Intrinsic and Extrinsic Modulation of Nitric Oxide Synthase Activity

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I. Introduction

Nitric oxide synthases (NOSs) are heme- and flavin-containing enzymes that catalyze the synthesis of NO through two serial monooxygenase reactions analogous to those of the NADPH-dependent cytochrome P450 oxidoreductase (CYPOR) systems. Electrons are transferred from NADPH, through the flavins FAD and FMN, to the heme iron, where molecular oxygen is bound and activated.

All NOSs share 50−60% overall amino acid sequence homology[1] and have similar cofactor requirements. The NOSs are functional dimers, with each monomer containing an N-terminal oxygenase domain with binding sites for arginine, tetrahydrobiopterin (H_4B), and a tetracoordinated zinc atom, and a reductase domain with an autoinhibitory region and binding sites for FMN, FAD, and NADPH connected by a linker containing a Ca^{2+}/calmodulin (CaM) binding site. nNOS and eNOS are directly activated by agonist-induced elevation of intracellular Ca^{2+}, binding of Ca^{2+} to CaM, and subsequent binding of CaM to NOS. The constitutive NOS isoforms are also indirectly regulated by H_4B synthesis and by other proteins through direct binding to NOS, subcellular localization, and phosphorylation in neurons, skeletal muscle (nNOS), or endothelial cells (eNOS), whereas iNOS is transcriptionally activated by endotoxins and cytokines in macrophages, hepatocytes, and vascular smooth muscle cells.

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This review, which is not intended to be comprehensive, is focused on the modes of regulation of the isoforms of NOS. As the structures of these enzymes have been revealed, albeit piecemeal in the form of domains of the intact proteins, and as the biochemistry and physiology of the NOS isoforms have revealed various interesting differences among them, it has become obvious that several regulatory mechanisms must be operating.

The elucidation of the three-dimensional structures of all three NOS isoform heme domain dimers by several laboratories[2−6] has presented meager insight into the differences in electron-transport capacities among them. This review will focus attention on the many differences in sequence and thus, potentially, structure and function demonstrated by our laboratory and others among the flavoprotein domains of the NOSs. While it would seem logical that an obvious control mechanism for an enzyme involved in oxygenation reactions would involve electron-transfer processes, only recently have experimental approaches been directed to the flavin-mediated enzymatic reactions.

While it is neither sufficient nor satisfying to implicate either the heme- or the flavin-binding domain in the overall regulation of the NOS isoforms, this review will direct attention to the consequences of activation or inhibition of flavin-mediated electron transfer in the efficiency of NO production. For example, Nishimura et al.[7] examined the effects of *stimulation* of the flavoprotein-mediated activities on the ultimate function of the heme-mediated oxygenation. They showed that increased electron transfer in the flavoprotein domain resulted in stimulation of NO production *only* when the NO produced was scavenged by other proteins or by increased superoxide production. These results clearly establish that overall production of NO by each of the NOS isoforms is finely orchestrated by the rate of electron flux into the heme domain and by the environment of the heme, which determines the ultimate fate of the product, NO.

CYPOR and the C-terminal 641 amino acids of nNOS share 36% identity and 58% close homology,[8] placing NOS reductase domains into the class of flavoprotein dehydrogenases containing both FAD and FMN on the same polypeptide chain, which also includes CYPOR, CYP102, mammalian dihydropyrimidine dehydrogenase, mammalian methionine synthase reductase, and sulfite reductase flavopro-

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tein. Despite the similarities of NOS to these enzymes, NOS is unique in that its reductase activity can be modulated by CaM. CaM induces a conformational change leading to increases in cytochrome *c* reduction, ferricyanide reduction, NADPH oxidation, and the rate of electron transfer through the flavins, all of which will be discussed below. This suggests that significant structure/function differences must exist in the flavin domain of NOSs as compared to the other flavoproteins in this class.

The various NOS isoforms are also intrinsically different from each other because the purified proteins vary greatly with regard to cytochrome *c* reduction rates, stopped-flow electron-transfer rates, heme−nitrosyl complex formation, and NO production rates. The turnover numbers for NO synthesis (at 25 °C) for the various NOS isoforms range from approximately 200 min^{-1} for iNOS[9] to 100 min^{-1} for nNOS[10] to 20 min^{-1} for eNOS.[10] Thus, structural differences must exist among the NOS isoforms which govern the intrinsic rates of electron flux through the enzymes.

The structures of the heme domains of all three NOS isoforms have been solved,[2−6,11] and all three exhibit a very similar overall α/β fold. There are no obvious major differences in the tertiary structures of the heme domains between the isoforms. Unfortunately, no complete structure is available for either the reductase domain or the holoenzyme of any of the NOS isoforms. A partial structure consisting only of the FAD domain of nNOS[12,13] has been solved and is very similar, but not identical, to the FAD domain of CYPOR.[14]

The independently expressed NOS reductase domains reduce cytochrome *c* in the absence of CaM at rates very similar to those of holoenzymes and are likewise stimulated by CaM to similar extents,[15] indicating that the response to CaM involving increased cytochrome *c* reduction is at least partially, if not wholly, contained within the reductase domain. Earlier studies using holo-nNOS that was devoid of heme and H_4B also demonstrated that the CaM effect, in the case of cytochrome *c* reduction but not NO synthesis, is within the reductase domain.[16] Chimeras of NOSs in which the reductase domains of the eNOS and iNOS proteins were replaced by that of nNOS have cytochrome *c* activity similar to that of the parent of the reductase domain (i.e., nNOS), supporting the observations above that the heme domain does not exert significant control over cytochrome *c* activity. More importantly, these experiments established that the maximal rate of NO synthesis was determined by the maximum intrinsic ability of the reductase domain to deliver electrons to the heme.[17] For this and all the reasons discussed above, attention was directed to the reductase domain in search of protein elements responsible for the control of the intrinsic catalytic abilities of each of the NOS isoforms.

Several comprehensive reviews of the structures and cofactor binding of the NOS heme domains have recently appeared[18,19] and are beyond the scope of the

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present review. This review will focus on two forms of regulatory aspects of NOS activity: (1) those intrinsic to the reductase domain of the enzyme and (2) those extrinsic to the protein, i.e., binding of or modification by other proteins.

II. Intrinsic Regulation

A. CaM Binding

All the NOSs require the binding of CaM for NO synthase activity. The reduction of heme in the absence of CaM is very slow[10] and does not support the production of NO from arginine. Flavin reduction in the absence of CaM is also much lower,[10,16,20] and cytochrome *c* reduction occurs at a rate about 1/10 that in the presence of CaM.[10,20,21] The rate of nNOS flavin reduction in the absence of CaM is very similar to that of heme reduction in the presence of CaM[10] and so is potentially fast enough to support heme reduction and NO synthesis; however, electrons simply are not transferred efficiently to the heme in the absence of CaM. Thus, CaM must play a role in facilitating electron transfer from the flavin to the heme domain. In addition, since CaM stimulates both phases of nNOS biphasic flavin reduction, i.e., the initial flavin reduction and comproportionation or transfer of electrons between the two flavins, by 6- and 2-fold, respectively,[10] and reduction of cytochrome *c* by 10-fold, it must also facilitate electron transfer through the flavin domain itself.

CaM can potentially influence two possible electron-transfer steps: (1) NADPH to FAD or (2) FAD to FMN. Matsuda and Iyanagi[20] investigated the transfer of electrons to and between the flavins using nNOS reductase domains with or without an intact CaM binding site to determine the effects of CaM on this process. They demonstrated that the air-stable semiquinone form of nNOS is 1 equiv more reduced than the fully oxidized form and, by monitoring the rates of flavin reduction in the presence and absence of CaM using stopped-flow and rapid scan spectrophotometry, determined that intramolecular transfer of electrons between the flavins of NOS is activated by CaM. They propose that, in the absence of CaM, intramolecular electron transfer is rate-limiting; when CaM is added, the rate of this process increases such that transfer of electrons from NADPH to the FAD becomes rate-limiting, as is the case with the CYPOR. This is mostly, but not totally, consistent with the observation that cytochrome *c* reduction by nNOS is stimulated 10-fold by CaM while ferricyanide reduction is stimulated much less (about 2-fold). The one-electron reduction of ferricyanide occurs mainly at the FAD and so is a one-step process (NADPH → FAD) while that of cytochrome *c* occurs mainly at the FMN, which is a two-step process (NADPH → FAD → FMN), so if CaM *only* increased the rate of intramolecular electron transfer, cytochrome *c* reduction would be stimulated but not ferricyanide reduction. The observation that ferricyanide reduction is also stimulated indicates some additional smaller effect of CaM on NOS electron transfer, perhaps on the introduction of the electron from NADPH to the FAD. CaM does *not* alter the affinity of nNOS for either NADPH[20] or cytochrome *c*.[10]

CaM can potentially effect an increase in electron-transfer rates by several different mechanisms. First, a conformational change in NOS induced by CaM may result in a realignment of the flavins, to positions more favorable to interflavin electron transfer and/or introduction of electrons from NADPH to FAD. Alternatively, the binding of CaM could modulate the redox potentials of the flavins. That potentiometric analysis of nNOS flavin cofactor redox potentials revealed no significant differences between the presence or absence of CaM[22] lends support to a conformational effect of CaM. There is, in fact, a measurable conformational change in the NOS flavin domain upon binding of CaM, as shown by changes in

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                                  Autoinhibitory loop insert
CYPOR (human): WL.QE.....................................TDVD  164
iNOS (murine): SLFML.....................................REL  609
eNOS (bovine): AL.MEMSGPYNSSPRPEQHKSYKIRFNSVSCSDPLVSSWRRKRKESSNTDSAGA  643
nNOS (rat):    AL.MEMRHP.NS..VQEERKSYKVRFNSVSSYSDSRKSSGDGPDLRDNFESTGP  873
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Figure 1. Sequence alignment of CYPOR and three NOS isoforms showing the putative autoinhibitory loop insert.[24]

intrinsic fluorescence,[21,23,24] which have been localized to the FMN domain,[25] and in the trypsin proteolysis pattern.[26,27]

CaM is a 17 kDa, dumbbell-shaped protein with N- and C-terminal globular domains connected by an α-helical linker.[28] Each globular domain or lobe consists of two EF hand motifs (helix−loop−helix structures), each of which can bind a Ca^{2+} atom in a cooperative manner. The lobes also exhibit different affinities for Ca^{2+}, with the affinity of the C-terminal lobe for its two Ca^{2+} atoms being 6-fold that of the N-terminal lobe, such that, at a given Ca^{2+} concentration, CaM will bind its site with zero, one, or two lobes.[29] In iNOS, this binding occurs at intracellular Ca^{2+} levels and is essentially irreversible without destruction of the enzyme. With eNOS and nNOS, binding of CaM is dependent on the concentration of Ca^{2+} present. In nNOS, at least, the C-terminal lobe binds first but cannot activate the enzyme. Binding of the N-terminal lobe, which occurs second, is essential for activation, and the C-terminal lobe may be required for stabilization of the CaM/nNOS complex.[30] It is possible that the C-terminal lobe of CaM is bound to nNOS and eNOS even at low Ca^{2+} concentration and that an increase in Ca^{2+} allows for N-terminal lobe binding and activation.[31,32]

The binding of CaM to peptides derived from the CaM binding sites of the various NOS isoforms generated K_D values of 2, 4, and 0.1 nM for nNOS, eNOS, and iNOS, respectively.[26,31,33−35] The K_D values for the Ca^{2+} dependence of NO synthesis were 300 and 150 nM for nNOS and eNOS, respectively; NO synthesis by iNOS was independent of exogenous Ca^{2+}.[36]

Although the K_D values for CaM binding and Ca^{2+} dependence correlate with the dependence of eNOS and nNOS on increased Ca^{2+} concentrations for activity and the Ca^{2+} independence and essentially irreversible binding of CaM by iNOS, the determinants of these characteristics are not restricted to the CaM binding sequence alone. iNOS chimeras containing the CaM binding sequence of nNOS or eNOS and those of nNOS or eNOS containing the iNOS CaM binding sequence all required an intermediate level of Ca^{2+}, demonstrating that the CaM binding sequence of iNOS was necessary but not sufficient to account for the tight binding and apparent Ca^{2+} insensitivity of the iNOS isoform as compared to eNOS and nNOS.[31,37] Lee et al.[38] identified Lys525 of iNOS, which is outside the actual CaM binding site, as a contributing factor for maximal Ca^{2+}-insensitive activity, but interestingly, this residue does not act by enhancing CaM binding at low Ca^{2+} concentration. Clearly, maximal Ca^{2+} insensitivity must be conferred by at least several disparate structural features throughout the molecule, not all of which have as yet been identified.

B. Autoinhibitory Domain

Alignment of the NOS sequences with those of CYPOR, with which they share 58% sequence similarity,[8] and several bacterial flavodoxins, which function as small electron-transfer proteins, highlighted a region of about 45 amino acids that represents a major insertion in eNOS and nNOS[27] (Figure 1). This insert is absent in iNOS, CYPOR, and all other flavodoxins and FMN-containing proteins examined and is the most obvious region of difference between the cNOSs and iNOS. As the most obvious difference in enzyme function between iNOS and the cNOSs is the tight control of cNOS catalytic activities by the transient binding of Ca^{2+}/CaM, the presence of this insert only in the cNOSs correlates with Ca^{2+}/CaM regulation. Although the crystal structure of an intact NOS FMN domain has yet to be solved, molecular modeling of this region based on solved crystal structures of CYPOR and the FMN-containing bacterial flavodoxins gave an indication of where this insert might be positioned in the cNOSs.[27] The backbone structures of the FMN domains of iNOS and CYPOR were virtually superimposable and consisted of a five-stranded parallel β-sheet with the FMN binding along one edge. The majority of the eNOS and nNOS backbone structures was also superimposable on CYPOR, with the glaring exception of the insert region corresponding to the replacement of a 5−10-residue loop in CYPOR with an approximately 50-residue loop in the cNOSs. The insert lies between two strands of β-sheet that form key interactions with the FMN. The 3D structural model predicted that this loop was positioned opposite the FMN moiety and immediately adjacent to the CaM binding site. Although the structure of the loop could not be approximated due to the lack of an analogous crystal structure, it was rather large (about 1/3 the size of the FMN domain) and appeared to be perfectly situated to interact with residues in or near the CaM binding site, or with CaM itself, thus serving as a control element that competed with and so inhibited CaM binding to its site.

The authors[27] present several lines of evidence to support this hypothesis: (1) Insert-derived peptides were potent inhibitors of nNOS and eNOS NO synthesis activity and CaM binding; this inhibition was relieved by the addition of excess CaM. (2) The rate of CaM dissociation from preformed nNOS/CaM complexes was increased in the presence of peptide. (3) Direct binding of the insert-derived peptide to CaM was undetectable, even with a large excess of peptide. (4) No inhibition was observed with the CaM-dependent phosphatase calcineurin, indicating that the target for the inhibitory peptide was located on NOS. The polypeptides derived from the autoinhibitory region of eNOS were far more potent inhibitors than those derived from nNOS; the most potent

was a hexapeptide, WRRKRK, derived from eNOS. Little inhibition by these peptides was observed with iNOS, arguably because the site of interaction with an autoinhibitory domain was not present. The mechanism presented argues that the insertion found in nNOS and eNOS functions as an autoinhibitory control element that competes with CaM for binding to NOS. In the absence of CaM, the insert binds to NOS in such a way as to obstruct CaM binding and enzyme activation. When CaM binds, the insert is displaced and the enzyme is activated. Evidence for a conformational change in this region upon CaM binding came from both proteolysis and fluorescence experiments. In the absence of CaM, limited proteolysis revealed a preferred cut site in the middle of the CaM binding region. In the presence of CaM, this site was protected and another site, located in the middle of the autoinhibitory domain, became sensitive to proteolysis.[26,27] In addition, binding of CaM promoted changes in both the intrinsic protein fluorescence, localized at least partially to the FMN domain,[25] and the intrinsic flavin fluorescence.[21,23,24]

Salerno et al.[27] found that peptides derived from the nNOS loop were much weaker inhibitors of nNOS and eNOS activity than peptides derived from the eNOS loop region. Lane and Gross[32] both deleted the RRKRK completely and substituted these basic residues with alanines, and found that, in either case, the rate of both cytochrome *c* reduction and NO synthesis by the resulting mutant eNOS was increased over that of wild-type eNOS. It is unclear, however, whether CaM was present during cytochrome *c* reduction in their experiments. Nishida and Ortiz de Montellano[39] also reported a slight increase in cytochrome *c* reduction by their alanine-substituted mutant in the absence of CaM and a significant increase in NO synthesis in its presence, although not as great as when the entire insert is removed. Such an increase in cytochrome *c* reduction was not observed in the presence of CaM; activity was, in fact, decreased as compared to that of the wild-type enzyme. Thus, these basic residues appear to be involved in the inhibitory effect although, clearly, they are not sufficient for a maximum result. These observations are consistent with the idea that the efficacy of the autoinhibitory loop inversely correlates with the intrinsic rate of NO synthesis or cytochrome *c* reduction by the NOSs. iNOS has no autoinhibitory loop and exhibits the highest turnover rates; nNOS, which has a weak autoinhibitory loop, synthesizes NO at a rate that is approximately 1/3 to 1/2 that of iNOS; eNOS, which contains the most potent autoinhibitory loop, synthesizes NO at a rate that is as low as 1/20 that of iNOS.

The idea of an autoinhibitory domain is very attractive because it provides an explanation for one of the basic differences between iNOS and the cNOSs—the differences in CaM affinity and Ca^{2+} requirements. As discussed earlier, these distinctions could not be explained solely by the variations in the amino acid sequences of the CaM binding regions in these enzymes but, rather, must include some additional element(s) on either the iNOS, to increase CaM affinity, or the cNOSs, to decrease CaM affinity. An autoinhibitory element on the cNOSs would compete with CaM binding, thus reducing the apparent affinity of the enzyme for CaM. The absence of this insert from iNOS, along with the CaM binding site itself, could then contribute to its enhanced affinity for CaM at low Ca^{2+} levels.

The Salerno et al. hypothesis[27] of the insert functioning as an autoinhibitory domain is eminently testable, and several laboratories created NOS constructs in which this region was deleted or swapped between isoforms. Daff et al.[40] constructed two deletion mutants in which either 40 or 42 residues were removed from the putative autoinhibitory domain of nNOS and expressed them in yeast. Unlike the wild-type nNOS, the mutant synthesized NO in the absence of exogenously added Ca^{2+}/CaM. Cytochrome *c* reduction in the absence of Ca^{2+}/CaM was also higher than that of the wild type, although about a 2-fold stimulation was observed in its presence, as compared to 10-fold for the wild type, making the cytochrome *c* reduction rates very similar for both mutants and the wild type when Ca^{2+}/CaM was present. They then examined NO production in the presence of 50 μM Ca^{2+} and increasing amounts of EGTA. As the EGTA concentration increased, the effective Ca^{2+} concentration decreased. When EGTA:Ca^{2+} was 1:1, the wild type nNOS was strongly inhibited, and activity decreased to 0 in the presence of 60 μM EGTA. Both mutants synthesized NO at their maximum rates at higher EGTA concentration, i.e., lower Ca^{2+} concentration, than the wild type and, even in the presence of 140 μM EGTA, still retained 30−40% of their maximal activity. Daff et al.[40] also examined the reduction of heme by NADPH under anaerobic conditions and found that it occurred in the wild-type nNOS only in the presence of Ca^{2+}/CaM, but occurred in the mutants even in its absence. These results supported the hypothesis that the insert in the wild-type nNOS played an important role in disabling the enzyme by inhibiting electron transfer from the FMN to the heme when Ca^{2+} was low, consistent with its role as an autoinhibitory domain.

Montgomery et al.[31] also removed 43 amino acids from the rat nNOS isoform and confirmed that this deletion reduced the Ca^{2+} requirement for NO synthesis by the holoenzyme or cytochrome *c* reduction by the reductase domain, although these investigators reported a 5-fold drop in the rate of NO synthesis as a result of the deletion. Both of these investigators[40,41] reported that the nNOS loopless mutant enzymes were deficient in FMN and required exogenous supplementation for activity, indicating that the binding interactions with FMN and/or enzyme stability were weakened by this deletion. In contrast to Daff et al.,[40] Montgomery et al.[41] report that NO synthesis activity at 10 nM Ca^{2+} was not detectable. However, they also reported greater instability of their mutants as compared to Daff et al.,[40] presumably due to the difference in residues removed. Daff et al.[40] left somewhat of a linker region, while Montgomery et al.[41] excised the loop completely. This may explain why one investigator reported NO

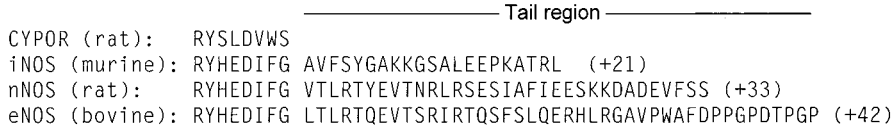

Figure 2. Sequence alignment of the C-terminal residues of CYPOR and three NOS isoforms showing the tail region of the NOSs.[17]

synthesis by the nNOS loopless mutant in the absence of CaM while the other did not.

Removal of either the CaM binding site or both the CaM binding site and the putative autoinhibitory insert from an nNOS reductase domain yielded an enzyme which reduced DCIP and cytochrome *c* similarly to wild-type nNOS in the absence of CaM,[41] suggesting that CaM was still required to align the reductase domain for optimal electron transfer through the flavins even in the absence of the insert.

Further support for the involvement of the putative autoinhibitory domain in Ca^{2+}/CaM regulation came from Nishida and Ortiz de Montellano,[36] who deleted the insert from wild-type eNOS and from chimeras created from the heme and CaM binding domains of nNOS or iNOS attached to eNOS reductase domains encompassing the FMN, FAD, and NADPH binding sites. They also constructed chimeras in which the heme and CaM binding domains were donated by eNOS or nNOS while the reductase domain was donated by iNOS, which has no autoinhibitory loops. Both eNOS/iNOS and nNOS/iNOS chimeras exhibited a 20-fold increase in affinity for Ca^{2+} as compared to wild-type nNOS, wild-type eNOS, or an nNOS/eNOS chimera, all of which contain an autoinhibitory loop, confirming that the Ca^{2+} dependence of NO production correlates with the presence or absence of the loop region. In the eNOS, nNOS/eNOS, and iNOS/eNOS loop-deletion mutants, the Ca^{2+} dependence of the enzyme was also lessened, consistent with the suppression of autoinhibition by the loop domain. Removal of this loop also increased the rates of both NO synthesis (2-fold) and cytochrome *c* reduction (2−3-fold in the presence of CaM, and 10−30-fold in its absence) by eNOS, indicating that this insert not only plays a role in CaM activation, but also contributes to the overall low activity of eNOS even in the presence of CaM. That is, the eNOS autoinhibitory loop is so effective that its suppression of electron transfer is only partially relieved even when CaM is fully bound. In the absence of Ca^{2+} and, presumably, CaM binding, the loopless mutant still retains 10% of the activity seen in the presence of CaM, which is equivalent to 30% of the wild-type activity in the presence of CaM.

Similar results were reported by Chen and Wu,[42] who deleted 45 amino acid residues from this region in human eNOS expressed in baculovirus cells. The resulting protein had several characteristics reminiscent of iNOS, i.e., endogenously bound CaM and a relative insensitivity to EGTA. This engineered enzyme did not require the addition of Ca^{2+} or CaM to attain maximum levels of NO synthesis and cytochrome *c* or ferricyanide reduction but, like iNOS, needed to be coexpressed with CaM to be properly folded and stabilized. In addition, the eNOS deletion mutant retained 60% of its NO sythesis ability in the presence of 5 mM EGTA, whereas wild-type eNOS was completely inhibited. The maximum rates of NO synthesis and cytochrome *c* reduction were 1.5 and 2.5 times higher than those of wild-type eNOS in the presence of CaM, again indicating that the insert plays some role in suppressing electron transfer between the two domains even in the presence of CaM, thus contributing to the low intrinsic activity of eNOS as compared to the other isoforms.

C. C-Terminal Tails

When compared to CYPOR, all of the NOS isoforms contain approximately 21−42 additional amino acids at the C-terminus, forming a "tail" not present in CYPOR[9,10] (Figure 2). On the basis of the crystal structure of the nNOS FAD domain,[12,13] which is very similar to that of CYPOR, the C-terminus not including the tail lies near the FMN/FAD junction. The crystallized nNOS FAD domain contained about 10 residues of the tail, but these residues were not visible in the structure, indicating that it is a flexible rather than ordered region. To determine the functional role of this tail region, Roman et al.[9,10] removed these residues from all three isoforms.

iNOS is perhaps the most similar of the NOS isoforms to CYPOR since it lacks the autoinhibitory domain discussed above and the reduction of cytochrome *c* by iNOS or its reductase domain (iNOSred) is comparable to that of CYPOR (∼3000 min^{-1}). Removal of the C-terminal 21 amino acids from either iNOS or iNOSred yields enzymes with markedly higher cytochrome *c* reductase and NADPH oxidation activities, 5−10-fold those of the intact proteins. The ability of the truncated iNOS to synthesize NO was also increased by about 20%.

The rate of iNOS flavin reduction under turnover conditions was examined using stopped-flow spectrophotometry in an attempt to dissect the mechanism determining the increased activities by the truncated iNOS. The biphasic rate of flavin reduction was increased 2-fold in the fast phase, representing the conversion of fully oxidized flavins to the fully reduced and semiquinone forms, and 5-fold in the slow phase, which probably represents transfer of electrons between flavins or comproportionation. Interestingly, the reoxidation of the flavin domain differed between the intact and truncated forms. Intact iNOS formed an air-stable, one-electron-reduced semiquinone form which persisted for at least 20 min, reminiscent of CYPOR, which displays the same behavior, albeit for a more prolonged period. The truncated iNOS, on the other hand, became fully reoxidized and never displayed a stable semiquinone form. The authors[9,10] postulate that the tail modulates the distance and/or angle between the two flavins, which, in turn, regulates electron flow be-

tween them. Removal of the tail may leave these flavins more exposed and thus more easily oxidized than those of CYPOR or the intact counterpart of iNOS. Thus, the tail performs a protective function, shielding the flavins from solution, as well as inhibiting electron flow through or between the flavins.

Removal of the 33 and 42 C-terminal residues of nNOS and eNOS, respectively, gave Roman et al.[10] a perspective on the complex interactions among the tail region, CaM binding, and the autoinhibitory domain. Truncated nNOS and eNOS catalyzed cytochrome *c* reduction 21- and 7-fold faster, respectively, than the intact proteins in the absence of CaM. While CaM is a nonessential activator of cytochrome *c* reduction by intact nNOS and eNOS, increasing activity by 10-fold, CaM becomes a partial noncompetitive inhibitor of the truncated enzymes, decreasing activity 35−50%. CaM, in fact, potentiates cytochrome *c* reduction by the truncated NOSs to the same level as the intact enzymes. Thus, for nNOS and eNOS in the presence of CaM, the tail does not limit cytochrome *c* reduction; CaM does.

The rates of nNOS and eNOS flavin and heme reduction under turnover conditions were also examined using stopped-flow spectrophotometry. In the absence of CaM, the rate of nNOS flavin reduction was increased 7-fold in the fast phase and 2−3-fold in the slow phase, but in the presence of CaM, both intact and truncated enzymes had similar rates for both phases. Comparable results were obtained for eNOS. Consistent with what was seen with iNOS, neither of the truncated isoforms exhibited the air-stable, one-electron-reduced semiquinone form characteristic of the wild-type enzymes in either the presence or absence of CaM.

D. Negative Intrinsic Modulation of NOSs

A picture of the complex interactions among CaM, the autoinhibitory domain, and the tail region has begun to emerge. CaM appears to function both by realigning the heme and reductase domains to allow electron transfer to the heme and by alleviating the repression of the two negative control elements in the NOS reductase domain: the autoinhibitory domain and the C-terminal tail region. In the absence of CaM, the autoinhibitory domain inhibits electron flow to the heme domain and both the autoinhibitory domain and the tail impede electron flow through the flavins. CaM binds, and the tail shifts somewhat, but inhibition is not completely alleviated; the tail region does not shift completely out of the way possibly because it still performs the necessary protective function of shielding the flavins from oxygen, thus decreasing the possibility of direct oxygen reduction to superoxide by the reductase domain. The autoinhibitory domain shifts away from its docking point, possibly the CaM binding site, but still remains in such a position as to partially hinder electron flow through the flavins. Thus, both elements continue to restrict electron flow even in the presence of CaM, thus setting the varied intrinsic rates of the NOS isoforms. The tail region appears to be the dominant negative regulator of electron transfer through the flavins in the absence of CaM, as indicated by the much higher rate of cytochrome *c* reduction in the truncated mutants than in either the intact or truncated enzymes in the presence of CaM. In the presence of CaM, however, the autoinhibitory domain is the dominant negative element for electron transfer through the flavins, which in turn influences electron transfer to the heme and ultimately, since the rate of NO synthesis is determined by the maximum intrinsic ability of the reductase domain to deliver electrons to the heme,[17,43] the maximal rate of NO production. Cytochrome *c* reduction by the iNOS truncated enzyme in the presence of CaM was stimulated above that of the intact iNOS, unlike the nNOS or eNOS truncations. Because the autoinhibitory domain is nonexistent in iNOS, this isoform is modulated only by the partial inhibition exerted by the tail region in the presence of CaM, an effect that is masked in eNOS and nNOS by the stronger negative effect of the autoinhibitory domain.

III. Extrinsic Factors

In addition to intrinsic protein controls on NOS activity, extrinsic elements play a major role in regulation, particularly in the case of eNOS. Direct interactions with other proteins or modifications such as phosphorylation are described below.

A. Phosphorylation in the Reductase Domain

Although the autoinhibitory domain and the tail region are regulatory elements intrinsic to NOS itself, both make attractive targets for in vivo modulation via interactions with other proteins or posttranslational modifications such as phosphorylation. Accordingly, several laboratories noted that both nNOS and eNOS contained serine/threonine protein kinase phosphorylation sites in the tail region of the reductase domain, Ser1177 for human eNOS and Ser1412 for rat nNOS, and in the autoinhibitory domain, Ser633 in eNOS and Ser847 in nNOS.[8,44−48]

Fulton et al.[44] demonstrated the positive regulation of bovine eNOS in vivo by phosphorylation via VEGF of Ser1179 in bovine endothelial cell culture by the serine/threonine protein kinase Akt. Dimmeler et al.[48] simultaneously published similar results using the human eNOS isoform, which is phosphorylated via sheer stress at the analogous residue, Ser1177, in HUVEC cells. In both cases, phosphorylation of this serine, which resides 16 residues after the start of the tail region, caused an increase in NO output and qualitatively shifted the dose/response curve for Ca^{2+} in NO synthase activity to a lower level. Mutation of this residue to alanine, S1179A or S1177A, obliterated phosphorylation and activation by Akt, and mutation to aspartate, S1179D or S1177D, mimicked the activation of eNOS observed upon phosphorylation.

Similar results were obtained by Chen et al.[45] using AMP-activated protein kinase (AMPK). These investigators reported a 1.5-fold increase in NO synthesis and a qualitative shift of the required Ca^{2+} concentration to a lower level when purified recombinant eNOS or purified rat heart eNOS was coincubated with AMPK, which specifically phosphorylated hu-

man eNOS at Ser1177. In rat heart extracts, AMPK co-immunoprecipitated with eNOS, indicating the likelihood of a close association of these proteins in vivo.

Positive regulation of eNOS by phosphorylation was also shown by Butt et al.,[47] who reported phosphorylation of purified human eNOS Ser1177 by both cAMP-dependent protein kinase (cAK) and cGMP-dependent protein kinase II (cGKII). Phosphorylation of eNOS resulted in a 4−6-fold increase in NO synthesis and caused partial Ca^{2+}/CaM-independent activation of the enzyme. That is, eNOS synthesized NO in the absence of exogenously bound Ca^{2+}/CaM. Since the enzyme was produced in *sf9* cells, CaM was present during purification and may have remained bound to eNOS even in the absence of Ca^{2+}, consistent with the observations of Fulton et al.,[44] Dimmeler et al.,[48] and Chen et al.[45] that the Ca^{2+} requirement of the phosphorylated enzyme was lowered.

McCabe et al.[49] investigated the in vitro effects of phosphorylation at this site by mutating bovine eNOS Ser1179 to aspartate (S1179D), which mimicked the negative charge afforded by phosphorylation, and expressing it in *E. coli*. The purified S1179D protein demonstrated a 2-fold increase in NO production and a 2−4-fold increase in cytochrome *c* reduction that was not due to an alteration in affinity for arginine, CaM, or cytochrome *c*. The enzyme also showed increased resistance to EGTA, indicating that, like the phosphorylated wild-type enzyme, lower levels of Ca^{2+} were required for enzyme activation. The negatively charged phosphate or aspartate at this position may be repelled by neighboring negative charges, thus repositioning the tail region. These results are consistent with the model in which the C-terminal tail inhibits electron flow through the flavin domain in the absence of CaM, but is shifted upon CaM binding so as to be less inhibitory.

The analagous mutation was made in nNOS (S1412D)[10,50] and yielded an enzyme with faster cytochrome *c* and ferricyanide reduction, slower NO synthesis, greater uncoupling of NADPH oxidation, faster heme reduction, faster flavin reduction, particularly in the second, slow phase, and faster heme−NO complex formation and accumulation. Adak et al.[50] concluded that the Ser1412 mutation stimulated electron transfer out of the reductase domain. This faster heme reduction increased the rate of NO formation but diminished release. Geminate recombination of the NO produced to the heme formed an inactive nitrosyl complex, thus explaining how faster electron flow through the reductase domain could lead to slower NO production. Thus, the rate of heme reduction displays an optimum for NO release during steady-state catalysis. In nNOS, unlike eNOS, which demonstrated higher NO synthesis when Ser1179 was phosphorylated or mutated to Asp, heme reduction already occurs at a near optimal rate.

Interestingly, Chen et al.[45] reported that, in the absence of CaM, AMPK also phosphorylated eNOS Thr495, which is located within the CaM binding site, inhibiting activation of NO production, and thus also implicating phosphorylation in negative modulation of eNOS activity. Michell et al.[51] describe the coordinated positive and negative control of eNOS by multiple protein kinases and phosphatases acting at Thr495 and Ser1177. They propose that protein kinase A (PKA) signaling promotes the dephosphorylation of Thr495 by phosphatase PP1 and phosphorylation of Ser1177 by multiple protein kinases (e.g., Akt, PKA, and AMPK), thus activating eNOS. Conversely, protein kinase C (PKC) signaling causes dephosphorylation of Ser1177 by phosphatase PP2A and phosphorylation of Thr495, thus inhibiting NO production by eNOS. A similar scenario was described by Harris et al.[52] and by Fleming et al.[53] with bradykinin-stimulated bovine and porcine, respectively, aortic endothelial cells. Both studies found that eNOS Thr495 was constitutively phosphorylated in these cells, but that bradykinin stimulation caused rapid dephosphorylation at this site and subsequently enhanced phosphorylation at Ser1177, thus explaining the agonist effect of bradykinin on NO production.

Adding yet another layer of complexity to the phosphorylation story, Butt et al.[47] reported phosphorylation of human eNOS at Ser633, which lies within the autoinhibitory domain. Fulton et al.[44] identified this residue as a possible consensus site for Akt phosphorylation, but did not detect phosphorylation at this site. The results of Butt et al.,[47] discussed above, were obtained with enzyme phosphorylated at both Ser633 and Ser1177, so *both* modifications could potentially have contributed to the increase in NO synthesis by eNOS observed. Hayashi et al.[46] also reported phosphorylation of nNOS Ser847, the analogue to eNOS Ser633, by CaM-dependent protein kinases Iα, IIα, and IV. The Ser847-phosphorylated nNOS demonstrated a 40−50% inhibition of NO synthesis and a 65% inhibition of cytochrome *c* reduction, which was at least partially due to suppression of CaM binding.

Although the in vivo situation is still unclear as to exactly which isoforms are phosphorylated, which sites are truly phosphorylated, what circumstances induce phosphorylation, and which kinases are involved, it is clear that modulation of enzyme activity by phosphorylation can and most likely does occur for at least the eNOS isoform.

B. Protein/Protein Interactions

Formation of NO by the NOSs is dramatically influenced by the cellular environment. One of the critical components of such an environment is the presence of other proteins near NOS. Protein/protein interactions play an important role in the control of NOS activity and, therefore, NO production (Chart 1). The critical role of calmodulin in NO production has already been described above. Two other protein/NOS interactions are of particular interest, those that are PDZ-motif-mediated in nNOS and caveolin-mediated (or caveolae-localized) in eNOS.[54]

Neuronal NOS must be tightly regulated because the detrimental or beneficial effect of NO depends mainly on its cellular localization and content.[55] PDZ motifs are modular protein/protein interaction do-

Chart 1. Protein/Protein Interactions of eNOS

PROTEINS WHICH BIND TO eNOS:

ACTIVATE eNOS	INHIBIT eNOS
Calmodulin, Hsp 90 (ENAP-1)	Caveolin-1 and caveolin-3, Bradykin-2 receptor, Dynamin-2

OTHER PROTEINS ASSOCIATED WITH eNOS

Angiotensin II (AT1) and endothelin-1 (ETB) receptors, Cationic amino acid transporter (y+)

mains of 80−120 amino acid residues. Originally called the GLGF repeat, on the basis of the amino acid sequence, later termed DHR (disks-large homologous region), and finally called PDZ motifs or domains,[56] their function is to direct intracellular protein into multiprotein complexes.[57] The N-terminus of nNOS contains the sequence GLGF and, therefore, a PDZ domain.[57] Hillier et al.[58] published the structure of an nNOS−syntrophin PDZ complex, which revealed that the domains interact in an unusual linear head-to-tail arrangement. In brain, the nNOS PDZ domain targets the enzyme to postsynaptic sites by binding to corresponding domains of PSD-95, PSD-93, and other proteins. PSD-95 binds to the *N*-methyl-D-aspartate (NMDA) receptor and mediates a link between this receptor and nNOS.[59,60] In the search to determine ligands for the nNOS PDZ domain, Stricker et al.[61] screened 13 billion distinct peptides and found that the nNOS PDZ domain binds tightly to peptides ending in the amino acid stretch DXV. Guided by this consensus sequence, glutamate and melatonin receptors were identified as possible candidates for nNOS interaction.[61] Schepens et al.[62] used two-hybrid methodology to address similar issues and showed that proteins bearing the C-terminal sequence G(E,D)XV were preferred targets for the nNOS PDZ domain. A melanoma-associated antigen, cyclophilin, and α1C-adrenergic receptor were identified as potential targets for nNOS.

The nNOS-associated adaptor protein CAPON (carboxy-terminal PDZ ligand of nNOS), which interacts with the nNOS PDZ domain through its C-terminus, represents another step in the control of NO signaling in the brain. CAPON competes with PSD-95 for interaction with nNOS and may, therefore, influence nNOS by regulating its ability to associate with PSD-95/NMDA-type glutamate receptor complexes.[63] Serving as an adaptor protein, CAPON specifically couples the G protein Dexras 1 to nNOS, forming a ternary complex and thus enhancing NO signaling.[64] NMR structure studies showed that the DXV-COOH peptide and a C-terminal peptide from CAPON bind to the same pocket of the nNOS PDZ domain.[65]

Another protein able to bind to nNOS via its PDZ domain is the muscle isoform of phosphofructokinase (PFK-M). The product of PFK, fructose-1,6-bisphosphate, is neuroprotective, and part of the neuroprotective effect of nNOS may come from its binding to PFK.[66]

One of the spliced forms of nNOS, nNOS-β, lacks the PDZ domain and is therefore not targeted to synaptic membranes, but retains full enzymatic activity.[55] Therefore, regulation of nNOS activity may be controlled by alternative splicing, resulting in alterations in protein/NOS interactions.

Residues Met228−His244 of the N-terminus of nNOS were also shown to bind the 8 kDa light chain of dynein, found originally as "protein-inhibiting nNOS" (PIN).[67] The structure of the PIN dimer bound to a 13-residue peptide from nNOS suggests that PIN is a linker protein that mutually orients two proteins with appropriate target sequences.[68]

Neuronal NOS is abundant in skeletal muscle. The PDZ-domain-mediated partner of nNOS in this tissue is syntrophin; both proteins are components of the dystrophin complex.[59] The area in which nNOS resides in plasmalemmal domains of skeletal muscle by virtue of its binding to dystrophin complexes[69] is also enriched in caveolin-3-coated caveolae.[70] All three NOS isoforms contain the conserved sequence FXXFXXXXW, the putative caveolin binding site, in their heme domains.[71] nNOS activity was clearly inhibited by a peptide derived from the caveolin-3 scaffolding domain.[72]

eNOS is anchored to caveolae in part because of its cotranslational N-myristoylation and posttranslational palmitoylation.[73,74] Caveolae are small invaginations on the surface of many cells and are considered to be a specialized form of raft.[75] In recent years, caveolae have been seen as organizing centers for signaling molecules, such as Src family kinases, endothelial NOS, epidermal growth factor receptor, platelet-derived growth factor receptor, phospholipase Cγ, protein kinase Cα and -β, Ras, trimeric G protein Gα subunits,[75−77] and certain heptahelical receptors, such as the bradykinin B2 receptors[78,79] and the Edg-1 receptor for sphingosine 1-phosphate.[80] It is likely that the number of such receptors associated with caveolae will grow. The principal structural components of caveolae are integral membrane proteins, caveolin-1, caveolin-2, and caveolin-3. Caveolins may interact with a whole variety of signaling molecules through their "scaffolding domains", a conserved domain of 20 amino acids (residues 82−101 in caveolin-1).[71]

eNOS was shown to interact directly with and be inhibited by caveolin-1 and caveolin-3 in vitro, in endothelial cells, and in cardiac myocytes.[73,81,82] The interaction of calmodulin and/or caveolin-1 with eNOS is mutually exclusive, suggesting that the intracellular calcium concentration governs eNOS activity by allowing binding of CaM and disposition of caveolin-1.[83] Direct interaction between caveolin-1 and the reductase domain of eNOS was also clearly demonstrated,[84] extending the effect of caveolin from the heme domain distally. This additional contact aids in understanding the observed interaction of eNOS and caveolin in view of the eNOS crystal structure, which revealed the lack of exposure, except for the C-terminal tryptophan, of the consensus sequence, FXXFXXXXW, for caveolin binding.[3]

The biologically active oligopeptide bradykinin (RPPGFSPFR) acts on the bradykinin 2 receptor (B2R) to increase intracellular Ca^{2+} levels, which, in complex with CaM, transiently activates eNOS.

Palmitoylation of eNOS is dynamically regulated by this process.[85] Venema's group reported that eNOS forms an inhibitory complex with B2R from which the enzyme is released in an active form upon receptor activation.[86] A B2R peptide spanning residues 310−329 blocks flavin to heme electron transfer in eNOS as well as in nNOS.[87] It was suggested that bradykinin plays a role in eNOS activation in bovine aortic endothelial cells by deinhibition of the enzyme through calcineurin-mediated dephosphorylation at Thr497.[52]

Michel's group recently introduced yet another aspect to the already complex regulation of eNOS. Physiologically relevant concentrations of sphingosine 1-phosphate (S1P) lead to eNOS activation via Edg-1 receptor stimulation, suggesting an S1P → PI-3 → Akt−eNOS phosphorylation pathway.[80,88] eNOS can also bind angiotensin II AT1 and endothelin-1 ETB receptors, but not the ATP P2Y2 receptor.[85,89] Recent studies showed that GST-dynamin binds recombinant eNOS and potentiates eNOS catalysis in solution, indicating that the protein can bind directly and in a functional manner in the absence of an adaptor protein.[90]

Nitric oxide production is increased when the molecular chaperone heat shock protein 90 (Hsp90) associates with eNOS.[91] The interaction between eNOS and Hsp90 and concomitant increase of eNOS activity in human endothelial cells are supported by vascular endothelial growth factor, histamine, and fluid shear stress. Pritchard et al.[92] showed that Hsp90 is essential for eNOS-dependent NO production and that inhibition of ATP-dependent conformational changes in Hsp90 uncouples eNOS activity and increases eNOS-dependent superoxide anion production.[83] nNOS−Hsp90 complexes also have been detected recently, and the role of Hsp90 in the incorporation of heme into nNOS in vivo was suggested.[93]

Very little information is available regarding iNOS protein/protein interactions. An interaction between iNOS and the Rho family GTPase Rac2 was identified; this interaction may increase iNOS activity.[94] Sustained NO production in macrophages requires the arginine transporter CAT-2,[95] suggesting that, as for CAT-1, direct interactions may take place.

IV. Conclusions

The NOS isoforms are modulated by a precise symphony of both intrinsic and extrinsic factors. The overall rate of NO production by each of the isoforms is finely tuned by an amazingly complex combination of intrinsic factors, including the heme environment, the dimerization state, CaM binding, the autoinhibitory domain, and the C-terminal tail region, and extrinsic factors, such as posttranslational modifications, phosphorylation, and specific interactions with other proteins. Further understanding of the intrinsic elements will come with structural studies of the reductase domain and the determination of more specific contacts among CaM, the autoinhibitory domain, the tail region, and the body of the enzyme itself.

V. Abbreviations

NOS	nitric oxide synthase
nNOS, eNOS, iNOS	neuronal, endothelial, and inducible isoforms, respectively
NO	nitric oxide
CYPOR	NADPH-dependent cytochrome P450 oxidoreductase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
H_4B	tetrahydrobiopterin
CaM	calmodulin
VEGF	vascular endothelial growth factor
HUVEC	human umbilical vein endothelial cells
AMPK	AMP-activated protein kinase
CAK	cAMP-dependent protein kinase
cGKII	cGMP-dependent protein kinase II
Hsp90	heat shock protein 90
PKA	protein kinase A
PKB	protein kinase B
B2R	bradykinin 2 receptor

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