Autoinhibition of Endothelial Nitric-oxide Synthase
IDENTIFICATION OF AN ELECTRON TRANSFER CONTROL ELEMENT*

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The primary sequences of the three mammalian nitric-oxide synthase (NOS) isoforms differ by the insertion of a 52–55-amino acid loop into the reductase domains of the endothelial (eNOS) and neuronal (nNOS), but not inducible (iNOS). On the basis of studies of peptide derivatives as inhibitors of NO formation and calmodulin (CaM) binding (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), the insert has been proposed to be an autoinhibitory element. We have examined the role of the insert in its native protein context by deleting the insert from both wild-type eNOS and from chimeras obtained by swapping the reductase domains of the three NOS isoforms. The Ca$^{2+}$ concentrations required to activate the enzymes decrease significantly when the insert is deleted, consistent with suppression of autoinhibition. Furthermore, removal of the insert greatly enhances the maximal activity of wild-type eNOS, the least active of the three isoforms. Despite the correlation between reductase and overall enzymatic activity for the wild-type and chimeric NOS proteins, the loop-free eNOS still requires CaM to synthesize NO. However, the reductive activity of the CaM-free, loop-deleted eNOS is enhanced significantly over that of CaM-free wild-type eNOS and approaches the same level as that of CaM-bound wild-type eNOS. Thus, the inhibitory effect of the loop on both the eNOS reductase and NO-synthesizing activities may have an origin distinct from the loop’s inhibitory effects on the binding of CaM and the concomitant activation of the reductase and NO-synthesizing activities. The eNOS insert not only inhibits activation of the enzyme by CaM but also contributes to the relatively low overall activity of this NOS isoform.

The enzymatic activities of the three NOS isoforms (1–7) differ in their Ca$^{2+}$-dependence: nNOS (NOS-I) and eNOS (NOS-II) are Ca$^{2+}$-independent constitutive isoforms, whereas iNOS (NOS-II), as typified by the inducible macrophage and hepatocyte form, is essentially Ca$^{2+}$-independent. This difference in the Ca$^{2+}$ dependence of the NOS isoforms is the result of a Ca$^{2+}$ requirement for the reversible binding of CaM to the constitutive isoforms (8, 9), in contrast to the almost Ca$^{2+}$-independent, high affinity binding of CaM to the inducible isoform (10).

The NOS isoforms are also differentiated by their maximum enzymatic activity, nNOS (8, 11–14) and iNOS (15–18) exhibiting much higher overall activities than eNOS (19–24). We have shown that the lower activity of eNOS is caused by a lower ability of its flavoprotein reductase domain to transfer electrons to the catalytic heme domain (25). However, the structural features that impair the reductase activity in eNOS, and hence lower the overall catalytic activity, remain unknown.

Recent evidence (26) suggests that the Ca$^{2+}$ dependence of nNOS and eNOS is caused by the presence of an autoinhibitory loop, absent in iNOS and P450 reductase, which interferes with the binding of CaM (Fig. 1). The presence of such an autoinhibitory moiety has precedence among CaM-binding enzymes. Indeed, the skeletal and smooth muscle myosin light chain kinases, multifunctional CaM-dependent protein kinase II, CaM-dependent protein phosphatase 2B, calcineurin, and phospholipase kinases are among the CaM-dependent enzymes that possess autoinhibitory structural elements (27). In these enzymes, CaM acts by displacing an autoinhibitory element that interferes with substrate access to the catalytic domain. In calcineurin, the autoinhibitory element is composed of two α helices of 23 amino acids located 54 amino acids after the CaM binding sequence (28, 29). In NOS and eNOS, the proposed autoinhibitory domain is composed of a 52–55-amino acid insert within the FMN binding domain located approximately 80 amino acid residues after the CaM binding sequence (Fig. 1). Location of the putative autoinhibitory element near the junction between the NOS oxygenase and reductase domains is consistent with a possible role in controlling electron transfer between these two domains.

To establish definitively whether the peptide insert in its native protein context functions as an autoinhibitory loop, we have constructed mutants of wild-type and chimeric NOS enzymes lacking the insert and have studied the consequences of deleting the loop on their Ca$^{2+}$ dependence and catalytic activities. Our results clearly identify the eNOS insert as an autoinhibitory loop that functions not only as an effector of the Ca$^{2+}$ dependence but also as an electron transfer control element that lowers the catalytic activity of eNOS relative to that of nNOS and iNOS.

EXPERIMENTAL PROCEDURES

Materials—Bovine endothelial NOS expression plasmid 6xHis-PCR Wori/beNOS was identical to that described previously (20). pBlue-script/iNOS was provided by Steve Black (University of California, San Francisco). The mouse macrophage NOS and human CaM expression plasmids were constructed as reported previously (18, 30). Enzymes used in DNA manipulation were from New England Biolabs (Beverly, MA). l-Arg was from Aldrich, (6R)-5,6,7,8-tetrahydrobiopterin from
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Alexis Biochemicals (San Diego), and HEPES buffer from Fisher. Recombinant human CaM was purified from Escherichia coli according to published procedures (31). DNA purification kits and Ni²⁺-nitrilotriacetic acid-agarose were purchased from QIAGEN (Chatsworth, CA). All other reagents and materials were from Sigma.

**DNA manipulations**—The genes encoding the N/E and I/E chimeras were prepared as described previously (25). The structures of all of the protein constructs employed in this study are shown schematically in Fig. 1. All PCR extensions were performed with Vent® polymerase, allowing for mutually primed synthesis of full-length AB using A, B, C, D, and E primers.

The E portion was generated by PCR primer extension using primer 6 (5'-GGACTGTCTTCGTGGATGG-3'), 3'-tagenesis (32) using template pBluescript/eNOS, primer 1 (5'-CCGGGCAAGCTTCACTG-3'), and primer 5 (5'-AGTCTATTAAAGAGGACTG TGGCAAGCGCTG-3') to produce 5'-NheI splice site (in bold), which introduced the 5'-NheI site into the parent chimera E/E plasmid, whose splice site produces a silent mutation, was produced by subcloning via NheI restriction sites (bold), which allowed for subcloning into pWori/N/E. These sites were used to produce pcWori/N/E by subcloning of the eNOS reductase domain PCR fragment into pWori/nNOS/nef1761, a ThaI site (Ser nNOS mutant possessing the NheI splice site (25)). Ser is the homologous amino acid found in eNOS. The INOS fragment containing the heme domain and CaM binding of I/E was generated by PCR extension using template pBluescript/INOS, primer 1 (5'-CAAGCTTATCCTACCGTCGACGGC-3'), and primer 2 (5'-NCGGGCGCATCTCCTCTCTAGAGCAGAAY-3'), which produced a 3'-XbaI site (bold) after the stop codon. These sites were used to produce pcWori/I/E by subcloning of full-length AB using the INOS reductase domain PCR fragment into pWori/E/N and pcWori/I/E, producing pcWori/I/E and pcWori/N/E, respectively.

The INOS reductase domain was generated by PCR primer extension using primer 5 (5'-CATGATGCCTTTATCGTCGACGGCAGAATATCCCTGCGCATCTCCTCTCTCCT-3'), and primer 6 (5'-TGTCGTATTAGGCAGTCTCCCGAGGCGAGTCCGGCTCGGTCGGCTCCTGGCAGGCGAGAATATCCCTGCGCATCTCCTCTCTCCT-3'), which produced a 3'-XbaI site (bold), respectively. These sites were used to subclone the macrophage reductase domain PCR fragment into pcWori/E/N and pcWori/I/E, producing pcWori/I/E and pcWori/N/E, respectively.

The eNOS loop deletion was made by overlap PCR extension mutagenesis (32) using template pcWori/I/E and primers 7–10 (5'-ATTAA CTGATCGTCGACGGCAGAATATCCCTGCGCATCTCCTCTCTCCT-3', 5'-GCGGCGCATCTCCTCTCTCCTCTCCTCGGCGAGAATATCCCTGCGCATCTCCTCTCTCCT-3', and 5'-GCGGGAAGGTTACGTCGACGGCAGAATATCCCTGCGCATCTCCTCTCTCCT-3'). Two PCR fragments were generated, primer 7 (which anneals before the NheI site), and 8 (mutagenesis primer), producing fragment A; and primers 9 (mutagenesis primer) and 10 (primer 10, which produces 20 bases after the unique KpnI site of eNOS), producing fragment B. A and B possessed a 15-base overlap, allowing for mutually primed synthesis of full-length AB using A, B, C, D, and E primers. Sequencing was performed to ensure against PCR errors in fragment AB. To produce pcWori/E/E, the parent chimeras E/E plasmid, whose splice site produces a silent mutation, was produced by subcloning via NheI and XbaI the E reductase domain fragment from pcWori/E/N into pcWori/E/E followed by subcloning of the above E fragment (AB, digested with NheI and KpnI) into pcWori/E/E.

**Protein Expression and Purification**—Expression and purification were performed as described previously (25).

**Activity Assays**—The rate of NO synthesis, determined from the oxidation of oxyhemoglobin to methemoglobin, and that of cytochrome c reduction were measured at 37 °C as reported previously (25). Where specifically indicated, an additional 100 mM KCl was included in the incubation mixture. Ferricyanide reduction was monitored at 420 nm using an extinction coefficient of 1.2 mA cm⁻¹ with an assay solution identical to that used for the other assays except that potassium ferriyanide was added to a final concentration of 0.8 mM in place of cytochrome c or oxyhemoglobin. Protein concentrations were estimated

![Fig. 1. Sequence alignment of various NOS isoforms and P450 reductase.](Image)

![Fig. 2. Schematic of wild-type, chimeric, and insert-deleted NOS proteins. Sequences are shown schematically (not to scale) of the wild-type, chimeric, and deletion proteins. Sequences in the chimeras are color-coded to match those of the wild-type sequences (black, nNOS; dark and light gray, eNOS; white, INOS). Splice sites in the chimeras (arrows) are indicated in the wild-type sequences (arrows) by a number corresponding to the last amino acid before the splice site. Deleted amino acids in eNOS, N/E, and I/E are indicated by diagonal shading, with a light gray shading used to indicate an eNOS reductase domain whose insert has been removed.](Image)
**RESULTS**

**Expression and Purification**—Expression and purification of the NOS proteins (for protein structures, see Fig. 2) was done as reported previously using Ni²⁺-nitrilotriacetic acid-agarose and 2',5'-ADP-agarose affinity chromatography (25). The final purity of all of the proteins was judged to be greater than 95% by SDS-polyacrylamide gel electrophoresis, and the spectra of all the proteins were consistent with the formation of heme-bound, functional proteins (not shown).

**Ca²⁺ Dependence**—The insert in the eNOS and nNOS reductase domains may function as an autoinhibitory element that impedes the Ca²⁺-dependent binding of CaM except at high Ca²⁺ concentrations (26). We therefore examined the NO-synthesizing activity of the enzymes as a function of the free-Ca²⁺ concentration. The effects of the insert in the reductase domain on the Ca²⁺ and CaM dependence of the activities of the recombinant proteins were measured in the presence of 500 nM CaM and various free Ca²⁺ concentrations (Fig. 3). The desired free Ca²⁺ concentrations were obtained by adding EGTA and Ca²⁺-EGTA as indicated by the Ca²⁺-EGTA dissociation constant at the given temperature and ionic strength (33). The Ca²⁺ concentration in these studies was controlled by adding CaCl₂ to the buffer. The results thus obtained with wild-type nNOS (Fig. 3A), from which an apparent K_D (K_{app}) of 300 nM for Ca²⁺ was calculated, were consistent with those reported by Ruan et al. (34). A slightly lower value of 150 nM was obtained for eNOS. The iNOS activity was independent of the free Ca²⁺ concentration, again in accord with Ruan et al. (34). The K_{Dapp} values for Ca²⁺ in the N/E, E/I, and N/I chimeras were 200, 10, and 10 nM, respectively (Fig. 3, A and B). For these three chimeras, the Ca²⁺ dependence correlates well with the presence (N/E) or absence (E/I, N/I) of the insert in the reductase domain.

**CaM Dependence**—Free calcium concentrations (26). We therefore examined the NO-synthesizing activity of the enzymes as a function of the free-Ca²⁺ concentration. The results thus obtained with wild-type eNOS by deletion of the putative autoinhibitory loop, had a K_{Dapp} value of 20 nM for Ca²⁺ rather than 150 nM and thus was activated by a 7-fold lower Ca²⁺ concentration. Likewise, activation of N/E required 4-fold less Ca²⁺ (50 nM) than the parent N/E chimera (200 nM). The lower K_{Dapp} values for Ca²⁺ in the proteins without the insert approach those for the E/I and N/I chimeras, which bear the insert-less NOS reductase domain (e.g. compare the open circles and open squares in Fig. 3A with the open diamonds and open squares in Fig. 3B). The E/I and E/Δ chimeras did not differ significantly in their Ca²⁺ dependence; both exhibited some activity even at a 0.1 mM free Ca²⁺ concentration, but their activity was not fully expressed at the lowest Ca²⁺ concentrations (Fig. 3B).

**NO Synthesis**—NO-synthesizing activity, monitored via the oxidation of oxyhemoglobin, and cytochrome c reduction were assayed in two different buffers. The first buffer, which included flavins and other necessary cofactors, was similar in its lack of added salt to buffers conventionally employed to assay the pure proteins (35, 36). The second buffer differed from the first only in that it contained 100 mM KCl to reproduce the conditions employed to assay Ca²⁺ dependence.

In the presence of CaM, the activity of the wild-type constitutive NOS isoforms is expected to approach zero when [Ca²⁺]_{free} = K_{Dapp} for Ca²⁺ (Fig. 3A). A similar Ca²⁺ dependence might be expected for the mutants without the insert, but the maximum NOS activities for the insert-less mutants might be expected to be the same as those for the corresponding CaM-bound wild-type proteins when [Ca²⁺]_{free} = K_{Dapp} for Ca²⁺. Under these conditions, the CaM-bound insert-less eNOS mutant should have the same activity as CaM-bound, wild-type eNOS. Contrary to this expectation, the proteins without the peptide insert have enhanced catalytic activities (Fig. 4). In the presence of a high salt concentration, the activities of eNOS, N/E, and N/E Δ (51.4 ± 2.5, 128 ± 19, and 55.4 ± 1.2 min⁻¹, respectively) are approximately double those for the parent eNOS, N/E, and N/E proteins (23.5 ± 2.3, 69.9 ± 2.0, and 32.6 ± 2.8 min⁻¹, respectively). Identical effects were seen with KCl or NaCl as the ionic strength buffer (not shown). Thus, in the presence of an approximately physiological salt concentration, the loop deletion mutants consistently expressed a higher activity than the corresponding parent proteins.

**FIG. 3. Calcium dependence of NO-synthesizing activity of NOS proteins. Panel A, wild-type isoforms and INOS reductase-containing proteins: nNOS (●), nNOS (○), eNOS (□), E/I (D), and N/I (○). Panel B, eNOS reductase-containing proteins, with the loop (filled symbols) and without the loop (empty symbols): N/E (●), N/E Δ (○), eNOS (□), eNOS Δ (●), I/E (▲), and I/E Δ (△). Relative rates are shown. Absolute rates used to normalize values to 100% are as follows (in nM): nNOS, 60; eNOS, 16; INOS, 97; eNOS Δ, 50; N/E, 21; N/E Δ, 115; I/E, 28; I/E Δ, 45; E/I, 36; N/I, 36.

Using an extinction coefficient of ε_ε = 100 mM⁻¹ cm⁻¹ for the NOS proteins and ε_ε = 3300 mM⁻¹ cm⁻¹ for human CaM.

**FIG. 4. NOS activity of NOS proteins in the absence (panel A) or presence (panel B) of 100 mM KCl. Error bars indicate the standard deviation for three replicate assays. Gray shading indicates that the protein possesses the loop.**
The activities (± KCl) of eNOSΔ, N/EΔ, and I/EΔ had activities of 2,150 ± 120 and 2,720 ± 370 min⁻¹, respectively, values that are 10–30-fold higher than those for the corresponding parent proteins (111 ± 4 and 119 ± 5 min⁻¹, respectively). In accord with this finding, the E/I and N/I chimeras with the iNOS loopless reductase have high cytochrome c-reducing activities in the absence of CaM (3,360 ± 150 and 4,390 ± 170 min⁻¹). The CaM-free activities of E/I and E/NΔ could not be determined because the proteins had to be coexpressed with CaM. As for overall NOS activity, the cytochrome c reduction rates of the proteins with the iNOS reductase suffered a rate decrease of 50–80% at high salt concentrations.

**CaM Dependence of Cytochrome c Reduction**—A comparison of the white and gray bars for each protein in Fig. 5 shows that the stimulation (“disinhibition”) by CaM is much lower for the loop-deleted mutants. The binding of CaM to eNOS and N/E, which have the wild-type eNOS reductase domain, enhances their activities 12- and 15-fold, respectively. In contrast, the binding of CaM to the corresponding loop-deleted mutants only enhances that of eNOSΔ by a factor of 0.5 and N/EΔ by a factor of 2. The comparison could not be made for I/E and I/EΔ because the proteins had to be coexpressed with CaM. The greater enhancement in the cytochrome c reductase activities of the CaM-free than CaM-bound NOS proteins caused by insert deletion in each instance makes the activities of the CaM-free and CaM-bound proteins nearly equal, as indicated by a lowering in the ratio of the rates (Table I).

The high reductase activities of the CaM-free, insert-deleted proteins are similar to those of the CaM-free chimeras with the insert-less iNOS reductase domain. For example, the cytochrome c reduction rates are comparably high for E/I in the presence and absence of CaM (3,360 ± 150 and 21,37 ± 27 min⁻¹, respectively), and the corresponding rates for N/I are 4,390 ± 170 and 1,654 ± 27 min⁻¹, respectively. Similarly, the isolated iNOS reductase domain expressed in E. coli has high reductase activity (43).

**Ferricyanide Reduction**—Ferricyanide reduction provides an independent and useful measure of reductase activity. Electron transfer from P450 reductase to ferricyanide occurs from the FAD cofactor, in contrast to electron transfer to cytochrome c, which occurs from the FMN cofactor (44). In the case of NOS, the binding of CaM accelerates electron transfer to both cytochrome c and ferricyanide (45). We have examined ferricyanide reduction by two proteins containing the eNOS reductase domain, eNOS and N/E, and by the corresponding loop deletion mutants, eNOSΔ and N/EΔ. In the absence of CaM, the consequences of removing the peptide loop were essentially the same for ferricyanide reduction as for cytochrome c reduction (Fig. 6.)
DISCUSSION

The Ca\textsuperscript{2+} dependence of NO synthesis is a major distinguishing factor among the NOS isoforms, nNOS and eNOS, which have an insert in the FMN binding domain, have a much higher Ca\textsuperscript{2+} requirement than iNOS, which does not have such an insert. As we show here, deletion of the putative autoinhibitory insert dramatically lowers the Ca\textsuperscript{2+} requirement for NO synthesis by both eNOS\textsubscript{D} and the N/E\textsubscript{D} (Fig. 3B). Furthermore, replacement of the reductase domains of eNOS and nNOS with the loopless iNOS reductase domain produces the loopless E/I and N/I chimeras that have a similarly shifted Ca\textsuperscript{2+} requirement (Fig. 3A). Thus, all of the NOS proteins without the insert, whether based on the wild-type or a chimeric structure, have a lower Ca\textsuperscript{2+} requirement for activity than the corresponding insert-bearing parent protein.

In addition to shifting the Ca\textsuperscript{2+} requirement, deletion of the loop unexpectedly produces a modest to large enhancement in the maximum rate of NO synthesis. Thus, the activities of eNOS\textsubscript{D}, N/E\textsubscript{D}, and I/E\textsubscript{D} are higher than those, respectively, of the parent eNOS, N/E, and I/E proteins (Fig. 4), the increase being larger in the presence of a physiological salt concentration. This increase in NO synthesis was caused by an increase in the reductase activity (see below), as found previously for other chimeric proteins (25).

Three key observations must be addressed regarding the effect of the loop on reductase activity. 1) Removal of the loop yields a protein with a reductase activity approaching that of the corresponding CaM-bound parent protein. 2) CaM is required for NO synthesis despite the increased reductase activity of the loop-free proteins. 3) The loopless proteins have a higher reductase activity than the corresponding loop-containing parent protein whether CaM is bound or not.

The binding of CaM to nNOS has been reported to stimulate electron transfer not only from the flavins to cytochrome c but also from NADPH to the flavins (45). The reduction of ferricyanide and cytochrome c by P450 reductase has been shown to occur at different sites (44). Cytochrome c accepts electrons exclusively from the FMN, whereas ferricyanide accepts electrons from the FAD and possibly also the FMN. Deletion of the loop increases the rates of cytochrome c (Fig. 5) and ferricyanide (Fig. 6) reduction by the NOS proteins in both the CaM-free and CaM-bound states. All of the proteins with a specific loop-deleted reductase domain have similar reductase activities if they are compared either in the CaM-free or CaM-bound state (Fig. 5). Furthermore, the reductase activities of these proteins in the absence of CaM are higher than that of the CaM-bound wild-type reductase (Fig. 5).

CaM enhances cytochrome c and ferricyanide reduction by the loop deletion mutants less effectively than by the parent proteins (Tables I and II). A restatement of this is that the CaM-free, loop-deleted proteins already reduce cytochrome c and ferricyanide at rates that more closely approach those for the CaM-bound proteins. A corollary of this is that loop removal increases the reductase activities of the CaM-free proteins to a greater extent than it does the activities of the CaM-bound enzymes. These differences, which are in accord with the finding that the CaM-free proteins are the most stimulated when the insert is deleted, establish that the insert in the eNOS and nNOS reductase domains fulfills an autoinhibitory role. In principle, autoinhibition should be relieved in the CaM-bound proteins whether they retain the autoinhibitory loop or not, so the difference between, for example, CaM-bound eNOS and CaM-bound eNOS\textsubscript{D} is expected to be small. An increase in the activities of the CaM-bound proteins caused by loop deletion is consistent with the fact that, even in the loop-free proteins, there is a small CaM-dependent increase in the rates of both cytochrome c and ferricyanide reduction. If this enhancement is caused by a conformational change that facilitates electron transfer to the heme domain, the conformational change must occur even in the loopless proteins to account for the CaM-de-
pendent incremental stimulation of activity.

P450 reductase and the NOS proteins without an autoinhibitory loop, including the isolated iNOS reductase domain, full-length iNOS, and the CaM-free N/I and E/I chimeras, exhibit a high electron transfer activity as measured by their ability to reduce cytochrome c, and, in the case of the full-length NOS isoforms, to produce NO (Figs. 3 and 4). Conversely, CaM-free NOS proteins with the autoinhibitory loop possess a lower electron transfer activity, as found for CaM-free nNOS, eNOS, and the N/E chimera. CaM-bound proteins with the intact eNOS reductase domain also have a relatively low electron transfer activity, although this activity is higher than that for the CaM-free proteins. In the case of nNOS and I/N, the electron transfer rates when CaM is bound approach those for P450 reductase and iNOS. Likewise, the loopless eNOSA and E/I, N/I, and N/E chimeras have enhanced reductase activities even when free of CaM. In contrast, the electron transfer activities of eNOS, I/E, and N/E (under salt-free conditions) are low even when CaM is bound. All of these observations are consistent with an autoinhibitory role for the loop with respect to the binding of CaM and the activation of electron transfer and NO synthesis. When the strongly inhibitory eNOS loop is removed from these proteins, the enzymatic activities approach, even if they do not equal, those of the loopless iNOS isoform.

The low activity of eNOS compared with nNOS, both of which have inserts in the FMN domain, suggests that only the eNOS autoinhibitory loop is sufficiently effective that its impairment of electron transfer is only partly relieved even when CaM is fully bound. The following evidence supports this inference. 1) The electron transfer activities of CaM-free eNOS and nNOS are lower than that of P450 reductase or the CaM-free iNOS reductase domain expressed as an isolated polypeptide (43). This diminution of the electron transfer activity is completely relieved when the loop in the NOS reductase domain is displaced by the binding of CaM but is only partially relieved in the eNOS reductase domain by either loop deletion or CaM binding. 2) CaM-free nNOS has a higher intrinsic reductase activity than CaM-free eNOS, suggesting that autoinhibition of electron transfer is less pronounced in CaM-free nNOS. 3) The eNOS reductase activity is low even when CaM is bound, suggesting that CaM-bound eNOS remains partially inhibited. This residual autoinhibition is alleviated when the insert is removed, as evidenced by the fact that the reductase activity of eNOSA approaches those of nNOS and iNOS (Fig. 5). 4) Deletion of the eNOS insert, as in eNOSA and N/ΔE, curtails the CaM dependence of the reductase activity. The enhanced (or disinhibited) activity of eNOSA and N/ΔE, observed even in the absence of CaM, is similar to that of E/I, N/I, and the isolated iNOS reductase domains, which have the naturally loopless iNOS reductase domain (43).

Thus, the peptide insert has two salient effects. First, the insert mediates a CaM-dependent inhibition that contributes to the Ca2+ dependence of eNOS and nNOS. Second, the insert lowers the intrinsic activity of the reductase domain. CaM-free eNOSA and N/ΔE therefore have higher reductase activities than even CaM-bound eNOS. These effects may be mediated by inter- or intramolecular mechanisms: either 1) the loop directly or through a conformational change sterically hinders access to the reductase by cytochrome c and ferricyanide, or 2) the loop induces a conformational change within the reductase domain which inhibits intradomain electron transfer; that is, removal of the loop increases intramolecular electron transfer, perhaps in a manner similar to that of the “hinge” domain in P450 reductase which has been suggested to improve the efficiency of electron transfer between the flavins. Another possibility is that the loop interferes with productive docking of the NOS oxygenase and reductase domains and that displacement of the loop by CaM eliminates this interference and allows proper docking. However, this possibility rationalizes the activation by CaM but does not explain the higher intrinsic activity of the loop-free reductase in the absence of CaM. The data, in accord with the observations of other groups (46), indicate that the reductase activity is controlled entirely within the reductase domain.

We provide strong evidence that the insert in the eNOS reductase domain is an autoinhibitory structural element that affects the Ca2+ dependence of eNOS and, by extension, also of nNOS. The insert is largely, but not solely, responsible for the Ca2+/CaM dependence of the constitutive NOS isoforms because CaM is still required for NO synthesis by the eNOSΔ and N/EΔ chimeras. Furthermore, its absence cannot be the sole determinant of the Ca2+ independence of iNOS because chimeras such as I/N (25) and I/E (Fig. 3) with a loop-containing reductase synthesize NO even in the presence of 5 mM EGTA, conditions under which the free Ca2+ concentration is far below the Kd for Ca2+ in eNOS and NOS. The autoinhibitory loop also plays a critical role in governing the net electron transfer activity of the reductase domain. In eNOS, the insert depresses enzymatic activity by inhibiting electron transfer from the reductase to the heme even when CaM is bound. The insert in nNOS may also act in an autoinhibitory manner but is much weaker, in accord with the peptide inhibition studies of Salerno et al. (26) and with the fact that the rate of NO synthesis by purified nNOS, albeit high, is still lower than that of iNOS (8, 12, 15, 16, 19, 20). This difference in the autoinhibitory potency of the eNOS and nNOS inserts may have evolved in response to the different requirements for the synthesis of NO satisfied by the different isoforms in their in vivo cellular context.

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