residues of CaM, weakening the ability of CaM to activate nNOS (*12*). In the absence of CaM, eNOS produces small amounts of superoxide (*52*, *53*). The inability of CaM$_{ox}$ and the CaM methionine to leucine mutants described here to fully activate eNOS strongly suggests that the oxidation of the methionine residues of CaM may be involved in the regulation of eNOS activity.

Overall, the greatest effect of these CaM mutants on nNOS was during cytochrome *c* reduction, indicating that the CaM mutants had an adverse effect on electron transfer within the reductase domain (Table 2). The single domain substitu-

tion of the CaM region containing Met^{144} and Met^{145} in a CaM−troponin c chimera also showed a difference in the rate of cytochrome *c* reduction and •NO synthesis when activated by the mutant CaM proteins (*35*). A possible explanation for our findings is that these CaM mutants bind with higher affinity to one of two sites normally occupied by wild-type CaM, presumably a site that links the reductase domain to the oxygenase domain (*12*, *46*) and not the site which influences electron transfer within the reductase domain. In contrast, all of the CaM mutants used in this investigation have the greatest effect on the rate of •NO synthesis by eNOS (Table 3). Therefore, these CaM mutants appear to hamper electron transfer from the reductase domain to the oxygenase domain of eNOS more so than electron transfer within the eNOS reductase domain.

Our results indicate that there are significant differences in the process of CaM binding and activation of the two cNOS enzymes. Studies using chimeras composed of different regions of all three isoforms have shown that the CaM binding sequence in iNOS is necessary but not sufficient to account for the tight binding and apparent Ca^{2+} insensitivity of the iNOS isoform relative to eNOS and nNOS (*54*, *55*). Significant differences were reported for the CaM-dependent activation of eNOS and nNOS deletion mutants in which the putative autoinhibitory domains had been removed from the cNOS enzymes (*29*, *56*, *57*). Thus, the interaction of CaM with NOS enzymes may be further complicated by the possibility that methionine oxidation may also affect CaM association within the putative autoinhibitory elements of the two isoforms.

The difficulty in predicting the CaM conformation and CaM interaction with its targets on the basis of sequence homology and peptide studies is exemplified by a recent report on the X-ray crystallographic structure of the anthrax oedema factor bound to CaM (*51*). Previously, a single CaM binding region had been identified in the anthrax oedema factor. The crystal structure showed that in addition to the previously proposed CaM-binding domain, three discrete regions within the oedema factor protein form a surface that recognizes the extended conformation of CaM (*51*). Therefore, until the structures of the entire NOS enzymes bound to CaM have been determined, it will be difficult to fully explain experimental observations showing differences in the activation of cNOS enzymes by CaM, let alone the results obtained for the iNOS isoform.

Physiological Relevance of CaM Oxidation. Oxidized CaM accumulates during aging, and has been suggested to be responsible, in part, for observed alterations in cellular Ca^{2+} homeostasis (*3*). However, prior measurements have quantitatively considered how methionine oxidation affects the ability of CaM to activate only a single protein (i.e., the plasma membrane Ca-ATPase). Oxidation of Met^{144} in CaM is primarily responsible for the 60% decrease in the level of enzyme activation (*32*). Oxidation of this site is responsible for a 30% reduction in the maximal extent of nNOS activation, but has essentially no effect on eNOS activity. Since methionine oxidation is reversible through the action of methionine sulfoxide reductases, these results support previous suggestions that the selective oxidation of critical Ca^{2+} regulatory proteins may modulate target protein activation in response to cellular redox conditions. Furthermore, the differential effects of methionine oxidation on nNOS and eNOS activity provide the first evidence that methionine oxidation differentially regulates target protein function. These results suggest that like the phosphorylation of CaM, site-specific oxidative modifications can differentially regulate target protein function, and suggest that methionine oxidation can modulate cell signaling under conditions of oxidative stress (*58*).

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