Characterization of the Roles of the 594–645 Region in Human Endothelial Nitric-oxide Synthase in Regulating Calmodulin Binding and Electron Transfer*

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It has been postulated that a segment (residues 594–645) inserted in the FMN subdomain of human endothelial nitric-oxide synthase (eNOS) plays a crucial role in controlling Ca\(^{2+}\)-dependent CaM binding for eNOS activity. To investigate its functions, we expressed human eNOS in a baculovirus system with deletion of a 45-residue segment from this region (residues 594–606 and 614–645, designated as Δ45eNOS), and characterized the purified mutant enzyme. In contrast with wild-type eNOS, Δ45eNOS exhibited characteristics resembling inducible NOS (iNOS). It contained an endogenously bound CaM, which was essential in folding and stabilizing this mutant enzyme, and retained 60% of L-citrulline formation in 5 mM EGTA. We also produced four N-terminally truncated reductase domains with or without the 45-residue segment, and either including or excluding the CaM-binding sequence. Basal cytochrome c reductase activity of reductase domains without the 45-residue segment was up to 20 fold greater than that of corresponding insert-containing domains, and higher than CaM-stimulated activity of the wild-type enzyme. A series of mutants with smaller fragment deletion in this region such as Δ594–604, Δ605–612, Δ613–625, Δ626–634, Δ632–639, and Δ640–645 mutants were further characterized. The crude lysate of mutants Δ613–625 and Δ632–639 did not show activity in the presence of Ca\(^{2+}\)/CaM, while other four mutants had activity comparable to that of WT eNOS. The purified Δ594–604 and Δ605–612 proteins had a 3–5-fold higher affinity for Ca\(^{2+}\)/CaM, but their L-citrulline forming activity was still 80% dependent upon the addition of Ca\(^{2+}\)/CaM. Both mutants exhibited a low level of the cytochrome c and ferricyanide reductase activities, which either did not respond to (Δ594–604) or slightly enhanced by (Δ605–612) the exogenous CaM. In contrast, activities of Δ626–634 and Δ640–645 like those of WT eNOS were largely Ca\(^{2+}\)/CaM-dependent. Thus, our findings indicate that the N-terminal half of the 594–645 segment containing residues 594–612 plays a significant role in regulating Ca\(^{2+}\)/CaM binding.

Nitric oxide (NO) is an important signal mediator in diverse physiological and pathological events of mammals (1–3). This molecule is coproduced with L-citrulline from L-arginine by three different nitric-oxide synthase (NOS) isoforms, all of which share similar biochemical composition and enzymatic characteristics, and possess a bidomain structure (4–8). The C-terminal half (reductase domain) contains the NADPH-, FAD-, and FMN-binding sites and the N-terminal half (oxygenase domain) contains the heme-, BH\(_4\)-, and L-arginine-binding sites (9–11). These two domains are connected by a calmodulin-binding region (6, 8).

Despite structural similarities, the NOS isoforms can be grouped into two distinct classes according to their mode of expression and dependence on Ca\(^{2+}\). Constitutive NOS (cNOS) isoforms including endothelial NOS (eNOS) and neuronal NOS (nNOS), are constitutively expressed as latent enzymes that require a higher concentration of Ca\(^{2+}\) for CaM binding and enzyme catalysis (12–13). In contrast, cytokine-induced NOS (iNOS) is active at a basal level of intracellular [Ca\(^{2+}\)], presumably due to high affinity of CaM binding. The Ca\(^{2+}\)/CaM-dependent NOS activity was assumed to be an intrinsic property determined by the primary sequences at the CaM-binding region. However, sequence analysis showed that all isoforms contained a similar canonical CaM-binding sequence rich in basic and hydrophobic residues, typical for Ca\(^{2+}\)/CaM-dependent proteins (14). Furthermore, chimeric eNOS and nNOS in which the CaM-binding region was replaced with the corresponding region of murine iNOS (residues 503–532) did not exhibit fully Ca\(^{2+}\)-independent NO activity (15–18).

Ruan et al. (15) performed truncation analysis and found an additional region spanning from residues 484–726 in murine iNOS, which was necessary for Ca\(^{2+}\)-independent NO activity. Sequence alignment among NOS isoforms suggests that a 50-residue segment present in the FMN-binding domain of human eNOS (residues 594–645) and nNOS (residues 834–882) but absent in that of iNOS represents a putative autoinhibitory element that impedes CaM binding to cNOSs at the basal level of intracellular [Ca\(^{2+}\)], a feature common to many Ca\(^{2+}\)/CaM-dependent proteins (19). This notion was supported by inhibition of CaM binding and catalytic activities of eNOS and nNOS with synthetic peptides corresponding to this segment of eNOS, and reversal of inhibition by exogenous CaM (20). However, it remains unclear how the presence of this segment in the constitutive NOS isoforms affects the Ca\(^{2+}\)/CaM dependence, and whether the segment has other roles in function of intact eNOS. To address these issues, we prepared a series of mutants by deleting this segment to various extent

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The abbreviations used are: NOS, nitric-oxide synthase; BH\(_4\), (6R, 5,6,7,8-tetrahydro-L-biopterin); CaM, calmodulin; DTT, dithiothreitol; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; PAGE, polyacrylamide gel electrophoresis; WT, wild-type.
from eNOS as well as N-terminally truncated reductase domains with or without this segment and CaM-binding sequence. The effects of various mutants on Ca\(^{2+}/\text{CaM}\) dependence, catalytic activities, and electron transfer are evaluated in this study.

**EXPERIMENTAL PROCEDURES**

**Materials—**arginine (58 Ci/mmol), ECL detection kit, CaM-Sepharose, and 2',5'-ADP-Sepharose products of Amersham Pharmacia Biotech. (6R)-S,6L,7S,8S-Tetrahydro-1-biotin (BH\(_4\)) 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 (Si9) cells, baculovirus transfer vector (pVL1392), and Baculoviral viral DNA were obtained from Pharmingen. Grace's insect cell culture medium was purchased from Life Technologies Inc. NADPH, CaM, anti-CaM monclonal antibody, and other reagents were purchased from Sigma.

**Constructs—**Fig. 1A shows alignment of a fragment among three human isoforms (21–23), cytochrome P450 reductase (24), and bacterial P450\(_{218}\) (25). An insertion of a 45-amino acid segment, which is located in the FMN subdomain (Fig. 1B), represents a major sequence change among the three isoforms. Fig. 1C is a schematic diagram showing the proteins that were constructed, expressed, and purified. The 45-amino acid insert was deleted by the method of polymerase chain reaction. Two steps of deletion were performed. The fragment (residues 614–645) of eNOS was first deleted by using two pairs of primer: 5'-TTCGTTGCTGTTGAAACGC and 5'-AAATGAATATGATATCTGTTG, and 5'-CTGTTCCGGACGAGGGGAGCTGTTGTAG and 5'-ATCTTATAACTCTTGTG, and 5'-TCAGTGGGCGCCGGTCC. The second reaction (residues 594–606) was then further deleted from the first deleted mutant by using two pairs of primer: 5'-GGAACGAGACGCTGGTTGCT and 5'-CCCTCATATTGCGTCTGAAAGCTCT-CC, and 5'-TATAATCCGGAATTCAACAGCATCTCCTGCA, and 5'-TATAATCCGGAATTCAACAGCATCTCCTGCA, and 5'-GGATGTTCCGGACGAGGGGAGCTGTTGTAG.

**Determination of Nitrate/Nitrite in Culture Medium—**To measure nitrate/nitrite in culture medium, was measured using a colorimetric assay kit from Cayman Chemical Co. (Alexis, San Diego, CA). Ten million Si9 cells were seeded in each 75 cm\(^2\) culture flask, each of which was cotransfected with the pgroESL or pACYC184-CaM plasmid (a generous gift from Dr. E. E. Strehler) in BL21 E. coli cells showing the proteins that were constructed, expressed, and purified.

**Assays of Enzyme Activity—**NOS activity was assayed by measuring conversion of L-[\(^3\)H]arginine to L-[\(^3\)H]citrulline as described by Bredt et al. (6) with slight modification. The reaction mixture containing 25 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 0.5 mM L-arginine, 10 mM 

**Comparison of Ca\(^{2+}/\text{CaM}\) Dependence—**To measure NOS activity at different 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 Ca\(^{2+}\)-(EGTA) = 43.7 mM at 37 °C in 25 mM Tris-HCl, pH 7.4, and 0.1 mM KCl.
Characterization of the Regulatory Element in eNOS

RESULTS

Sequence alignment shows that human eNOS contains a 45-residue insert (residues 594–606 and residues 614–645, Fig. 1A) in the FMN subdomain (Fig. 1B), which is also present in nNOS but absent in iNOS or other related flavoproteins. An eNOS mutant with the deletion of this 45-amino acid segment designated as Δ45eNOS (Fig. 1C) was constructed and expressed in Sf9 cells. When a comparable amount of protein was expressed, the NO2-/NO3- concentration in the cultured medium of the Sf9 cells 74 h after infection with Δ45eNOS recombinant viruses was about 50-fold higher than that produced by Sf9 cells infected with WTeNOS recombinant viruses (82 μmol/min/mg versus 1.5 μmol/min/mg). These results suggest that Δ45eNOS is active at the basal level of intracellular [Ca2+] (Fig. 2).

WTeNOS and Δ45eNOS proteins were purified to near homogeneity and shown to have molecular masses of 133 and 123 kDa, respectively (Fig. 3, lanes 1 and 2). The absorbance spectrum of purified Δ45eNOS exhibited a Soret peak at ~401 nm, a charge-transfer band at 647 nm, and a flavin absorbance shoulder between 450 nm and 475 nm (Fig. 4A). Addition of 1 mM imidazole shifted the Soret peak to 428 nm, and the low spin heme-imidazole complex could be converted back to high spin heme (397 nm) upon addition of 1 mM L-arginine (Fig. 4A). The Δ45eNOS displayed spectral peaks at 401 nm, 594–645, and 594–606 (Fig. 4A, inset). The dithionite-reduced, CO-saturated Δ45eNOS displayed spectral peaks at 445 and 550 nm (Fig. 4B). These spectral properties are similar to those of WTeNOS (30), indicating that deletion of 45 residues did not perturb the heme electronic environment.

The cofactor requirement of purified Δ45eNOS (Fig. 5, hatched bar) was compared with that of WTeNOS (Fig. 5, filled bar). In the presence of all essential cofactors, the specific activity of l-citrulline formation was 170 nmol/min/mg for Δ45eNOS versus 130 nmol/min/mg for WTeNOS. Omission of BH4 resulted in a 65–75% reduction in l-citrulline formation.
for both WTeNOS and Δ45eNOS, with the residual activity presumably due to the endogenous BH4 in the purified proteins. However, omission of CaM from the reaction mixture completely eliminated l-citrulline formation for WTeNOS without an apparent effect for Δ45eNOS. It was reported that murine iNOS retained 50–85% of enzyme activity in 5 mM EGTA (15–16, 18), whereas eNOS activity was lost in the presence of 2 mM EGTA (5, 16). Catalytic activity was thus measured in the presence of 5 mM EGTA. No activity was detected for the WTeNOS, but ~60% of activity was still retained for Δ45eNOS. This behavior indicates that the activity of Δ45eNOS is largely Ca2+/CaM-independent and WTeNOS is fully Ca2+/CaM-dependent (Fig. 5).

To determine whether the Ca2+/CaM independence of Δ45eNOS was attributed to an intrinsically bound CaM, the purified proteins were subjected to SDS-PAGE, followed by immunoblot with anti-CaM monoclonal antibody. An endogenously bound CaM was found in Δ45eNOS but not in WTeNOS (Fig. 6, lanes 1 and 2). We have tried to obtain CaM-free Δ45eNOS to test whether Δ45eNOS could generate NO in the absence of CaM by using a bacterial expression system. WTeNOS and Δ45eNOS were cloned into a bacterial expression pCW ori vector, which was cotransfected with the pgroESL or pACYC184-CaM plasmid into BL21 E. coli cells. WTeNOS purified from either system was similar and active, whereas Δ45eNOS had to be coexpressed with pACYC184-CaM, suggesting that Δ45eNOS requires CaM to properly fold and stabilize its conformational structure (data not shown).

In order to elucidate the reciprocal relationship between the 45-residue segment and canonical CaM-binding region, we next prepared N-terminally truncated constructs of the eNOS reductase domains containing FMN- and FAD-binding regions, and either including (CaM/FMN/FAD construct, residues 482–1204) or excluding (FMN/FAD construct, residues 511–1204) the CaM-binding sequence. Two other reductase domains were constructed by deleting this 45-residue segment from the CaM/FMN/FAD construct (Δ45CaM/FMN/FAD) and the FMN/FAD construct (Δ45FMN/FAD). These four reductase domains were expressed in the S9/baculovirus system and purified to near homogeneity (Fig. 3). We generally obtained about 30 mg of purified reductase domains from 4 × 10⁹ S9 cells. The apparent molecular masses on SDS-PAGE analysis were ~80 kDa (CaM/FMN/FAD), ~75 kDa (Δ45CaM/FMN/FAD), ~77 kDa (FMN/FAD), and ~72 kDa (Δ45FMN/FAD) (Fig. 3, lanes 3–6). An endogenously bound CaM was not detected in these purified domains (Fig. 6, lanes 3–6).

Each of the constructs displayed almost a 1:1 FAD to FMN ratio (~0.8 eq) (data not shown), indicating that removal of the 45-residue segment did not perturb FMN binding affinity. The absorbance spectra of the purified reductase domains had absorption peaks at 454 and 381 nm (Fig. 7, A–D). Both CaM/FMN/FAD (Fig. 7A) and FMN/FAD (Fig. 7C) domains, which contained the 45-residue segment exhibited an additional prominent peak at 590 nm in the resting state, attributable to an air-stable flavin semiquinone radical, whereas both domains without the 45-residue segment (Δ45CaM/FMN/FAD and Δ45FMN/FAD) did not exhibit the semiquinone peak in the resting state (Fig. 7, B and D). Addition of NADPH produced a spectrum characteristic of the air-stable semiquinone form in all reductase domains (Fig. 7, A–D). Presumably this portion is due to partial reduction of FMN or FAD in contrast to a complete reduction to flavin hydroquinone by dithionite.

The NADPH-dependent electron transfer rates to ferricyanide and cytochrome c were determined using the purified proteins in the absence (basal) or presence of Ca2+/CaM (Table I). Cytochrome c and ferricyanide reductase activities in the WTeNOS were increased 3–5-fold in the presence of CaM. Both activities in Δ45eNOS did not respond to exogenous CaM and were ~2-fold higher than CaM-stimulated activities of WTeNOS. CaM/FMN/FAD domain (residues 482–1204) displayed a similar rank order of basal and CaM-stimulated ferricyanide reductions. The cytochrome c reduction of this domain was enhanced 3.5 times by addition of Ca2+/CaM. Basal and CaM-stimulated cytochrome reductions in the CaM/FMN/FAD domain were both 4-fold higher than that of WTeNOS and also higher than that previously reported for a slightly shorter reductase domain (residues 491–1204, Ref. 9). The reason for the later difference is unknown. The corresponding reductase domain lacking the 45-residue insert (Δ45CaM/FMN/FAD) had an even higher ferricyanide reductase activity, which was slightly stimulated by CaM. Adding Ca2+/CaM to this domain enhanced cytochrome c reduction by 3-fold. The FMN/FAD reductase domain had a low level of ferricyanide and cytochrome c reduction rates, which were not significantly changed by CaM, consistent with its not containing a CaM-binding
sequence. The D45FMN/FAD reductase domain had ferricyanide and cytochrome c reductase activities which were 3- and 20-fold, respectively, greater than those of the parent FMN/FAD domain, and which was not enhanced by exogenously added CaM.

The functional significance of this segment was further evaluated using a series of mutants with smaller fragment deletion in this region such as D594–604, D605–612, D613–625, D626–634, D632–639, and D640–645. We expressed these mutants in the Sf9/baculovirus system. At 72 h after infection with recombinant baculoviruses, the concentration of NO22/NO32 in the culture media was determined. The D594–604 and D605–612 mutants produced a considerable quantity of NO22/NO32 close to D45eNOS, while the other mutants produced either undetectable (mutant D613–625 and D632–639) or a small quantity (mutants D626–634 and D640–645) of NO22/NO32 similar to WTeNOS (Fig. 8A), indicating that like D45eNOS, mutants D594–604 and D605–612 are active at the basal level of intracellular [Ca2+]. The L-citrulline formation of cell lysates of each mutant was determined in the presence of optimal concentration of Ca2+/CaM and other essential cofactors. The results show that all the mutants except D613–625 and D632–639 are at least as active as WTeNOS. Mutants D613–625 and D632–639 lost the activity completely despite equivalent expression of the proteins (Fig. 8B), suggesting a global conformational change in these two mutants; they were not further studied. Four active mutants (D594–604, D605–612, D626–634, and D640–645) were thus purified, and the spectra were consistent

**FIG. 4.** Optical absorption spectra of Δ45eNOS. Panel A, solid line denotes the resting form of Δ45eNOS (1.5 μM); dotted line, 1 mM imidazole-induced shift of the Soret peak to 428 nm; dashed line, conversion of the low spin form at 428 nm to the high spin form at 397 nm by adding 1 mM L-arginine to the imidazole-heme complex. Inset shows the spectrum ΔA45eNOS as a function of the free L-arginine concentration. Panel B, binding of CO to the reduced Δ45eNOS. The solid line denotes the resting form of 2 μM Δ45eNOS, and the dashed line denotes the dithionite-reduced CO-bound form.

**FIG. 5.** Cofactor dependence for L-citrulline formation of wild-type eNOS (filled bar) and Δ45eNOS (hatched bar). All denotes a reaction mixture containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2 mM DTT, 50 μM L-arginine, 1 μM of L-[^3H]arginine, 100 μM Ca2+, 0.5 μM CaM, 100 μM NADPH, 100 μM BH4, and 10% glycerol. -BH4, omission of BH4 from the reaction mixture; -CaM, omission of Ca2+/CaM from the reaction mixture; +EGTA, complete reaction mixture plus 5 mM EGTA. Each bar represents mean ± standard deviation of triplicate experiments.
with those seen in WTeNOS and Δ45eNOS (data not shown).

The Ca\(^{2+}\) and CaM dependences of the purified mutant and wild-type enzymes were determined. Dependence of \(\Delta\)-citrulline formation on CaM was titrated by adding increasing concentrations of CaM along with 100 \(\mu\)M Ca\(^{2+}\) and other cofactors in the reaction mixture. The CaM concentration-response curves are shown in Fig. 9A. Δ45eNOS was active without exogenous CaM and addition of CaM did not increase the activity, while WTeNOS required about 50 \(\mu\)M of CaM to reach half-maximal activity (EC\(_{50}\)). The EC\(_{50}\) values for Δ594–604 and Δ605–612 mutants were ~15 and ~10 \(\mu\)M, respectively, whereas the EC\(_{50}\) values for Δ626–634 and Δ640–645 were higher, 22 and 30 \(\mu\)M, respectively. The results suggest that these four shorter deletion mutants have an increased CaM binding affinity when compared with WTeNOS but the binding was weaker than Δ45eNOS. This notion was supported by the absence of an intrinsically bound CaM in all shorter deletion mutants (data not shown).

Dependence on [Ca\(^{2+}\)] was similarly evaluated in the presence of 500 \(\mu\)M CaM and Ca\(^{2+}\) dependence curves were shown in Fig. 9B. The procedure to obtain the desired free Ca\(^{2+}\) by adding EGTA and Ca\(^{2+}\)-EGTA was similar to the previously reported procedures (15, 31). Δ45eNOS retained ~60% activity in the absence of Ca\(^{2+}\), but activity increases with increasing Ca\(^{2+}\) concentration. The WTeNOS was inactive without added Ca\(^{2+}\), and the EC\(_{50}\) was estimated to be 150 \(\mu\)M. The Δ594–604 and Δ605–612 mutants had a detectable but low activity in the absence of Ca\(^{2+}\) and the EC\(_{50}\) values were 25 and 35 \(\mu\)M, respectively. The Δ626–634 and Δ640–645 had no activity without addition of Ca\(^{2+}\), and the EC\(_{50}\) values were estimated

Table I

| Constructs | \(\Delta\)-Citrulline formation\(^b\) | Cytochrome \(c\) reductase\(^c\) | Ferricyanide reductase\(^d\) |
|------------|----------------------------------|---------------------------------|-------------------------------|
|            | \(\text{nmol/mg/min}\) | \(\text{nmol/mg/min}\) | \(\text{nmol/mg/min}\) |
| Wild-type  | ND\(^e\) | 138 ± 21 | 2.2 ± 0.4 | 6.8 ± 0.4 | 24.5 ± 1.0 |
| Δ45eNOS    | 173 ± 32 | 170 ± 28 | 5.1 ± 0.7 | 5.0 ± 0.2 | 26.7 ± 3.2 | 30.3 ± 2.8 |
| Δ594–604   | 12 ± 2   | 114 ± 35 | 0.6 ± 0.1 | 0.62 ± 0.2 | 7.7 ± 1.5 | 8.7 ± 1.8 |
| Δ605–612   | 15 ± 6   | 107 ± 24 | 1.3 ± 0.4 | 1.3 ± 0.4 | 6.7 ± 1.9 | 12 ± 1.8 |
| Δ626–634   | 1.7 ± 0.2 | 120 ± 18 | 1.6 ± 0.3 | 1.6 ± 0.3 | 6.4 ± 2 | 20 ± 4 |
| Δ640–645   | 2.9 ± 0.2 | 120 ± 27 | 1.8 ± 0.3 | 1.8 ± 0.3 | 6.2 ± 0.7 | 23 ± 2 |
| CaM/FMN/FAD| 2.9 ± 0.4 | 9.7 ± 2.1 | 22.9 ± 4.6 | 30.8 ± 0.6 |
| Δ45CaM/FMN/FAD | 7.4 ± 1.5 | 22.1 ± 0.3 | 42.2 ± 3.5 | 47 ± 2.8 |
| FMN/FAD    | 0.5 ± 0.1 | 0.7 ± 0.2 | 6.9 ± 1.1 | 8.7 ± 1.2 |
| Δ45FMN/FAD | 10.8 ± 0.2 | 10.1 ± 0.4 | 23.7 ± 2.1 | 27.2 ± 2.9 |

\(^a\) Constructs are shown in Fig. 1.

\(^b\) The \(\Delta\)-citrulline formation was assayed in a mixture containing 25 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 0.5 mM calmodulin, 100 \(\mu\)M CaCl\(_2\), 100 \(\mu\)M \(\beta\)-NADPH, 100 \(\mu\)M BH\(_4\), 50 \(\mu\)M \(\Delta\)-arginine, 1 \(\mu\)Ci of \(L\)-[\(\text{\(^{3}\)}\]H]arginine, and 10% glycerol.

\(^c\) Cytochrome \(c\) reduction was determined in a mixture containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10% glycerol, 0.5 mM calmodulin, 100 \(\mu\)M CaCl\(_2\), 50 \(\mu\)M cytochrome \(c\), and 100 \(\mu\)M \(\beta\)-NADPH, and the absorbance at 550 nm was recorded.

\(^d\) Ferricyanide reduction was determined in a reaction mixture containing 0.5 mM ferricyanide, and the absorbance at 420 nm was measured.

\(^e\) ND, undetectable.
to be 65 and 55 nM, respectively (Fig. 9B). We also determined the cytochrome c and ferricyanide reductase activities for the shorter deletion mutants (Table I). Both reductase activities were enhanced 3–4-fold for the mutants D626–634 and D640–645, and 1.5-fold for the mutant D605–612 by exogenously added Ca\(^{2+}\)/CaM. In contrast, mutant D594–604 had a low level of ferricyanide and cytochrome c reduction rates, which were not significantly changed by CaM.

**DISCUSSION**

Although x-ray crystallography results have shown that the oxygenase domains of iNOS and eNOS have similar structures (32, 33), there are clear differences in the biochemical and biophysical properties between these two NOS isoforms. Salerno et al. (20) proposed a ~50-residue insert unique to eNOS isoforms accounting for the difference in Ca\(^{2+}\)/CaM dependence. They have shown that synthetic peptides derived from the insert of eNOS (residues 594–645) are able to block CaM binding and enzyme catalysis (20). However, the exact role of this insert in intact eNOS remains unclear. In this study, we expressed the 45-residue deletion mutant of eNOS in the Sf9/baculovirus system and purified the expressed protein to homogeneity. The D45eNOS mutant had the expected molecular mass, and exhibited optical spectral properties and dimeric structure comparable to the wild-type eNOS. Deletion of the 45-residue fragment from eNOS, hence, did not alter the global structure or the active center of eNOS but drastically changed its dependence on Ca\(^{2+}\)/CaM for CaM binding, electron transfer, and catalytic activity.

The D45eNOS expressed in Sf9 cells is highly active, producing a large quantity of \(\cdot\)NO measured as NO\(_2^-\)/NO\(_3^-\) in the culture medium. The purified D45eNOS contained endogenously bound CaM and retained 60% of L-citrulline formation activity even in the presence of 5 mM EGTA. The D45eNOS, hence, resembles iNOS with respect to all the Ca\(^{2+}\)/CaM dependence characteristics. These results support the notion that this 45-amino acid segment impedes binding of CaM to eNOS at the basal level of intracellular [Ca\(^{2+}\)] (20). Since intrinsically active D45eNOS expressed in Sf9 cells produces a large quantity of \(\cdot\)NO continuously, which is likely to be toxic to the cells (16), it is conceivable that a higher expression level and enzyme activity of D45eNOS might be achieved by adding NOS inhibi...
itors. However, even with this constraint, the overall L-citrul-
line formation and cytochrome c activities of Δ45eNOS were
still 1.5 and 2.5 times higher as compared with those of CaM-
stimulated wild-type eNOS. Thus, removal of this insert re-
sulted in not only an iNOS-like NO activity but also an in-
creased electron transfer rate. As both redox potential and
orientation between each redox partner influence the electron
transfer rate (34), deletion of this insert may either change the
relative orientation or shift the redox potential between heme
and FMN, or FMN and FAD redox centers in eNOS.

Studies have reported that the activating effect of CaM on
nNOS can be fully accounted by its interaction with the reduct-
ase domains (35). The rate-limiting step for electron transfer in
NOS was proposed to be within the reductase domain rather
than in the oxygenase domain (18). As this 45-residue insert is
also located in the FMN subdomain (20), to determine whether
this insert is involved in electron transfer within the reductase
domain, we generated a series of reductase domains either with
or without this insert and the CaM-binding sequence. Our
results showed that the full-length reductase domain (CaM/
FMN/FAD, residues 482–1204) and the reductase domain with-
out the CaM-binding sequence (FMN/FAD, residues 511–1204)
contained a considerable amount of air-stable semiquinone
radical after isolation. In contrast, the other two reductase
domains without the 45-residue insert (Δ45CaM/FMN/FAD and
Δ45FMN/FAD) did not show any flavin semiquinone radical
after purification, implying that semiquinone radical in
Δ45CaM/FMN/FAD and Δ45FMN/FAD reductase domains is
less stable in the air, and the insert would play a role in
stabilizing the semiquinone radical. Treatment of several re-
ductase domains with a slight excess of NADPH produced an
increase in absorbance at 590 nm, indicating the ability of
CaM binding to cause a conformational change in
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its structure. Four active proteins, Δ594–604, Δ605–612,
Δ626–634, and Δ640–645, were purified and characterized.
The results have indicated the following. 1) All four proteins
did not show Ca2+-independent activity, but required lower
Ca2+ and CaM concentrations for maximal activity than WT-
NOS (Fig. 9). Because of having a higher affinity for CaM
binding, cells expressing mutants Δ594–604 and Δ605–612
continuously produce NO and spontaneously accumulate NO2–/
NO in the culture medium (Fig. 8A). Therefore, segment 594–
612 plays a more important role in regulating CaM binding and
enzyme activity. This is not consistent with the peptide studies
(20), which proposed RRKRK motif as essential for blocking
CaM binding and eNOS activity. The RRKRK motif is situated
in the segment 626–634, but not in the segment 594–612. Our
results indicate that a free peptide might not truly reflect the
specific interaction of corresponding residues in the intact pro-
tein. 2) In addition to having much lower Ca2+ requirement for
maximal NO synthesis, deletion of residues 594–604 unex-
expectedly resulted in a low level of ferricyanide and cytochrome
c reduction rates, which was not stimulated by exogenous CaM.
This observation led to the proposal that segment 594–604 has
two effects. First, it covers or allosterically perturbs the CaM-
binding site in eNOS, thereby obstructing CaM binding, and
dinection of residues 594–604 greatly increases sensitivity to
Ca2+/CaM for NO synthesis. Second, the activation of eNOS
reductase activities by CaM requires the interaction of 594–
604 segment with CaM and nNOS activity. Removal of the
RRKRK motif is required for mediating the electron transfer from the reductase
to oxygenase domain, and the basal level of electron transfer
within reductase domain is sufficient for NO synthesis. Al-
though the reductase activity of Δ594–604 is low and not
sensitive to the exogenous CaM, its L-citrulline formation is
Ca2+/CaM-dependent and approaches the level of WT
NOS upon Ca2+/CaM binding (Table 1). Moreover, Δ45eNOS activity
could not be detected when it was expressed in bacteria without
CaM (data not shown), probably because of perturbation of its
global structure. The result suggests that CaM is crucial for
proper folding and stabilization of Δ45eNOS. These data were
similar to the report that an active mouse iNOS in E. coli
required coexpression with CaM (40).

Immunoblot analysis using an anti-CaM antibody in our
study showed that Δ45eNOS contained an endogenously bound
CaM, while WT NOS, other deletion mutants, and N-termi-
nally truncated reductase domains did not. It appears that the
tightly-bound CaM to NOS requires the absence of entire 45-
residue segment like Δ45eNOS and iNOS. Removal of the
45-residue insert eliminates the steric hindrance, contributing
to an enhanced CaM affinity at a basal level of intracellular
[Ca2+]. However, the Δ45CaM/FMN/FAD domain, which
contained the CaM-binding site but lacked the 45-residue insert,
did not contain endogenous CaM, suggesting that CaM binding
requires not only the canonical CaM-binding sequence but also
sequences in the reductase domain and the oxygenase domain.
Absence of oxygenase domain in Δ45CaM/FMN/FAD protein
would therefore weaken the interaction of CaM with eNOS,
resulting in reversible CaM binding.

In conclusion, we have demonstrated by deletion experiments that the 45-amino acid insert in the FNM subdomain of eNOS influences CaM binding and Ca$^{2+}$-dependent NO production. It also plays a regulatory role in controlling electron transfer within the reductase domain, contributing to a low intrinsic reductase activity. Data from this report have provided valuable information on the regulation of Ca$^{2+}$/CaM binding as well as the reductase and oxygenase activities of eNOS by a 45-residue region in the eNOS molecule.

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