Structural Elements Contribute to the Calcium/Calmodulin Dependence on Enzyme Activation in Human Endothelial Nitric-oxide Synthase

Pei-Feng Chen and Kenneth K. Wu

From the Vascular Biology Research Center and Division of Hematology, Department of Internal Medicine, The University of Texas Health Science Center at Houston, Houston, Texas 77225

Two regions, located at residues 594–606/614–645 and residues 1165–1178, are present in the reductase domain of human endothelial nitric-oxide synthase (eNOS) but absent in its counterpart, inducible nitric-oxide synthase (iNOS). We previously demonstrated that removing residues 594–606/614–645 resulted in an enzyme (Δ45) containing an intrinsic calmodulin (CaM) purified from an SF9/baculovirus expression system (Chen, P.-F., and Wu, K.K. (2000) J. Biol. Chem. 275, 13160–13169). Here we have further elucidated the differential requirement of Ca2+/CaM for enzyme activation between eNOS and iNOS by either deletion of residues 1165–1178 (Δ14) or combined deletions of residues 594–606/614–645 and 1165–1178 (Δ45/Δ14) from eNOS to mimic iNOS. We measured the catalytic rates using purified proteins completely free of CaM. Steady-state analysis indicated that the Δ45 supported NO synthesis in the absence of CaM at 60% of the rate in its presence, consistent with our prior result that CaM-bound Δ45 retained 60% of its activity in the presence of 10 mM EGTA. Mutant Δ14 displayed a 1.5-fold reduction of EC50 for Ca2+/CaM-dependence in L-citrulline formation, and a 2–4-fold increase in the rates of NO synthesis, NADPH oxidation, and cytochrome c reduction relative to the wild type. The basal rates of double mutant Δ45/Δ14 in NO production, NADPH oxidation, and cytochrome c reduction were 3-fold greater than those of CaM-stimulated wild-type eNOS. Interestingly, all three activities of Δ45/Δ14 were suppressed rather than enhanced by Ca2+/CaM, indicating a complete Ca2+/CaM independence for those reactions. The results suggest that the Ca2+/CaM-dependent catalytic activity of eNOS appears to be conferred mainly by these two structural elements, and the interdomain electron transfer from reductase to oxygenase domain does not require Ca2+/CaM when eNOS lacks these two segments.

Nitric-oxide synthase (NOS) catalyzes the synthesis of NO through a series of electron transfers from the C-terminal reductase domain, which harbors the FAD and FMN cofactors and the NADPH binding site, to the N-terminal oxygenase domain, which contains the heme catalytic center, the H2B cofactor, and the arginine binding sites (1–8). An electron generated by NADPH is transferred in tandem to FAD and FMN and then to the heme, which has been proposed to be facilitated by CaM bound to a site situated between these two domains (9). The NOS family comprises three isoforms that share domain structures, sequence homology, and catalytic properties (10–12). Despite these similarities, there are considerable differences among the NOS isoforms with respect to their cellular expressions, Ca2+/CaM-dependent CaM activation, and rate of electron transfer. Two isoforms, i.e. nNOS and eNOS, are constitutively expressed NOSs (cNOSs), but their expressed enzymes are latent until CaM binding is elicited by an elevated intracellular calcium level (13). In contrast, iNOS is absent or expressed in low abundance at the resting state, and its expression is induced by cytokines and endotoxins. The expressed iNOS is catalytically active, thought to be due to its high affinity for CaM binding even at a basal level of intracellular calcium (14). Among the three isoforms, eNOS has lower electron transfer rate and catalytic activity than nNOS and iNOS (15), suggesting other control mechanisms for eNOS catalysis.

The x-ray crystallographic analysis of the oxygenase domains of NOS isoforms has revealed a striking degree of conservation at the active-site structure (16–18). The results from studies of chimeric enzymes in which the oxygenase domain was swapped to the reductase domain of another isoform suggested that divergence in the reductase domain rather than in the oxygenase domain accounted for the differences in Ca2+/CaM sensitivity and the rate of electron transfer between the cNOSs and the iNOS (15). An ~50-amino acid fragment (Fig. 1) present in the FMN-subdomain of human eNOS (residues 594–645) and nNOS (residues 834–882), but absent in the corresponding part of iNOS, was proposed as an autoinhibitory element that impedes the electron transfer of cNOSs in the absence of CaM (19, 20). Deletion of this region rendered the mutant enzymes less dependent on Ca2+ concentration, with a faster rate of electron flow (21–23). We previously characterized an eNOS mutant (24) in which residues 594–606 and 614–645 were deleted (Δ45eNOS) with preservation of residues 607–613 because it was conserved between the sequences of cNOSs and iNOS (Fig. 1). The Δ45eNOS contained an endogenous CaM bound to the protein isolated from an SF9/baculovirus expression system. This mutant was completely CaM-independent as well as significantly Cu2+-independent in L-citrulline formation and exhibited a higher rate of cytochrome c reduction in a CaM-independent manner (24). The results confirm that residues 594–606 and 614–645 in
eNOS control calcium sensitivity for CaM-dependent enzyme activation. However, because this mutant still requires Ca\(^{2+}\) to achieve a maximal catalytic activity, the calcium requirement for electron transfer and NO production may be controlled by other intramolecular mechanisms.

Recent studies have implicated the C-terminal region of all NO synthases as an additional regulatory element in modulating electron transferring. The C terminus of eNOS contains a conserved serine (Ser\(^{1179}\)) in human eNOS and Ser\(^{1417}\) in nNOS with a kinase-dependent phosphorylation motif (RSRXX(5/7)T) that has been noted in eNOS to be phosphorylated in response to a number of stimuli (35–32). Phosphorylation has been shown to trigger eNOS activation at a lower Ca\(^{2+}\) concentration and to increase the rate of NO production by 2-fold in vivo (25–26). Mutation of this serine to Asp in eNOS (33) and nNOS (34), which mimics phosphorylation by introducing a negative charge, also causes a faster electron flow through enzymes. By sequence comparison with cytochrome P450 reductase (CPR), Roman et al. (35, 36) proposed that the 21–42-amino acid C-terminal extension tail present in all NO synthases but absent in CPR was involved in modulating electron transfer. Their experimental data demonstrated that deletion of the entire C-tail from rat nNOS (33 residues) or bovine eNOS (42 residues) greatly increased electron transfer into and between flavins in the absence of CaM. Paradoxically, their cytochrome c reductase activities were suppressed rather than enhanced by exogenously added CaM, and their CaM-induced NO synthesis activities were only 50% that of CaM-bound wild-type enzymes (36). In contrast, Lane and Gross (37) did not delete the entire C-terminal tail but instead partially removed the Ser\(^{1179}\) and the subsequent 26 C-terminal amino acids from bovine eNOS (Δ27). This mutant exhibited a 5-fold reduction in EC\(_{50}\) for calcium and a 2–4-fold increase in maximal catalytic activities. Both reductase and oxygenase activities of Δ27 were enhanced 3-fold by exogenously added CaM (37). These findings underscore a complex control process of the C-terminal tail, especially with respect to the influence of CaM on electron transfer.

Sequence alignment reveals that a segment at the proximal C-terminal tail is conserved in the sequences of human eNOS (residues 1165–1178) and nNOS (residues 1404–1417) but absent in iNOS. Besides the autoinhibitory loop of residues 594–606/614–645, this segment indicates another, more obvious dissimilarity between eNOSs and iNOSs. We postulated that this conserved region in conjunction with the autoinhibitory loop might confer the dramatic differences in Ca\(^{2+}\) sensitivity and the rate of electron flux between eNOS and iNOS. To test this hypothesis, we assessed the changes in electron transfer and NO synthesis caused by deletion of eNOS sequence (residues 1165–1178, Δ14eNOS). We were particularly interested in learning whether combined deletions of Δ394–606/614–645 and Δ1165–1178 would yield an eNOS mutant protein resembling iNOS in terms of the CaM requirement for reductase and oxygenase activities. Wild-type and mutant eNOSs expressed in an Sf9/baculovirus system were purified by adding an adequate amount of chelators to remove calcium, and the reductase and NO synthesis activities were measured. All of the purified proteins were free of the endogenous CaM. The results showed that a combined Δ455/Δ14eNOS mutant had a significant increase in the rates of cytochrome c reduction, NADPH oxidation, and NO synthesis in a completely Ca\(^{2+}\)/CaM-independent manner.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-[2,3,4,5-\(^{3}H\)]Arginine (58 Ci/mmol), the ECL detection kit, and 2’,5’-ADP-Sepharose were products of Amersham Biosciences. (6R)-5,6,7,8-Tetrahydro-1-biotoririne (H,B) was obtained from Research Biochemical International. AG 50W-X8, cation exchange resin, Bradford protein dye reagent, and electrophoretic chemicals were products of Bio-Rad. *Spodoptera frugiperda* (Sf9) cells, baculovirus transfer vector (pVL1392) and BaculoGold viral DNA were obtained from BD Pharmingen. Grace’s insect cell culture medium was purchased from Invitrogen. NADPH, CaM (catalog no. P-1431), anti-CaM monoclonal antibody, and other reagents were obtained from Sigma.

**Expression and Determination of Nitrate/Nitrite in Sf9 Culture Media**—The cDNAs of WT-eNOS and deletion mutants (Δ45, Δ14, and Δ45/Δ14) were inserted into the EcoRI site of pVL1392 transfer vector, which was used to generate recombinant viruses in an Sf9/baculovirus system. The nitrate/nitrite accumulation in culture medium was measured using a colorimetric assay kit from Cayman Chemical Co. Ten million Sf9 cells were seeded in each T\(_{75}\) culture flask, which was incubated for 24 h at 27 °C. The supernatant was collected by using a Durotech MR5000 microplate reader, and NO\(_{3}\)/NO\(_{2}\)\(^{–}\) was quantified using NaNO\(_{3}\) as standard.

**Purification of CaM-free Proteins of WT-eNOS and Deletion Mutants**—To generate the CaM-free mutant proteins, cells were harvested at 72 h postinfection, washed twice with calcium-free phosphate-buffered saline, pH 7.2, and resuspended in Buffer A (25 mM Tris-HCl, pH 7.5, 0.2 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 mM pepstatin A, 1 mM leupeptin, 1 mM antipain, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). Cells were sonicated four times for 30 s and then centrifuged at 15,000 \(x\) g for 60 min at 4 °C. The supernatant was loaded onto a 2’,5’-ADP-Sepharose affinity column (1.5 x 5 cm) pre-equilibrated with Buffer A. The column was washed with 20 column volumes of Buffer A and then with 10 column volumes of Buffer B (25 mM Tris-HCl, pH 7.5, 0.2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 M NaCl, and 10% glycerol). The protein was eluted with Buffer B containing 20 mM 2’-AMP and concentrated by Centriprep-30 (Amicon). The concentrated protein was applied onto a gel filtration chromatography (1 x 120 cm, Ultrogel AcA34) and eluted with a buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM dithiothreitol, and 10% glycerol. SDS-PAGE and Immunoblotting—Protein concentration was estimated using an extinction coefficient of Soret absorption peak = 100 mm\(^{2}\) cm\(^{-1}\) for the NOS proteins (15) and also was determined by the method of Bradford (42). SDS-PAGE was performed on a 7.5% gel using the Laemmli procedure (43) and stained with Coomassie Blue R250. For CaM immunoblot, the purified protein (15 μg) was subjected to SDS-PAGE in a 15% gel under reducing condition and then transferred to a polyvinylidene difluoride membrane. The membrane was blotted with monoclonal antibody raised against CaM (Sigma, catalog no. C-7055). Goat anti-mouse IgG-horseradish peroxidase conjugate was used as secondary antibody detected by the ECL-CP141 (Amersham Biosciences).

**Ca\(^{2+}\)-dependent Measurement**—To measure NO activity at different free Ca\(^{2+}\) concentrations, a 100 mM stock of Ca\(^{2+}\)-EGTA (Molecular Probes, Inc.) was used to obtain the desired free Ca\(^{2+}\) solution as calculated according to manufacturer’s procedure using the K\(_{d}\) value of 10 mM and 107.9 mM at 27 °C in 10 mM Mops, pH 7.2, and 10 mM KCl. The conversion of \(L\)-[\(^{3}H\)]arginine to \(L\)-[\(^{3}H\)]citrulline was measured as described by Breit and Snyder (13) with slight modifications. The reaction mixture (100 μl) containing 10 mM MOPS, pH 7.2, 100 mM KCl, 0.3 μM calmodulin, 100 μM \(\beta\)-NADPH, 100 μM H\(_{2}\)B, 50 μM l-arginine, 1 μCi of \(L\)-[\(^{3}H\)]arginine
nine, 10% glycerol, and various concentrations of free Ca2+ was incubated with 50–100 ng enzyme at 37 °C for 5 min.

**CaM-dependent Measurement**—CaM-dependent NOS activity was tested at 37 °C for 5 min by measuring t-arginine conversion to citrulline formation in a reaction mixture containing 10 mm MOPS, pH 7.2, 100 mm KCl, 3.9 mm free Ca2+ (prepared from 100 mm stock of Ca8+-EGTA, Molecular Probes, Inc.), 100 μM t-arginine, 50 μM free Ca2+, and various concentrations of CaM.

**Steady-state Catalysis**—NO synthesis, NADPH oxidation, cytochrome c reduction, and ferricyanide oxidation were determined by measuring the optical absorbance change using a Shimadzu-2501 PC spectrometer equipped with a temperature controller. A 10-mm light path cuvette was used. The amount of reduced ferricyanide was quantified from the NO-mediated conversion of oxyhemoglobin to met-hemoglobin by monitoring the absorbance increase at 401 nm using an extinction coefficient of 6.22 mM cm−1.

**Nitrite/Nitrate Accumulation in Sf9 Culture Medium**—Studies have shown that cells expressing iNOS spontaneously produce a large quantity of NO measured as NO2−/NO3− in their culture medium at a resting state of Ca2+, whereas cells expressing eNOS produces only a trace amount of nitrate in the absence of agonists (19). To determine whether the deletion mutants behaved like iNOS, the constructs of wild-type and mutant eNOS were expressed in Sf9 cells under identical conditions for 72 h. Western blot with anti-eNOS antibody was used to estimate the amount of protein expressed and showed that both Δ45 and Δ45/Δ14 were expressed at a level approximately half that of wild-type and Δ45/Δ14eNOS (data not shown). Even with this constraint, the total amount of NO2−/NO3− from cells expressing Δ45 and Δ45/Δ14 was higher than that from cells expressing Δ14, respectively, whereas cells expressing wild-type eNOS was barely detectable (Fig. 2). The results suggest that all mutants with either a single deletion or a combined deletion of residues 594–606/614 and residues 1165–1178, respectively, from human eNOS. A combined deletion mutant (Δ45/Δ14) was also constructed.

**RESULTS**

**Nitrate/Nitrate Accumulation in Sf9 Culture Medium**—Studies have shown that cells expressing iNOS spontaneously produce a large quantity of NO measured as NO2−/NO3− in their culture medium at a resting state of Ca2+, whereas cells expressing eNOS produces only a trace amount of nitrate in the absence of agonists (19). To determine whether the deletion mutants behaved like iNOS, the constructs of wild-type and mutant eNOS were expressed in Sf9 cells under identical conditions for 72 h. Western blot with anti-eNOS antibody was used to estimate the amount of protein expressed and showed that both Δ45 and Δ45/Δ14 were expressed at a level approximately half that of wild-type and Δ45/Δ14eNOS (data not shown). Even with this constraint, the total amount of NO2−/NO3− from cells expressing Δ45 and Δ45/Δ14 was higher than that from cells expressing Δ14 (81, 37, and 62 μM for Δ45, Δ45/Δ14, and Δ14, respectively), whereas that from cells expressing wild-type eNOS was barely detectable (Fig. 2). The results suggest that all mutants with either a single deletion or a combined deletion of residues 594–606/614 and residues 1165–1178, respectively, from human eNOS. A combined deletion mutant (Δ45/Δ14) was also constructed.

**Nitrate/Nitrate Accumulation in Sf9 Culture Medium**—Studies have shown that cells expressing iNOS spontaneously produce a large quantity of NO measured as NO2−/NO3− in their culture medium at a resting state of Ca2+, whereas cells expressing eNOS produces only a trace amount of nitrate in the absence of agonists (19). To determine whether the deletion mutants behaved like iNOS, the constructs of wild-type and mutant eNOS were expressed in Sf9 cells under identical conditions for 72 h. Western blot with anti-eNOS antibody was used to estimate the amount of protein expressed and showed that both Δ45 and Δ45/Δ14 were expressed at a level approximately half that of wild-type and Δ45/Δ14eNOS (data not shown). Even with this constraint, the total amount of NO2−/NO3− from cells expressing Δ45 and Δ45/Δ14 was higher than that from cells expressing Δ14 (81, 37, and 62 μM for Δ45, Δ45/Δ14, and Δ14, respectively), whereas that from cells expressing wild-type eNOS was barely detectable (Fig. 2). The results suggest that all mutants with either a single deletion or a combined deletion of residues 594–606/614 and residues 1165–1178, respectively, from human eNOS. A combined deletion mutant (Δ45/Δ14) was also constructed.
To further determine whether the WTeNOS and mutant enzymes contained an endogenous CaM, the purified proteins were subjected to SDS-PAGE followed by immunoblot with anti-CaM monoclonal antibody. None of the proteins purified in the presence of EGTA and EDTA contained an intrinsic CaM (Fig. 3B, lanes 1–4), whereas a Δ45 protein prepared in the absence of chelators and run parallel in SDS-PAGE had a detectable CaM (Fig. 3B, lane 5).

Requirement for Ca^{2+} and CaM in l-Citrulline Formation—Dependence of l-citrulline formation on CaM was titrated by adding increasing concentrations of CaM (Sigma, catalog no. P-1431) along with 39 μM free Ca^{2+} and other cofactors in the reaction mixtures. CaM concentration response curves for wildtype and mutant eNOS are shown in Fig. 4A. The CaM-free Δ45 exhibited a constitutive activity in the absence of CaM, which was increased by increasing CaM concentration, whereas WTeNOS and Δ14 had a very low basal activity and required about 85 and 58 nM CaM, respectively, to reach half-maximal activity (EC_{50}). Interestingly, Δ45/Δ14 was active in the absence of CaM, and its activity was reduced by about 25% in the presence of saturating Ca^{2+}/CaM.

Dependence on [Ca^{2+}] was similarly titrated in the presence of 300 nM CaM, and the response curves are shown in Fig. 4B. The desired concentration of free Ca^{2+} was obtained by adding varied ratios of K-EGTA and Ca^{2+}-EGTA as described previously (24). In the absence of Ca^{2+}, Δ45eNOS had a considerable level of activity, which was increased by exogenous Ca^{2+}. WTeNOS was inactive without added Ca^{2+}, and the EC_{50} for Ca^{2+} was estimated to be ~160 nM. The activity of the Δ14 mutant was barely detectable in the absence of Ca^{2+}, and the EC_{50} value was 90 nM. In contrast, the Δ45/Δ14 mutant was fully active in the absence of calcium, and the activity was reduced by 20% at saturating CaM/Ca^{2+}. The Ca^{2+} and CaM response curves for all of the mutants coincided (Fig. 4A versus B).

These results indicated that Δ45/Δ14 became constitutively active and did not require Ca^{2+}/CaM for l-citrulline formation.

Steady-state Enzymatic Activities of WTeNOS Versus Deletion Mutants—The electron flow in NOS is from NADPH to FAD, to FMN, and finally to the oxygenase heme. The steady-state catalysis of each subdomain, analyzed in the absence or presence of CaM, was expressed in nanomoles of product formation/nanomole of heme/min (min^{-1}).

Ferricyanide Reduction—The NOS is able to reduce artificial electron acceptors such as cytochrome c and ferricyanide. Ferricyanide accepts electrons either from FAD or FMN, whereas cytochrome c accepts electrons exclusively from FMN (46). Ferricyanide reduction thus provides a useful tool for evaluating the effect of site-directed deletion on the FAD subdomain activity. As shown in Fig. 5A, the basal rate of ferricyanide reduction for all mutants was 1.6-fold higher than that of WTeNOS (2900 versus ~4700 min^{-1} for WTeNOS versus all mutants, respectively). In contrast, the CaM-stimulated maximal rates (6300–6700 min^{-1}) were similar for wild type and all mutants, indicating that the effect of deletion on ferricyanide reduction is more pronounced for the CaM-free than the CaM-bound state.
Cytochrome c Reduction—As electron transfer to cytochrome c occurs exclusively from FMN, cytochrome c assays were performed to determine the effects of mutating these elements on FMN subdomain (Fig. 5B). Unlike ferricyanide reduction, all mutants displayed the maximal rates of cytochrome c reduction severalfold greater than WTeNOS either in the CaM-free or CaM-bound enzyme. The addition of CaM increased cytochrome c reduction by 4.5-fold for both WTeNOS (90 versus 434 min⁻¹ in the absence and presence of CaM, respectively) and Δ14 (402 versus 1573 min⁻¹ in the absence and presence of CaM, respectively), whereas CaM slightly enhanced the rate of Δ45 (731 versus 787 min⁻¹ in the absence and presence of CaM, respectively) and decreased the rate of Δ45/Δ14 by 25% (1389 versus 1059 min⁻¹ in the absence and presence of CaM, respectively). The results suggest that these two peptide regions play a role in controlling electron flow from the FMN moiety to the heme of cytochrome c.

NADPH Oxidation—NADPH is oxidized and consumed after electron flux from reductase to oxygenase domain where heme is reduced with O₂ as an electron acceptor (47). We measured the rate of NADPH oxidation in wild-type and mutant eNOSs (Fig. 6A). For mutant Δ45, the rate of NADPH oxidation was increased 1.6-fold in the presence of CaM (33 versus 54 min⁻¹). The basal rate of Δ14 was slightly higher than that of WTeNOS (9.8 versus 4.2 min⁻¹) but was markedly increased by the presence of CaM, at a rate far exceeding that of wild type (108 versus 32 min⁻¹). In the absence of CaM, the rate of Δ45/Δ14 was 18-fold higher than that of wild type (75 versus 4.2 min⁻¹), but its rate was reduced by 36% when CaM was present (48 min⁻¹). Changes in NADPH oxidation by deletion of these two regions parallel those in cytochrome c reduction (Fig. 6A versus Fig. 5B), suggesting that the control mechanism of these two peptide regions for electron flow from FMN to oxygenase domain is similar to that from FMN to an artificial electron acceptor, cytochrome c.

NO Synthesis—We next determined whether a higher rate of electron flow in the mutants correlated with a higher efficiency of NO production. We compared the rate of NO production between each mutant and WTeNOS. As shown in Fig. 6B, WTeNOS and Δ14 produced a very low level of NO barely
beyond the detection limit in the absence of CaM. With CaM, the maximal NO synthesis of \( \Delta 14 \) was 2.5-fold higher than that of wild type (61 versus 27 min\(^{-1}\)). In contrast, \( \Delta 45 \) and \( \Delta 45/\Delta 14 \) produced a significant amount of NO (17 versus 36 min\(^{-1}\) for \( \Delta 45 \) and \( \Delta 45/\Delta 14 \), respectively) in the absence of CaM. Upon the addition of CaM, there was a 1.8-fold increase in NO synthesis by \( \Delta 45 \) (32 min\(^{-1}\)) and a 40% reduction by the double mutant \( \Delta 45/\Delta 14 \) (23 min\(^{-1}\)). The rate of NO production in all three mutants is generally correlated with the rates of electron transfer from FMN to cytochrome c and oxygenase heme.

**FIG. 5.** Ferricyanide reduction (A) and cytochrome c reduction (B) by WT eNOS and deletion mutants. The ferricyanide and cytochrome c reductions were determined as described under “Experimental Procedures.” Each bar represents the mean ± S.D. of triplicate experiments.
DISCUSSION

The molecular basis for the dramatic difference of calcium sensitivity in enzyme activation between the cNOSs and iNOS has not been fully elucidated. Three segments including the CaM binding sequence, the autoinhibitory loop in the FMN subdomain, and a C-terminal extension tail were reported to play a pivotal role in regulating Ca\(^{2+}\)/CaM-mediated electron flux. Modifications at those sites affect enzyme activity by reducing Ca\(^{2+}\) dependence relative to wild-type cNOSs. However, the CaM-binding domain, the autoinhibitory element, or the C-terminal tail alone does not confer Ca\(^{2+}\)-independent activity similar to that of iNOS. Here we have focused on the aforementioned two regions (residues 594–606/614–645 and residues 1165–1178 in human eNOS) that exist in the reductase domain of cNOSs but have no counterpart in iNOS. We made the constructs by deleting either or both fragments from eNOS to mimic iNOS. Our prior work with deletion of residues 594–606/614–645 yields a mutant enzyme (Δ45) containing a trapped CaM isolated from an Sf9/baculovirus system, and because of this it is not clear whether the 45-residue segment actually serves a regulatory role in CaM dependence. We thus prepared CaM-free enzymes by adding an adequate amount of chelators during the purification process. The availability of CaM-free wild-type and mutant eNOSs allowed us to evaluate the effect of Δ45 and Δ14 mutations on CaM-dependent electron transfer and catalytic properties.

CaM-free Δ45 had a lower heme content and lower catalytic activities than CaM-bound Δ45 (24), indicating that the trapped CaM stabilized the mutant protein. Steady-state analysis demonstrated that Δ45 in the absence of CaM catalyzed ferricyanide reduction, NADPH oxidation, and NO synthesis at rates higher than wild type. However, Δ45 was regulated by Ca\(^{2+}\)/CaM. Ca\(^{2+}\)/CaM increased the rates by 30–40% over those in the absence of Ca\(^{2+}\)/CaM. The rate of cytochrome c

![Fig. 6. NADPH oxidation (A) and NO synthesis (B) by WT eNOS and deletion mutants. NADPH oxidation and NO synthesis were measured as described under “Experimental Procedures.” Each bar represents the mean ± S.D. of triplicate experiments.](image-url)
reduction of Δ45 was higher than wild type, but Ca\(^{2+}\)/CaM had little influence on the rate of cytochrome c reduction by Δ45. This is consistent with our previously published study (24) as well as that of Nishida and Ortiz de Montellano (21). Taken together, the results indicate that Δ45 deletion significantly increased Ca\(^{2+}\)-sensitivity with a parallel reduction in CaM requirement for electron transfer and catalytic activity. Thus, residues 594–606/614–645 control Ca\(^{2+}\)/CaM binding and repress electron transfer.

The Δ14eNOS with deletion of residues 1165–1178 did not show Ca\(^{2+}\)/CaM-independent activity but required a lower Ca\(^{2+}\)/CaM concentration in achieving maximal activity than WT-eNOS. This is also supported by the spontaneous accumulation of a considerable amount of NO\(_2\)/NO\(_3\) in the culture medium of Si9 cells expressing Δ14, indicating that the Δ14 segment partially confers Ca\(^{2+}\) requirement in eNOS catalysis. Truncation of Δ14 increased CaM-induced NO synthesis and both basal and CaM-bound cytochrome c reduction 2–4-fold over wild type. CaM-stimulated NO synthesis and NADPH oxidation by Δ14eNOS approach those of CaM-bound nNOS (21); thus the Δ14-segment can be inferred to negatively control electron transfer through enzymes (36) that contributes to the relatively low catalytic activities of eNOS. Analysis of an x-ray crystal structure of the nNOS FAD domain lacking the C-terminal 22 residues (48) has revealed that the adjacent C-terminal 10 residues are disordered in the crystal structure, suggesting that the C-terminal tail probably lies between the flavins and/or between the FAD and NADPH, modulating the electron transfer into and out of FMN module. We extended the flavins and/or between the FAD and NADPH, modulating the electron transfer through enzymes (36) that contributes to the relatively low catalytic activities of eNOS. Analysis of an x-ray crystal structure of the nNOS FAD domain lacking the C-terminal 22 residues (48) has revealed that the adjacent C-terminal 10 residues are disordered in the crystal structure, suggesting that the C-terminal tail probably lies between the flavins and/or between the FAD and NADPH, modulating the electron transfer into and out of FMN module. We extended

The above described studies demonstrated that the Δ45 exhibited a considerable amount of reductase and oxygenase activities in the absence of Ca\(^{2+}\)/CaM, whereas Δ14 was largely enhanced by CaM in those reactions. However, the rates of cytochrome c reduction, NADPH oxidation and NO synthesis catalyzed by Δ14 were 2–4-fold higher than those catalyzed by Δ45 and wild type in the presence of CaM. The result supports the model of Roman et al. (36) that the Δ45-segment plays a pronounced role in regulating Ca\(^{2+}\)/CaM-dependence for inter-domain activation, whereas Δ14 is involved in limiting the rate of electron transfer into and out of FMN module. We extended our finding by combined deletion of both fragments, resulting in a mutant enzyme (Δ45/Δ14) with the basal rates of cytochrome c reduction, NADPH oxidation, and NO synthesis 2–4-fold over CaM-induced wild type. Intriguingly, CaM binding to this double mutant was observed to inhibit rather than enhance those reactions, indicating a completely Ca\(^{2+}\)/CaM-independent electron transfer between FMN and the hemes of cytochrome c and eNOS oxygenase domain. This observation appears to contradict the viewpoint that CaM binding to its canonical binding site is essential for electron transfer from the reductase to oxygenase domain. Our results are consistent with the recent catalytic model (36). In the absence of CaM, the Δ45 autoinhibitory loop and C-terminal tail function as barriers sitting near each side of flavin wall and/or between the FAD and NADPH, that almost completely block the electron transfer; therefore there is almost no detectable NO synthesis activity in CaM-free WTeNOS. When two segments are deleted, the barriers are removed such that the rate of electron transfer is greatly enhanced, even exceeding the rate of CaM-induced WTeNOS. The binding domains of these two elements on eNOS might either overlap or allosterically interact with CaM binding site(s). Once CaM binds to wild type, the CaM-driven conformational change partially lifts the inhibitory inserts to allow limited electron transfer within the reductase domain and from the reductase to the oxygenase domain. In fact, the wild-type and the double mutant (Δ45/Δ14) enzymes in the CaM-bound state are very similar in ferricyanide reduction, NADPH oxidation, and NO synthesis, indicating that the addition of CaM to the double mutant appears to retighten a loosened electron transfer, and those activities are reduced back to normal wild-type levels. Therefore, the primary mechanism of CaM to gate electron flux is to tether the redox partners of the interdomain or intradomain close together for electron transfer despite the presence or absence of these two inhibitory elements. Three-dimensional structure information on the reductase domains of eNOSs is essential to define how the interaction among CaM, the autoinhibitory loop, and the C-terminal tail regulates eNOS catalysis.

REFERENCES
1. Förstermann, U., H. H. W.Schmidt, H. H. W., Pollock, J. S., Sheng, H., Mitchell, J. A., Warner, T. D., Nakane, M., and Murad, F. (1991) Biochem. Pharmacol. 42, 1849–1857
2. Nishida, C. (1992) FEBS Lett. 3051–3064
3. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1141–1145
4. Stuehr, D. J., and Griffith, O. W. (1990) Adv. Enzymol. 65, 287–346
5. Marletta, M. A. (1993) J. Biol. Chem. 268, 12231–12234
6. Sheta, E. A., McMillan, K., and Masters, B. S. (1994) J. Biol. Chem. 269, 15147–15153
7. Ghosh, D. K., and Stuehr, D. J. (1995) Biochemistry 34, 801–807
8. Chen, P.-F., Tsai, A.-L., Berka, V., and Wu, K. K. (1996) J. Biol. Chem. 271, 14153–14159
9. Abu-Soud, H. M., and Stuehr, D. J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10769–10772
10. Pollock, J. S., Förstermann, U., Mitchell, J. A., Warner, T. D., Harald, H. H., Schmidt, H. H. W., Nakane, M., and Murad, F. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10480–10484
11. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
12. Xie, Q. W., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T., Nathan, C. (1992) Science 256, 225–228
13. Bredt, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 682–685
14. Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C. (1992) J. Biol. Chem. 267, 599–604
15. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 273, 5566–5571
16. Raman, S. C., Li, H., Martasek, P., Kral, V., Masters, B. S., and Pouls, T. L. (1998) Cell 95, 839–850
17. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
18. Ruan, J., Xin, Q., Hu, C., Wolfe, G. C., and Nathan, C. (1996) J. Biol. Chem. 271, 22679–22686
19. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777
20. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 274, 14692–14698
22. Daff, S., Sagami, S., and Shimizu, T. (1999) *J. Biol. Chem.* **274**, 30589–30595
23. Montgomery, H. J., Romanov, V., and Guillemette, J. G. (2000) *J. Biol. Chem.* **275**, 5052–5058
24. Chen, P.-F., and Wu, K. K. (2000) *J. Biol. Chem.* **275**, 5052–5058
25. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *Nature* **399**, 597–601
26. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) *Nature* **399**, 597–601
27. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) *J. Biol. Chem.* **274**, 30589–30595
28. Montgomery, H. J., Romanov, V., and Guillemette, J. G. (2000) *J. Biol. Chem.* **275**, 5052–5058
29. Haynes, M. P., Sinha, D., Russell, K. S., Collinge, M., Fulton, D., Morales-Ruiz, M., Sessa, W. C., and Bender, J. R. (2000) *Circ. Res.* **87**, 677–682
30. Montagnani, M., Chen, H., Barr, V. A., and Quon, M. J. (2001) *J. Biol. Chem.* **276**, 12420–12426
31. Montagnani, M., Chen, H., Barr, V. A., and Quon, M. J. (2001) *J. Biol. Chem.* **276**, 30392–30398
32. Harris, M. B., Ju, H., Venema, V. J., Liang, H., Zou, R., Michell, B. J., Chen, Z. P., Kemp, B. E., and Venema, R. C. (2001) *J. Biol. Chem.* **276**, 16587–16591
33. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) *J. Biol. Chem.* **275**, 6123–6128
34. Roman, L. J., Miller, R. T., de La Garza, M. A., Kim, J. J., and Masters, B. S. (2000) *J. Biol. Chem.* **275**, 21914–21919
35. Roman, L. J., Martasek, P., Miller, R. T., Harris, D. E., de La Garza, M. A., Shea, T. M., and Masters, B. S. (2000) *J. Biol. Chem.* **275**, 29225–29232
36. Lane, P., and Gross, S. S. (2002) *J. Biol. Chem.* **277**, 19087–19094
37. Janssen, S. P., Shimouchi, A., Quartermous, T., Bloch, D. E., and Bloch, K. D. (1992) *J. Biol. Chem.* **267**, 14519–14522
38. Nakane, M., Schmidt, H. H., Pollock, J. S., Förstermann, U., and Murad, F. (1993) *FEBS Lett.* **316**, 175–180
39. Geller, D. A., Lowenstein, C. J., Shapiro, R. A., Nussler, A. K., Di Silvio, M., Wang, S. C., Nakayama, D. K., Simmons, R. L., Snyder, S. H., and Bilias, T. R. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 3491–3495
40. Lane, P., and Gross, S. S. (2002) *J. Biol. Chem.* **277**, 19087–19094