Disabling a C-terminal Autoinhibitory Control Element in Endothelial Nitric-oxide Synthase by Phosphorylation Provides a Molecular Explanation for Activation of Vascular NO Synthesis by Diverse Physiological Stimuli*

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Calmodulin-dependent activation of endothelial nitric-oxide synthase is generally considered to follow a transient increase in intracellular calcium levels. However, a number of physiological stimuli (e.g. endothelial shear-stress, insulin) are known to activate endothelial nitric oxide (eNOS) via a non-classical, “calcium-independent” pathway. Recent findings demonstrate that such stimuli elicit the phosphorylation of a C-terminal residue in eNOS (Ser1179 in the bovine isoform), rendering eNOS active at resting levels of intracellular calcium. However, the mechanistic basis for this mode of eNOS activation remains unknown. Protein modeling led us to consider that the C terminus of eNOS may fulfill an autoinhibitory function that can be disrupted by phosphorylation of serine 1179. To test this possibility we contrasted the phenotype of wild type bovine eNOS with that of a mutant lacking C-terminal residues 1179–1205 (CΔ27 eNOS). Despite no observed difference in calmodulin affinity, CΔ27 eNOS exhibited a 5-fold reduction in EC50 for calcium and a 2–4-fold increase in maximal catalytic activities. In these phenotypic properties, CΔ27 accurately mimics phospho-Ser1179 wild type eNOS. We conclude that the C terminus imposes a significant barrier to the activation of eNOS by calmodulin binding and that this barrier can be functionally disabled by Ser1179 phosphorylation-elicited enzyme activation.

Nitric-oxide synthases (NOSs) comprise a family of three mammalian gene products that each possess an N-terminal heme-containing oxygenase domain and a C-terminal flavin-containing reductase domain, bridged by a canonical calmodulin (CaM)-binding polypeptide (1). NOS isoforms are functionally distinguished by their modes of regulation. Two Ca2+-dependent mammalian isoforms of NOS, neuronal (nNOS) and endothelial (eNOS), remain dormant until Ca2+/CaM binding is elicited by transient elevation of intracellular Ca2+. In contrast, Ca2+-independent NOS (iNOS) is continuously active, due to a remarkably high affinity for CaM even at low resting levels of intracellular Ca2+. The three isoforms are further differentiated by their maximal rates of NO synthesis; nNOS and iNOS exhibit several-fold greater activity than eNOS (3). The lesser activity of eNOS has been attributed to an inherently slower rate of electron flux between reductase domain flavin cofactors, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (3).

We previously demonstrated that the FMN-binding subdomain of Ca2+-dependent isoforms of nitric-oxide synthase (cNOS) contains a 45-amino acid insertion peptide that functions as an autoinhibitory control element (ACE) (4). Limited proteolysis studies revealed that the ACE is physically displaced upon CaM binding to cNOS and that synthetic ACE-derived peptides inhibit both CaM binding and NOS activation. Homology-based molecular modeling provided structural predictions consistent with an interaction between the ACE and bound CaM on cNOS. A model was posited whereby the ACE reversibly docks with a site on eNOS that impedes CaM binding and hence, enzyme activity. Binding of CaM to cNOS was postulated to displace the ACE by virtue of domain overlap, thereby eliciting enzyme activation. Support for this prediction was subsequently provided by demonstrations that deletion of the entire ACE from eNOS (5, 6) or nNOS (7, 8) results in a marked reduction in the Ca2+ concentration required for activation of NO synthesis. Deletion of the ACE from eNOS also caused an increase in the maximal rate of NO synthesis to a level comparable with that of wild type nNOS and iNOS (5).

Recognition sites on cNOSs that interact with the ACE to modulate activity have not been elucidated. That such sites exist within the reductase domain is suggested by the finding that Ca2+/CaM-dependent control of electron flux remains intact in isolated eNOS- and nNOS-derived reductase domains (9, 10). The present study was initiated to characterize a hypothesized ACE interaction site.

Despite the lack of a solved high-resolution structure for a complete NOS reductase domain, three-dimensional homology-based models of NOS reductase subdomains have been developed considering the many related proteins in the protein structure database (4), and a partial structure of an nNOS reductase domain has recently been reported (11). Only two regions of eNOS cannot be reliably modeled due to the lack of suitable structural homologs. These completely unknown structural elements are: (I) the aforementioned 45-amino acid...
ACE peptide that in the FMN-binding subdomain and (2) a C-terminal peptide that is partially sequence-conserved within NOS isoforms and which extends beyond the C terminus of the highly conserved NOS-homolog, cytochrome P-450 reductase (CPR). Notably, the two C-terminal residues of CPR, Trp-Ser, are substituted in all NOSs with Phe-Gly, followed by 21–42 amino acids depending on NOS isoform. The peptide extension of NOSs is found in no other FAD-containing flavoprotein that otherwise bears NOS-homology.

The C-terminal peptide of NOSs is not essential for NO synthesis but is profoundly important for efficient NOS catalysis. Deletion of the entire C-terminal extension (i.e. all residues beyond the CPR C-terminal homolog, Phe-Gly) was reported to have no significant effect (12) or increase by 20% NO synthesis activity by murine iNOS (13) while decreasing NO synthesis by bovine eNOS and rat nNOS to levels 33 and 45% of that observed with full-length enzymes, respectively (14). When devoid of bound Ca$^{2+}$/CaM, C-terminally truncated eNOS and nNOS were observed to produce low levels of NO (6–7% of that with full-length NOSs containing bound Ca$^{2+}$/CaM) while reducing artificial electron acceptors at a 7–21-fold accelerated rate (14). Paradoxically, CaM binding to C-terminally truncated eNOS and nNOS was observed to inhibit, rather than enhance, reductase activities (14). Given the profound consequences of C terminus removal on NOS enzymatic activities, a regulatory function for the eNOS C terminus is conceivable.

Studies have shown that endothelial shear-stress elicits Akt/PKB-dependent phosphorylation of a conserved serine that lies 27 amino acids from the C terminus of eNOS (Ser$^{1179}$ in bovine eNOS); this modification triggers eNOS activation even at low physiological levels of Ca$^{2+}$ and enhances maximal catalytic activity by 2-fold in vivo (15, 16). Further studies have implicated a role for Ser$^{1179}$ phosphorylation of eNOS in the stimulation of vascular NO synthesis by estrogen (17, 18), vascular endothelial cell growth factor (19), insulin (20), sphingosine-1-phosphate (21), and bradykinin (22). In addition to Akt/PKB, protein kinase A (23, 24) and AMP-activated protein kinase (25) have been implicated as mediators of eNOS Ser$^{1179}$ phosphorylation in response to physiological stimuli.

Herein, we have expressed and characterized a truncated bovine eNOS mutant lacking Ser$^{1179}$ and the subsequent 26 C-terminal amino acids. Evidence is presented in support of a key regulatory role for the unphosphorylated C-terminal peptide in maintaining eNOS in a catalytically inactive state at basal levels of Ca$^{2+}$. Activation of eNOS by phosphorylation of Ser$^{1179}$ in vivo can be explained by a loss of autoinhibitory C-terminal peptide function and consequent activation of electron transfer into and between reductase domain flavins.

MATERIALS AND METHODS

Materials—Calmodulin was obtained from Calbiochem (La Jolla, CA) and (6R)-5,6,7,8 tetrahydrobiopterin from Schirks Laboratories (Jona, Switzerland). $^{125}$I-labeled CaM was purchased from PerkinElmer Life Sciences. Terrific broth, isopropyl-$\beta$-thiogalactopyranoside and chloramphenicol were purchased from Invitrogen. 25′-ADP-Sepharose 4B was purchased from Amersham Biosciences, and GFB membrane-clad 96well microfiltration plates were from Millipore (Bedford, MA). Bovine heart cytochrome $\epsilon$, $\beta$-lactoglobulin, CaM-Sepharose resin and all other chemicals were purchased from Sigma.

eNOS Expression Purification—eNOS was purified from Escherichia coli harboring pGroELS and pCW-eNOS expression vectors (26). An overnight culture of pCW-eNOS containing E. coli strain TG1 (100 mg/ml Tris-HCl, 100 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 100 mM NaCl, 1 mM phenylmethylsulfonfyl fluoride, 5 mg/liter pepstatin A, 5 mg/ml leupeptin and 20 mM CHAPS), subjected to lysis by pulsed sonication, and then centrifuged to sediment particulate matter (120,000 $\times$ g, for 1 h). The supernatant was applied to a 2′-ADP-Sepharose 4B column that had been pre-equilibrated in buffer B (100 mM NaCl, 1 mg/ml dithiothreitol, 10% glycerol, 100 mM NaCl). The column was then washed with 20-column volumes of buffer B followed by 20-column volumes of buffer B containing high salt (600 mM NaCl). eNOS protein was eluted with high salt buffer B that additionally contained 10 mM NAPDH. This NaCl concentration of the eNOS-containing eluate was reduced to 100 mM by repeated concentration/dilution in buffer B using a centrifugal filter device (Viomax 100K, Millipore). The concentrated and desalted NAPDH eluate was further purified by affinity chromatography on a calmodulin-Sepharose column as previously described (27). Protein purity was assessed by SDS-PAGE and Coomassie staining. Quantification of eNOS protein was determined spectrophotometrically based on heme content, calculated using an extinction coefficient of 74 mmol $^{-1}$ cm$^{-1}$ for As$_{46}^{N}A_{580}$ of the dithionite-reduced CO-bound eNOS heme-chromophore (28).

Mutagenesis of eNOS—Mutagenesis of full-length bovine eNOS cDNA was performed using a QuikChangeTM site-directed mutagenesis kit (Stratagene; La Jolla, CA). Briefly, the mutagenesis of eNOS cDNA was carried out in the pCW-eNOS expression vector using two synthetic oligonucleotide primers (forward: 5′-CTCTCTGCAGGGAATACCAAGGTACGTTACAATGCTGCTGAGG-3′; reverse: 5′-GCTATACGTTACGAGTATCGTTCCCTGTCAGGGAAAATCACGG-3′) to introduce a stop codon (TAG) in place of the codon that encodes Ser$^{1179}$ in wild type eNOS. Introduction of the desired stop codon was confirmed by dyeoxidireonucleotide sequencing performed by the Cornell University DNA Sequencing Core Facility.

Analysis of $^{125}$I-labeled Calmodulin Binding to eNOS—CaM binding to eNOS was analyzed in 96-well microfiltration plates containing GFB membrane filter-bottoms that had been washed with binding buffer (10 mM MOPS, 100 mM KCl, 100 mM CaCl$_{2}$, and $\beta$-lactoglobulin 0.5%, pH 7.2). Incubations contained binding buffer, BH$_{4}$ (10 mM) and dithiothreitol (1 mM), supplemented with desired concentrations of $^{125}$I-labeled CaM in a total volume of 100 mM. In some cases, Ca$^{2+}$-buffer solutions were additionally added. Binding reactions were initiated by the addition of $^{125}$I-labeled CaM at the desired concentrations and allowed to proceed for 20 min at 25°C. Binding was terminated by rapid vacuum filtration, and filters were washed twice with 100 mM ice-cold assay buffer before air drying under vacuum. Bound radioactivity was determined in a Microbeta Plus 96-well liquid scintillation counter (Wallac) after addition of 25 μl of scintillation mixture to each well (Optiphase Supermix, Wallac). Specific binding of CaM was calculated as the amount of radioactivity lost when samples were incubated with the calcium-chelator EGTA (5 mM) or upon inclusion of a 1000-fold molar excess of unlabelled CaM. Equilibrium binding constants and association and dissociation kinetic rates were quantified by computer-assisted non-linear least squares regression analysis using Prism 2.0 (GraphPad Software Inc.).

Aspects of NO Synthesis—NO synthesis was deduced using the Griess assay for quantification of nitrate, a stable oxidation product of NO. Assays were carried out in 96-well microtiter plates using a 100-μl total sample volume. All wells contained 1-arginine (1 mM), calmodulin (100 mM), CaCl$_{2}$ (100 μM), BH$_{4}$ (10 μM), Tris-HCl (50 mM, pH 7.6), and eNOS at the desired concentrations. Reactions were initiated by the addition of NADPH (1 mM). After one h at 37°C, 10 μl of lactate dehydrogenase was added (20 μl of lactate dehydrogenase slurry in 0.5 ml of 500 mM pyruvate), and samples were incubated at 37°C for 15 min. Griess reagent (freshly made 1:1 mix of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine) was added as a 100-μl volume and A$^{550}$ was determined within 10 min. The level of nitrite in samples was assessed by comparison with sodium nitrite standards. For experiments that assess the Ca$^{2+}$ dependence of NO synthesis, cells were kept at a constant Ca$^{2+}$ concentration as described previously and an aequorin calcium sensor was substituted (10 mM MOPS, 100 mM KCl, pH 7.2) containing varying ratios of EGTA-Ca$^{2+}$ to give desired Ca$^{2+}$ concentrations (described below).

Preparation of Calcium Standard Solutions—Stock solutions of “zero” free Ca$^{2+}$ (10 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2) and 40 mM free Ca$^{2+}$ (10 mM MOPS, 100 mM KCl, 10 mM EGTA, 10 mM CaCl$_{2}$, pH 7.2) were prepared. Solutions of defined free Ca$^{2+}$ (0–40 μM)
were then prepared by mixing varying ratios of the two solutions (29). The free Ca\(^{2+}\) concentration was quantified by ratiometric fluorometry using fura-2.

**Reduction of Cytochrome c and Ferricyanide by eNOS—** Assays of eNOS reductase domain activity were carried out in 96-well microtiter plate format in a total reaction volume of 100 \(\mu\)l. Reaction progress was monitored continually for 30 min at 15-s intervals by following the rate of increase in absorbance at 550 and 405 nm using the same Ca\(^{2+}\) concentration as in the above experiments. Activity assays were performed to assess the dependence on CaM binding, maximum CaM binding was elicited by addition of 0.83 mM Ca\(^{2+}\). After addition of the desired amount of recombinant eNOS (full-length or C\(27\)) or water to blank wells, substrate reduction was initiated by addition of NADPH (100 \(\mu\)M final concentration).

**RESULTS**

Sequence alignment of the C-terminal region of rat CPR with NOS isoforms reveals that the C terminus of all NOSs extends beyond the site of CPR termination (Fig. 1). The fact that CPR is functionally homologous to the NOS reductase domain implies that the C-terminal extension in NOSs is not required for catalytic function. Indeed, it had been demonstrated for murine iNOS that 21 amino acids can be deleted from the C terminus without significant loss of activity (12). Additionally, deletion of C-terminal segments of nNOS and eNOS have been recently reported to perturb electron transfer and alter calmodulin-dependent regulation (14). Significant identity in the C termini of eNOS isoforms and conservation among all isoforms suggests a functional role. Because physiological activation of eNOS has been shown to involve Akt/PKB-mediated phosphorylation of a Ser residue 27 amino acids distal to the C terminus in bovine or human eNOS (15, 16), we hypothesized that this mode of eNOS activation may be a molecular consequence of conserved C-terminal inhibitory functions. To evaluate this possibility, we contrasted the functional properties of full-length eNOS with those of a truncation mutant lacking the 27 C-terminal amino acids (C\(27\), see Fig. 1).

C-terminal truncation of eNOS was conferred by introducing a stop codon in lieu of Ser\(^{179}\), the site of in vitro phosphorylation and catalytic activation by Akt/PKB in bovine eNOS. This resulted in C\(27\), a truncated eNOS protein that is deleted in 27 of the 42 C-terminal amino acids, the residues that extend beyond the homologous termination site in CPR.

**Expression and Purification of Full-length and C\(27\) eNOS—** A two-step purification involving sequential affinity chromatographies on ADP-Sepharose and CaM-Sepharose resins resulted in >90% homogeneity for both full-length and C\(27\) as estimated from Coomassie-stained SDS/PAGE (not shown). Extraction of C\(27\) (but not full-length eNOS) from bacterial pellets was significantly improved by inclusion of CHAPS detergent (20 mM) in the bacterial lysis buffer; CHAPS was included in both full-length and C\(27\) eNOS preparations to allow for direct comparison of enzymatic properties. Spectrophotometry demonstrated that CO-bound absorption spectra of dithionite-reduced C\(27\) and full-length eNOS are indistinguishable, indicating that C-terminal truncation does not compromise heme-coordination. Accordingly, spectral assessment of the heme-chromophore was used to quantify eNOS protein mass. Overall, eNOS yields ranged from 2–10 mg of purified protein per liter of bacterial culture.

**Ca\(^{2+}\) Dependence for Calmodulin Binding and Activation of Full-length and C\(27\) eNOS—** Experiments were performed to compare the Ca\(^{2+}\) dependence for activation of full-length and C\(27\) eNOS (80 nM) by CaM (100 nM). As shown in Fig. 2A, truncation of the C terminus of eNOS resulted in a 5- to 6-fold diminution in the Ca\(^{2+}\) concentration required for half-maximal activation of NO synthesis relative to full-length eNOS (EC\(_{50}\) values for free Ca\(^{2+}\) = 83 and 461 nM, respectively). Notably, C\(27\) eNOS was >80% active at 150 nM Ca\(^{2+}\), which supported <10% of maximal activity with full-length eNOS. This relative “Ca\(^{2+}\)-independence” of C\(27\) eNOS contrasts with the complete Ca\(^{2+}\)-independence of murine iNOS activity at all concentrations of Ca\(^{2+}\) tested (Fig. 2A).

We next compared the Ca\(^{2+}\) dependence for activation of full-length and C\(27\) eNOS. Despite the substantial difference between C\(27\) and full-length eNOS in their Ca\(^{2+}\) concentration dependence for enzymatic activation, the Ca\(^{2+}\) dependencies for binding of 125I-labeled CaM were not markedly different. Notably, assessment of eNOS activity (Fig. 2A) and 125I-labeled CaM binding (Fig. 2B) were each performed using the same Ca\(^{2+}\) buffer solutions under identical assay conditions, permitting direct comparison of observed Ca\(^{2+}\) dependencies. These findings revealed a fundamental difference between the enzymes; whereas full-length eNOS binds 125I-labeled CaM at Ca\(^{2+}\) concentrations that are significantly lower than that required for physiological enzyme activation (>5-fold difference in EC\(_{50}\) values), the Ca\(^{2+}\) dependence for 125I-labeled CaM binding and activation of C\(27\) eNOS are indistinguishable at Ca\(^{2+}\) concentrations above those typically found in resting cells (100 nM) (30).

**Affinity and Kinetics of CaM Binding to Full-length and C\(27\) eNOS—** An increased affinity for Ca\(^{2+}\)/CaM binding could potentially explain the reduced Ca\(^{2+}\) dependence for activation of C\(27\) compared with full-length eNOS. To test this possibility, we defined the concentration dependence for Ca\(^{2+}\)/CaM to both activate and bind to each of C\(27\) and full-length eNOS; experiments were performed by varying CaM in the presence of excess Ca\(^{2+}\) (100 \(\mu\)M). Activity assays
failed to demonstrate the predicted leftward shift in the concentration dependence of Ca$^{2+}$/CaM for eliciting NO synthesis by C$\Delta$27 eNOS compared with full-length eNOS; indeed, a small but paradoxical rightward shift was observed (Ca$^{2+}$/CaM EC$_{50}$ = 35 nM for C$\Delta$27 eNOS and 23 nM for wild type eNOS; see Fig. 3A). Saturation analysis revealed an identical affinity for binding of Ca$^{2+}$/125$I$-labeled CaM to C$\Delta$27 versus full-length eNOS (EC$_{50}$ = 13 and 14 nM, respectively; see Fig. 3B). Based on estimated calculations of free Ca$^{2+}$/125$I$-labeled CaM, correcting for the component that contributes to total binding, we calculate $K_a$ values for binding to C$\Delta$27 and full-length eNOS of 3 and 4 nM, respectively.

The equilibrium binding constant, $K_a$, equals the ratio of two kinetic rate constants, dissociation ($k_{off}$) and association ($k_{on}$). As equilibrium binding of Ca$^{2+}$/125$I$-labeled CaM to eNOS was found to be unaltered by truncation of the 27 C-terminal amino acid residues, contrary to our prediction, we sought verification of this finding by analysis of CaM binding kinetics. The rate of dissociation of 125$I$-labeled CaM-eNOS at 25°C was evaluated for complexes that had been formed in the presence of maximal levels of Ca$^{2+}$. Measurements were initiated by addition of a 3000-fold molar excess of unlabeled CaM to prevent reassociation of 125$I$-labeled CaM-eNOS complexes following their dissociation. As shown in Fig. 4 (main panel), the rate of dissociation of C$\Delta$27 eNOS-CaM complexes was not discernibly different from that exhibited by full-length eNOS-CaM complexes; indeed, fitted curves were virtually superimposable. Linear analysis of this data indicated half-times for 125$I$-labeled CaM dissociation of 21.4 and 21.0 min for full-length and C$\Delta$27 eNOS, respectively. As we observed no difference in CaM dissociation rates, we next compared the rates of CaM association with full-length and C$\Delta$27 eNOS at 25°C (Fig. 4, inset). The kinetics of CaM association was indistinguishable for wild type and C$\Delta$27 eNOS, although the rapidity of binding preceded accurate determination of $k_{off}$ values. Nonetheless, as both association and dissociation rates are indistinguishable for C$\Delta$27 and full-length eNOS, we conclude that truncation of the C-terminal amino acids from eNOS does not significantly influence CaM binding to eNOS. Accordingly, the diminished Ca$^{2+}$ requirement that we observed for activation of C$\Delta$27 eNOS versus full-length eNOS occurs by a mechanism independent of a detectable alteration in CaM binding to eNOS. Catalytic Activities of Full-length and C$\Delta$27 eNOS—Three distinct catalytic activities of eNOS were analyzed and contrasted for differences between full-length and C$\Delta$27 enzymes (Fig. 5). These three activities were determined in the absence and presence of bound CaM, and together they serve to define kinetic rates of electron flux within eNOS to heme (NO synthesis, panel A), FAD (cytochrome c reduction, panel B), and FMN (ferricyanide reduction, panel C) (31). As shown in Fig. 5A, neither full-length nor C$\Delta$27 eNOS supported detectable
levels of NO synthesis in the absence of added CaM. Nonetheless, for CaM-bound enzymes, truncation of the C terminus of eNOS resulted in a 2-fold increase in maximal NO synthesis versus full-length (V\textsubscript{max} = 152 ± 14 and 303 ± 4 nmol/min/mg for full-length and C\textsuperscript{A27} eNOS, respectively). Given that the rate-limiting step for NO synthesis by CaM-bound eNOS resides in the reductase domain (3, 5) we monitored the rate that artificial electron acceptors could be reduced by electrons derived from reductase domain flavins. Although significant basal cytochrome c and ferricyanide reduction was observed with both, maximal activity was 3–4-fold greater with C\textsuperscript{A27} (Fig. 5, B and C). Upon binding of CaM by either C\textsuperscript{A27} or full-length eNOS, cytochrome c and ferricyanide reduction was accelerated to a rate that was 2–3-fold greater than that measured in the absence of bound CaM. These findings reveal that truncation of the eNOS C terminus markedly accelerates both basal and maximal CaM-induced electron flux to FAD and FMN, in addition to potentiating CaM-induced NO synthesis.

**Inhibition of eNOS Activity and Calmodulin Binding by Autoinhibitory Domain-derived Peptides**—The relative decrease in Ca\textsuperscript{2+} dependence for enzyme activation and increase in maximal catalytic rates with C\textsuperscript{A27} compared with full-length eNOS are similar to those previously described for eNOS mutants in which the ACE peptide in the FMN domain was deleted (5). Accordingly, we wondered whether there was functional overlap between the ACE and C-terminal peptides, perhaps via their interaction to regulate eNOS activity. In a previous study we showed that synthetic peptides derived from the autoinhibitory domain of eNOS were inhibitors of eNOS activity and \textsuperscript{125}I-labeled CaM binding putatively as a consequence of interaction with an unspecified ACE docking site (4). If the C terminus of eNOS contributes to the docking site of the ACE, then truncation of the C terminus would predictably diminish the ability of an ACE-derived peptide to inhibit both NOS activity and Ca\textsuperscript{2+}/\textsuperscript{125}I-labeled CaM binding affinity. To examine this possibility, we compared the concentration dependence of a synthetic ACE-derived peptide (bovine eNOS-(617–639)) for inhibiting maximal activity (Fig. 6A) and Ca\textsuperscript{2+}/CaM binding (Fig. 6B) with C\textsuperscript{A27} and full-length eNOS.

**DISCUSSION**

The initial purification of nNOS (32) and eNOS (33) was enabled by the discovery that these enzymes depend on Ca\textsuperscript{2+}/
CaM for activity; CaM was later found to be a tightly bound subunit of iNOS (2). Subsequent cloning of all three NOS isoforms revealed a conserved bidomain structure consisting of an N-terminal oxygenase domain and a C-terminal reductase domain, bridged by a 25–35-amino acid CaM-binding domain. From the cloned sequences it was apparent that the reductase domain of all the NOS isoforms bears striking homology to mammalian CPRs (34, 35). Despite this overt resemblance, the FMN-domain autoinhibitory peptide results in an enzyme with diminished sensitivity to inhibition by a synthetic ACE-derived peptide. These features of CA27 eNOS provide marked insights into the molecular mechanism of eNOS phosphoregulation involving Ser1179.

Ser1179 in bovine eNOS has previously been shown to undergo reversible phosphorylation, a posttranslational modification of eNOS with a now-established physiological role in activating vascular NO production by endothelial shear-stress, estrogen, and various growth factors (17–20, 22). An inability of physiological stimuli to elicit phosphorylation of Ser1179 has been implicated as the basis for vascular complications of diabetes, a consequence of a hyperglycemia-induced O-linked N-glucosamine modification of Ser1179 (36). Notably, phosphorylation of Ser1179 in bovine eNOS by Akt/PKB (15, 16), AMP-activated protein kinase (25) or protein kinase A (23–25) has been reported to elicit a relatively “Ca2+-independent” activation of eNOS and an increase in the rate of NO synthesis by the enzyme in vivo, in accord with the in vitro phenotype we observed with CA27 eNOS. Moreover, in vitro analysis of the enzymatic properties of recombinant Akt-phosphorylated bovine eNOS (15) as well as a stable “phospho-mimic” of eNOS wherein Ser1179 was replaced with Asp (37), has revealed changes in catalytic function that are essentially indistinguishable from those that we observe with CA27 eNOS. As the functional consequences of eNOS phosphorylation are elucidated by removal of Ser1179, along with all C-terminal amino acids, the phenotype of Akt-PKB-phosphorylated bovine eNOS can be explained most simply by loss of inhibitory actions imposed by the non-phosphorylated C terminus. Thus, we propose that phosphorylation of Ser1179 serves to activate eNOS indirectly, by triggering dysinhibition, i.e. obstructing molecular interactions involving the C terminus that would otherwise impede electron flux through the reductase domain.

An attractive possibility is that the autoinhibitory loop in the FMN-binding domain is the target of inhibitory interactions imposed by residues in the C-terminal peptide extension. If so, phosphorylation of Ser1179 (or removal of the C-terminal extension) may activate eNOS by abrogating critical interactions with the autoinhibitory loop. Two findings provide evidence consistent with an interaction between the C terminus and the FMN domain autoinhibitory peptide. First, deletion of the FMN-domain autoinhibitory peptide results in an enzyme with a decreased Ca2+-dependence for activation and a 2–3-fold increase in the rate of NO synthesis (5), a near-identical phenotype to that observed for CA27 eNOS. If the C terminus contains the binding site for the autoinhibitory domain, this would explain the similar phenotypes; inhibition can be equivalently relieved by removal (5, 6) or modification (15, 16, 38) of either autoinhibitory peptide. Second, we found that truncation of the C terminus of eNOS imparts a diminished sensitivity to inhibition by a synthetic autoinhibitory loop-derived peptide. The efficacy of such peptides for inhibiting CaM binding and activation of eNOS was reported to result from binding to residues that dock with the endogenous autoinhibitory peptide loop (4).
It is commonly assumed that CaM binding to eNOS necessarily elicits eNOS activation. This simplistic view conflicts with our finding using full-length eNOS, where we observe a large disparity between the Ca$^{2+}$-dependence curves for CaM binding and activation of eNOS, a phenomenon also observed for nNOS. A reasonable explanation for this disparity is provided by considering the activation of CaM itself. CaM consists of two lobes held together by a flexible linker domain. Upon binding of Ca$^{2+}$, the lobes of CaM undergo a conformational change, which reveals sites of interaction with target peptides. Notably, the two lobes of CaM are activated at different levels of free Ca$^{2+}$, the C-terminal lobe at ~80 nM free Ca$^{2+}$ (39). It has been shown previously that binding of the N-terminal lobe of CaM is specifically responsible for the activation of nNOS (40). Hence it is possible for CaM to be tethered solely by its C-lobe to eNOS, a situation that could engender tight binding without eliciting enzyme activation. As the levels of Ca$^{2+}$ required for C-lobe interaction with eNOS are in the range of those found basally in cells (~100 nM), a more physiological representation may be that CaM is largely bound to eNOS in endothelial cells at rest. Activation of NOS catalysis would presumably occur only after stimulus-evoked increases in cellular Ca$^{2+}$ trigger the compartmentation of Cytochrome P450 reductase, thereby permitting eNOS activation to occur in tandem with CaM binding.

It is notable that electron flux through the reductase domain of eNOS is distinguished from other isoforms in being relatively slow, explaining a severalfold diminished intrinsic rate of NO synthesis relative to nNOS and iNOS. Because truncation of the C-terminal 27 amino acids of eNOS restored NO synthesis and reductase domain activities to a rate commensurate with that of other isoforms, the dampened catalytic activity of eNOS appears to be conferred by features of the C-terminal peptide. As shown in Fig. 1, the entire peptide extension of eNOS (42 amino acids) is considerably longer than that of nNOS (33 amino acids) and double that of iNOS (21 amino acids). Hence we speculate that regions of the C terminus unique to eNOS are responsible for “capping” the rate of electron transfer into and between flavins and heme. The N-terminal 17 amino acids of the extensions in eNOS and nNOS are highly conserved (58% identity), whereas the next 16 amino acids exhibit a much lower degree of identity (12%), and there are nine amino acids at the C terminus of eNOS for which there are no corresponding residues in the shorter extension of nNOS. Both the extra nine amino acids unique to eNOS and the 16 amino acid residues that are poorly conserved in nNOS are candidates for the electron flux-capping structural motif of eNOS.

While the C-terminal peptide extension in eNOS and nNOS inhibits electron transfer within the reductase domain, a portion also appears to be required for efficient interdomain electron transfer. Indeed, removal of the entire 42 amino acid extension of eNOS was reported to decrease by 70% the maximal rate of NO synthesis by CaM-bound eNOS, despite markedly increasing electron transfer into and between reductase domain flavins (14). In contrast, we found that the more modest truncation of only 27 C-terminal amino acids increased the maximal rate of NO synthesis by CaM-bound eNOS in addition to promoting reductase domain activities. Together, the new and published findings implicate C-terminal amino acids 1162–1179 in playing a role in regulating interdomain electron transfer between FMN and heme.

Although it can be argued that the C-terminal peptide serves to regulate Ca$^{2+}$/CaM-stimulated activities of eNOS (and nNOS, by inference), it is unclear what function the abbreviated C-terminal extension would play in iNOS, an isoform that has Ca$^{2+}$/CaM continuously bound (2). One answer arises from consideration of the enzymatic properties of an iNOS mutant enzyme lacking the C-terminal tail in its entirety. Notably, complete C-terminal truncation of iNOS was shown to increase electron flux through the reductase domain by 7–21-fold, while resulting in only a 20% increase in NO output (13). A similar situation is evident in an nNOS Ser1412Asp mutant (analogous to the eNOS1179Asp mutant) in which the marked enhancement in the rate of electron flux does not translate directly into an enhanced rate of NO synthesis. Indeed in this case maximal CaM-bound reductase activity of the mutant enzyme actually cause a reduction in the rate of NO synthesis (41). These altered catalytic activities are indicative of NOS uncoupling, i.e. dissociation of NADPH consumption from NO production. Uncoupling results in the production by iNOS of superoxide anion, a free-radical that reacts with NO at a near-diffusion-limited rate, forming peroxynitrite (42). Accordingly, the minimal iNOS C-terminal extension may play an important function in limiting the maximal rate of electron flux into the oxygenase domain, thereby preserving efficient coupling of NADPH consumption to NO synthesis and minimizing production of deleterious oxidants that would otherwise scavenge NO. By this view, it is conceivable that the C-terminal peptide has
evolved in iNOS to promote the release of bioactive NO rather than alternative reaction products. Perhaps ancestral iNOSs functioned by generating NO- and O2-derived species before the evolution of a C-terminal extension that enabled efficient production of NO of NO.

Insight into the possible mechanism for limitation of electron flux by the C-terminal peptide of eNOS is offered by consideration of the high resolution crystal structure of rat CPR (43). Although this exercise provides no direct structural information about the C terminus of eNOS, the relative positioning of the C terminus in CPR is telling. Notably, the C terminus of CPR curls back toward the main body of the enzyme and ends in a region proximal to the binding sites for both NADPH and FAD (see Fig. 7). The penultimate residue of CPR is Trp, which lies at the interface between bound NADPH and FAD. In this setting, the aromatic ring of Trp is coplanar with the isoalloxazine ring of FAD and positioned in a manner in which the π-cloud of electrons could reasonably impact on electron transfer between NADPH and FAD. All NOs contain the conservative substitution of Phe in place of Trp in CPR (see Fig. 1). Given this arrangement, one can readily imagine that interactions of the C terminus may orient this aromatic residue and thereby serve to regulate electron flux through the reductase domain. Importance of this Phe in NOS catalysis has already been demonstrated for iNOS; while activity is not diminished by removal of all subsequent C-terminal amino acids, deletion of this Phe results in 71% loss in maximal activity.

Consideration of the structure of CPR suggests an additional mechanism by which the C-terminal extension could serve to inhibit eNOS activity, obstruction of the trans-flow of electrons from the reductase domain in one monomer of an eNOS dimer to the oxygenase domain in the companion monomer. X-ray crystal structures of CPR and a partial structure of the reductase domain in one monomer of an eNOS dimer to the oxygenase domain in the companion monomer are consistent with the possibility that C-terminal residues of NOSs extend into the region of the dimer interface. If so, interactions with the FMN domain autoinhibitory loop may serve to modulate FMN-heme contacts required for NO synthesis. In one model, the C-terminal extension would impose a “wedge” that impedes electron transfer between the FMN of one monomer and the heme of its counterpart. Information regarding the C terminus and the autoinhibitory domain will be essential to define the actual mode(s) of regulation carried out by these domains.

Taken together, our findings reveal that the C-terminal peptide of eNOS governs the rate of NO synthesis and the Ca2+ dependence of enzyme activation by CaM. Because phosphorylation of this peptide has been shown to occur in vivo, resulting in phenotypic changes essentially identical to those observed with Ca2+-eNOS, it is appropriate that the C-terminal peptide be recognized as a second autoinhibitory control element (ACE2). In conjunction with the previously recognized ACE peptide loop in the FMN domain (ACE1) and the CaM-binding peptide itself, we propose that ACE2 contributes to a tripartite control mechanism that is fundamental to the physiological control of eNOS by Ca2+/CaM. Crystalllographic and/or NMR protein structural data will be invaluable to rigorously assess this possibility and shed light on the precise molecular mechanism by which eNOS activity is governed by ACE2. Unfortunately, it is unlikely that structures of ACE1 or ACE2 will be immediately forthcoming (nor the structural relation of either ACE to eNOS-bound CaM), given that these peptides are anticipated to be highly mobile regulatory elements that deflect attempts at structural specification. Once specified, however, atomic level knowledge of ACE1 and ACE2 structure, function, and interactions should inform on the fundamental molecular control mechanism that gates eNOS activity.
Disabling a C-terminal Autoinhibitory Control Element in Endothelial Nitric-oxide Synthase by Phosphorylation Provides a Molecular Explanation for Activation of Vascular NO Synthesis by Diverse Physiological Stimuli
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