Electron Transfer by Neuronal Nitric-oxide Synthase Is Regulated by Concerted Interaction of Calmodulin and Two Intrinsic Regulatory Elements*

Linda J. Roman¹ and Bettie Sue S. Masters

From the Department of Biochemistry, the University of Texas Health Science Center, San Antonio, Texas 78229

The nitric-oxide synthases (NOSs)² comprise a family of enzymes that catalyze the formation of nitric oxide and L-citrulline from L-arginine through a series of monooxygenation steps using NADPH as the electron donor. There are three different isozymes encoded by different genes, neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). Nitric oxide has been implicated in hemodynamic control, neurotransmission, and the immune response, depending on the cell type in which it is being expressed (for review see Refs. 1–8).

All three NOS isoforms are modular, cofactor-containing enzymes. They can be roughly divided into an oxygenase domain, containing heme, tetrahydrobiopterin, and the arginine-binding site, and a reductase domain, containing the flavins FMN and FAD, as well as the NADPH-binding site. These domains are connected by a calmodulin (CaM)-binding site, which is always occupied under physiological conditions by CaM in iNOS, whereas CaM binding to nNOS and eNOS requires an increase in intracellular calcium.

CaM controls NOS activity by regulating the rate of electron flux, by enhancing electron flow through the flavin domain as well as switching on transfer of electrons from the flavin to the heme domain. The flow of electrons is in the form of a hydride ion from NADPH to FAD, from FAD to FMN, and as an electron from FMN to the final acceptor, i.e. the heme domain of the adjacent monomer in the NOS dimer (9–11) or exogenously added cytochrome c. Recent models (12, 13) propose that bound NADPH locks the enzyme into a state in which the FMN domain is closely associated with the FAD and NADPH binding domains, and the FMN is deeply buried in the protein, inaccessible to other electron acceptors. In this state, electrons are passed in a facile manner from FAD to FMN. In order for electrons to be passed from FMN to the final acceptor, according to this model, the FMN domain must be unlocked and must move to another position so that it is accessible to the final electron acceptor. CaM facilitates this release of the FMN domain from the closed to the open position, in part by accelerating the release of NADP⁺ (12, 14). The mechanism behind this control by CaM is unclear; however, the CaM response is encoded by the reductase domain, as the isolated reductase domain retains stimulation by CaM (15–17).

In addition to the CaM-binding site, there are at least two CaM-sensitive elements in the NOS reductase domains, the autoregulatory region (AR, originally identified as an ~40-residue sequence in the FMN-binding subdomain (18)), which was proposed to compete with CaM binding, and the C-terminal tail region (19, 20), which impedes electron flow between the flavins in the absence of CaM. Several investigators have deleted the AR, creating an enzyme with reduced CaM control of reductase activity and decreased calcium sensitivity of both reductase activity and NO synthesis (21–25). Deletion of the tail yields an enzyme with greatly enhanced electron transfer through the reductase domain in the absence of CaM. In the

¹To whom correspondence should be addressed: Dept. of Biochemistry, the University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78229; Tel.: 210-567-6979; E-mail: roman@uthscsa.edu.
²The abbreviations used are: NOS, nitric-oxide synthase; nNOS, eNOS, iNOS, neuronal nitric-oxide synthase, endothelial nitric-oxide synthase, inducible nitric-oxide synthase; NO, nitric oxide; CaM, calmodulin; CYPOR, cytochrome P450 oxidoreductase; HPLC, high pressure liquid chromatography; TnC, troponin C; SI, small insertion.
Regulation of Electron Transfer in the nNOS Reductase Domain

presence of CaM, activity is equivalent to that of wild-type enzyme (19, 20, 26).

In this study, we describe the characteristics of an enzyme in which both the AR and tail regions have been deleted. This enzyme has completely lost CaM modulation of electron transfer through the reductase domain, although transfer from the reductase to the oxygenase domain remains almost totally CaM-dependent. We also propose a model in which these elements interact directly with each other and CaM, incorporating the concept of the NADPH "lock" proposed by Daff and co-workers (12) and the tethered shuttle mechanism of the FMN domain proposed by Ghosh and Salerno (13), to explain the physical basis of electron transfer control through the reductase domain.

EXPERIMENTAL PROCEDURES

Chemicals—(6R)-5,6,7,8-Tetrahydrobipterin was from Research Biochemicals International (Natick, MA). All other chemicals were from Sigma and were of the highest grade available.

Enzymes—Pfu Turbo polymerase was from Stratagene (La Jolla, CA); shrimp alkaline phosphatase was from U. S. Biochemical Corp., and restriction enzymes were from Promega (Madison, WI) or New England Biolabs (Beverly, MA).

Plasmids—The plasmid nNOSpcW was constructed by Roman et al. (27) using pCWori+ (28). The plasmid encoding calmodulin, CaM ACMIP, was kindly provided by Anthony Persechini of the University of Missouri, Kansas City.

Recombinant DNA Manipulations—nNOS-AR and nNOS-AR-tr1 were created as follows; the codons encoding the initial 877 amino acids of recombinant rat nNOS, minus the coding sequence for the autoregulatory insert (residues Pro832–Asn868), were amplified by PCR, using nNOS minus the coding sequence for the autoregulatory region (residues Pro832–Asn868), were amplified by PCR, using nNOS and ligated with NdeI/XbaI-digested pCW vector. This ligation was used to transform XL10-gold cells (Stratagene), and colonies were screened by restriction digest. The correct construct was then used to cotransform E. coli BL21 cells along with the groELS plasmid (27).

Protein Expression and Purification—nNOS, nNOS-tr1, nNOS-AR, and nNOS-AR-tr1 were expressed and purified as described previously (27), except that cultures were grown in 500 ml of medium in Fernbach flasks, all of the column equilibration and wash buffers contained 100 mM NaCl, and the protein was eluted with buffer containing 500 mM NaCl and 15 mM 2',3'-AMP. Calmodulin was prepared by the method of Zhang and Vogel (29). CaM-CC (30) was kindly provided by Dr. Anthony Persechini of the University of Missouri, Kansas City.

Analysis of FMN and FAD Content—Flavin content of these proteins was analyzed using a Waters analytical HPLC system, equipped with a 2487 absorbance detector and 515 HPLC pumps. A Nova-Pak C18, 60 Å, 4-μm (3.9 × 150 mm) column was fitted with a guard column packed with Nova-Pak C18 Guard-Pak HPLC column inserts. Integration and analysis of the chromatograms were done with the Millennium32 chromatograph manager (Waters). The column was equilibrated with the mobile phase buffer (filtered and degassed) consisting of 10 mM (NH4)2HPO4 adjusted to pH 5.5 with H3PO4 and acetonitrile in the ratio of 10:1.2 (v/v) as reported (31). The flow rate was 1.00 ml/min, and flavins were monitored at 473 nm. Elution profiles of the flavins were calibrated using 20-μl injections of 10 μM standard solutions of FMN, FAD, and an equimolar mixture of these two, made in HPLC-grade water. The protein solutions at concentrations of 10 μM in water were heated for 10 min in a boiling water bath, chilled for 5 min on ice, and then centrifuged at 14,000 × g for 10 min using an Eppendorf 5415C centrifuge. The supernatant was transferred to another tube, and 20-μl aliquots were analyzed by HPLC. Concentrations of FMN and FAD solutions were determined from their absorbance spectra according the values reported (32) (FMN, ε455 = 12,500 M−1 cm−1; FAD, ε450 = 11,300 M−1 cm−1). In the standards and proteins, FAD eluted at 1.45 min (relative area 58%) and FMN at 1.90 min (relative area 42%). Injections were done in triplicate and agree with each other. All enzymes had FMN: FAD:heme ratios of 1:1:1, except nNOS-AR, which exhibited a mild FMN depletion (0.9:1:1).

Spectrophotometric Methods—CO difference spectra were performed as described. The molar protein concentrations for nNOS and nNOS deletion mutants were determined based on heme content via reduced CO difference spectra, where ε = 100 mm−1 cm−1 for A445–470 and thus reflect only active enzyme. All spectral analyses were performed using a Shimadzu model 2401PC UV-visible dual-beam spectrophotometer.

Activity Measurements—Nitric oxide formation (hemoglobin capture assay) and cytochrome c reduction were measured at 23 °C as described (33), with the exception that both assays were performed in a buffer containing 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl. Rates of NO synthesis and cytochrome c reduction were determined using extinction coefficients of 60...
Regulation of Electron Transfer in the nNOS Reductase Domain

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JOURNAL OF BIOLOGICAL CHEMISTRY

In addition to the CaM-binding site, two major elements that transduce the CaM effect through nNOS have been identified, the AR and the C-terminal tail region (tr1), shown in Fig. 1a. The nNOS AR is a 37-amino acid insert in the FMN domain, encompassing residues 832–868, which crystal structure data has shown to be relatively unstructured, with only a 9-residue helical region in the middle of the insert being detectable (34). The nNOS C-terminal tail is a 33-amino acid extension, encompassing residues 1397–1402, of which residues 1401–1412 form an α-helical region that lies across the FMN/FAD interface (34), and residues 1397–1400 interact with FMN and NADPH binding domains; the remaining C-terminal amino acids cannot be seen in the structure. To investigate the roles of these regions in CaM-dependent regulation, we characterized deletion mutants of each of these elements, as well as a double deletion in which both elements are absent (Fig. 1b).

**Cytochrome c Reduction**—The flow of electrons through the nitric-oxide synthase reductase domain is NADPH to FAD to FMN. As in the case with CYPOR, it is the NOS hydroquinone that donates electrons to cytochrome c (35); the NOS FMN semiquinone or a NOS in which FMN has been removed cannot reduce cytochrome c (36). The rate-limiting step in this process is the transfer of electrons from FAD to FMN (37). Therefore, cytochrome c reduction is a measure of electron flux through the entire reductase domain. Cytochrome c reduction by the various deletion mutants determined in the presence and absence of CaM is shown in Fig. 2a.

As expected, cytochrome c reduction by nNOS-wt is stimulated 10-fold by CaM, whereas nNOS-tr1 is inhibited about 60% by CaM. When the autoregulatory region is deleted, CaM modulation of reductase activity is decreased but still evident; nNOS-AR is stimulated only about 2-fold by CaM. When both the autoregulatory region and the C-terminal tail are removed, CaM no longer affects cytochrome c reduction, indicating CaM modulation of electron flow through the reductase domain is implemented entirely by these two regulatory elements. These observations suggest an interaction between these regions in the inhibited enzyme conformation.

**Ferricyanide Reduction**—Ferricyanide receives reducing equivalents in electron transfer from the NOS and CYPOR FAD moieties. Although it cannot be completely discounted that ferricyanide can receive electrons from FMN also, chimeric constructs between CYPOR and nNOS strongly implycate FAD as the primary donor. Unlike CYPOR, which has a $K_m$...
for ferricyanide of 32 μM, the $K_m$ of nNOS for ferricyanide is in the 1–2 mM range in the absence of CaM but is decreased by about 50% (~750 μM) in its presence (38). Concentrations of ferricyanide over 2 mM have very high absorbance, making it difficult to assay reduction at large enough concentrations of ferricyanide, so turnover numbers must be extrapolated from plots of initial rate versus substrate concentration, which also will yield apparent $K_m$ values.

Table 1 shows the data derived for ferricyanide reduction in the presence and absence of CaM for nNOS-wt and the various deletions. Neither nNOS-wt nor any of the deletion mutants exhibit alterations of $k_{cat}$ values in the presence or absence of CaM, indicating that the rate of electron flow from NADPH to FAD to ferricyanide is unaffected by CaM. The $K_m$ values, however, are affected differentially by CaM depending on the presence or absence of the C-terminal insert. nNOS-wt and nNOS-AR, both of which have intact C termini, demonstrate CaM regulation of $K_m$ values, whereas nNOS-tr1 and nNOS-AR-tr1, both of which are missing their C termini, have the same $K_m$ values in both the presence and absence of CaM. These data indicate that in the absence of CaM the C terminus of nNOS may interfere with ferricyanide access; in the presence of CaM the C terminus is realigned to alleviate this interference.

Nitric oxide Synthesis—Evidence indicates that nitric oxide synthesis requires electron flow through the reductase domain of one monomer into the heme domain of another (10, 11, 39); this process is blocked in the absence of bound CaM. In the presence of CaM, heme reduction is allowed and NO synthesis occurs. Fig. 2b shows the effect of CaM on NO synthesis in the presence and absence of the AR and/or C-terminal regulatory elements. CaM is required in all cases for maximal NO synthesis activity, and deletion of AR and/or the C terminus has a minimal (~25%) effect on the rate of NO synthesis. Interestingly, in the absence of the C terminus, a small amount of NO is produced in the absence of CaM, although the rate is stimulated 8–10-fold in its presence. The observation that CaM is required for or greatly stimulates NO production indicates an additional role for CaM other than stimulation of electron flow through the reductase domain and, possibly, the presence of another regulatory element to mediate this process.

Mutant CaM Support of NO Synthesis—CaM is a dumbbell-shaped protein with N- and C-terminal globular domains connected by an α-helical linker. The C-terminal lobe binds to the N-terminal region of the nNOS CaM-binding site and the N-terminal lobe binds to the C-terminal region of nNOS CaM-binding site (40). A CaM molecule in which the N-terminal lobe was replaced by a copy of the C-terminal lobe (CaM-CC (30)), such that both globular domains consisted of C-terminal lobes, was examined for its ability to support NO synthesis by nNOS and the deletion mutants. In agreement with previous investigations, the data presented in Fig. 3 demonstrate that CaM-CC activates wild-type nNOS activity to only 17% of the maximum. It is therefore striking that CaM-CC activates nNOS-AR and nNOS-AR-tr1 synthase activities as well as native CaM and activates nNOS-tr1 synthase activity nearly as well as native CaM.

NADPH Oxidation—In the absence of substrate, NADPH oxidation by nNOS results in superoxide formation. In the absence of CaM, superoxide formation occurs via the flavins of
Regulation of Electron Transfer in the nNOS Reductase Domain

FIGURE 3. Support of NO synthesis by CaM and CaM-CC. Solid bars, CaM; hatched bars, CaM-CC. NO synthesis in the presence of CaM was set to 100% for each of the constructs; the activities were 30 ± 2, 32 ± 5, 20 ± 1, and 18 ± 4 min⁻¹ for nNOS, nNOS-tr1, nNOS-AR, and nNOS-AR-tr1, respectively. NO synthesis in the presence of CaM-CC was calculated as the percentage of CaM-supported activity. Assays were performed at least in triplicate and as described under "Experimental Procedures." Final enzyme concentration was 100 nM.

The reductase domain, because electrons are not passed to the heme. In the presence of CaM, the nNOS heme is reduced, and superoxide formation occurs at this site; the rate is ~7-fold higher than in the absence of CaM. Fig. 2c shows NADPH oxidation by nNOS and each of the deletion mutants in the presence and absence of CaM. In all cases, NADPH oxidation is stimulated by CaM, although to a lesser extent in the deletion mutants than in wild-type nNOS (7-fold versus 4.3, 4.5, and 3.1-fold for wild-type, nNOS-tr1, nNOS-AR, nNOS-AR-tr1, respectively). As shown for CYPOR (41, 42), FAD produces O₂ reduction, but NOS is stimulated to produce more reduced oxygen species by CaM. These data strongly support production of reactive oxygen species by FAD in the absence of CaM and the additional reduction of O₂ by heme and/or FMN in the presence of CaM. The presence or absence of the regulatory elements had little effect on the rates of NADPH oxidation as compared with the wild-type enzyme, in the absence of CaM, indicating that NADPH to FAD electron transfer is not modulated by either of the regulatory elements.

Flavin Reoxidation—Wild-type nNOS and the deletion mutants described herein are all purified with the flavins in the fully oxidized state. Following catalysis, during which the reductase domain cycles between one- and three-electron reduction, the nNOS reductase domain assumes a one-electron-reduced, air-stable FMN semiquinone form (37, 43). Eventually, over a period of hours for wild-type nNOS, the flavins will become fully oxidized. In contrast, the FMN semiquinone in nNOS-tr1 is very unstable, and the enzyme quickly regains its fully reoxidized state (20). Flavin reoxidation traces are shown for nNOS-AR and nNOS-AR-tr1 in Fig. 4; those of nNOS and nNOS-tr1 were published previously (20). The enzymes are initially in their resting states, with flavins fully oxidized. NADPH is added, catalysis begins, and the flavin absorbance drops. When the NADPH is exhausted, the flavin absorbance returns to a plateau, the absorbance of which corresponds to the air-stable form. If the absorbance plateaus at the same absorbance as it started, the flavins have been fully reoxidized. If it regains only a portion of the absorbance change, the semiquinone form is present. Ferricyanide is then added to the stable form to fully oxidize the flavins, confirming the oxidation state of the stable form. As shown in Fig. 4, nNOS-AR and nNOS-AR-tr1 both form the stable semiquinone form observed for the wild-type nNOS. It is surprising that nNOS-AR-tr1 forms the stable semiquinone because nNOS-tr1 does not. This indicates that the accessibility of the flavins to the solvent is probably different in nNOS-tr1 and nNOS-AR-tr1. Indeed, nNOS-AR-tr1 is a much more stable enzyme, in terms of maintaining catalytic activities, than is nNOS-tr1.

DISCUSSION

The most recent hypothesis regarding electron transfer in the NOSs is that bound NADPH locks the enzyme into a state in which the FMN domain is closely associated with the FAD and NADPH binding domains, the so-called "closed" conformation, where the FMN is deeply buried in the protein and inaccessible to other electron acceptors (12). In this state, electrons can be passed from FAD to FMN but not to outside electron acceptors. According to this hypothesis, in order for electrons to be passed from FAD to FMN but not to outside electron acceptors. This indicates that the accessibility of the flavins to the solvent is probably different in nNOS-tr1 and nNOS-AR-tr1. Indeed, nNOS-AR-tr1 is a much more stable enzyme, in terms of maintaining catalytic activities, than is nNOS-tr1.

The observation that perturbation of either AR or the C-terminal tail results in partial loss of the CaM effect on cytochrome c reduction and that deletion of both elements results in CaM independence supports a model in which AR and the C-terminal tail act in concert to respond to the binding of CaM. Little data exist on the exact site(s) of interaction of either of these elements, particularly AR. The crystal structure of the nNOS reductase domain (34) confirms our original prediction that the C-terminal tail lies at the interface between the FMN and FAD subdomains (19, 20). However, only residues 1397–1413 can be seen in the structure; the terminal 16 residues are not observable, implying flexibility in this region. Only about 14 residues of the AR are evident in the structure, forming a short helix; the remaining 28 amino acids cannot be seen, again suggesting flexibility in this region. Given the proximity and flexibility of these
two regulatory elements, we propose that they interact directly to stabilize the closed position of the FMN subdomain. Furthermore, we propose that the AR also interacts directly with CaM to stabilize the open conformation of the FMN subdomain (Fig. 5).

In the absence of CaM, AR will bind preferentially to the C-terminal tail, aligning the FAD and FMN for electron transfer between them (Fig. 5, upper left). Binding of NADPH also helps stabilize this conformation, because of direct ionic interactions of the tail with NADPH, as well as contacts of the 2' phosphates of NADPH with several conserved residues of nNOS (34), particularly Arg1400 (44). NADPH is oxidized, and NADP⁺ is released, altering the position of the tail, which no longer has NADP(H) with which to interact, and destabilizing the AR/tail interaction. The FMN domain is now free to either move into a partially open, "intermediate" conformation, allowing electron transfer to cytochrome c, but not the nNOS heme (Fig. 5, lower middle), or to transiently form the fully open complex. Because cytochrome c reduction in the absence of CaM is 100× faster than NO production, even infrequent adoption of the open conformation may allow measurable cytochrome c reduction but not measurable reduction of the NOS heme. This process is impeded by the very slow release of NADP⁺ in the absence of CaM (12, 14). In the presence of CaM, NADP⁺ release is accelerated (12), destabilizing the AR/tail interaction more quickly, permitting AR binding to CaM and stabilizing the productive open conformation of FMN, which allows for transfer of electrons from FMN to the nNOS heme (Fig. 5, upper right). NADPH rebinds and realigns the tail, to which the AR binds, once again stabilizing the closed state of the FMN subdomain.

During ferricyanide reduction, the C-terminal tail competes with ferricyanide binding in the absence of CaM, as inferred from the decrease in apparent $K_m$ value in the presence of CaM, but the turnover number is independent of the presence of CaM. FeCN is a very small molecule, and it is reduced by the FAD rather than the FMN, so adoption of the fully open conformation may not be necessary to allow access for electron transfer. Indeed, when the C-terminal tail is deleted, both the rate of turnover as well as apparent $K_m$ values are unchanged whether CaM is present or not.

When the C-terminal tail is deleted, cytochrome c reduction in the absence of CaM is very much higher than wild type in the absence of CaM, whereas in the presence of CaM, nNOS-tr1 reduces cytochrome c similarly to wild-type nNOS. NO production, however, is still mostly dependent on the presence of CaM. These data are consistent with our model; in the absence of CaM, the FMN domain has increased access to cytochrome c, because the fully locked conformation is less stable in the absence of the tail, but little NO is produced until CaM binds because FMN needs to be in the fully open AR/CaM-bound position. Cytochrome c reduction in the absence of the C terminus is inhibited in the presence of CaM because the binding of AR to CaM to stabilize the open conformation aligns the FMN domain in the same way whether the tail is present or not.

nNOS-tr1 is the only construct tested in which the flavins become fully oxidized, whereas nNOS-AR-tr1 forms the air-stable semiquinone form. This may be explained by the destabi-
Regulation of Electron Transfer in the nNOS Reductase Domain

In nNOS-AR-tr1, CaM regulation through the reductase domain is eliminated, but CaM still stimulates NO production. In this case, an additional CaM-sensitive element in the connecting region, originally identified by Zhang et al. (51) as the “β-finger,” may play a role in either stabilizing the open conformation or in destabilizing a conformationally restricted state (52). This 15-residue β-finger structure, called the SI for “small insertion,” is adjacent to the edge of the FMN-binding subdomain, proximate to the AR and, by inference, the CaM-binding site (52). This element, like AR, is inhibitory for electron transfer and NO production (52, 53) in the absence of CaM. Its function appears to be masked in the presence of the AR, and it is only when AR is deleted that the regulatory effects of SI become apparent (53). As shown in Fig. 5, simple movement of the FMN domain may place the SI provocatively close to the FMN domain and/or CaM. Such proximity might allow for an interaction with either to further stabilize the open conformation. In the presence of AR, this stabilizing effect might be minimal, but in the absence of the AR, such an interaction may be the only stabilizing element for the open conformation and thus explain why NO production is still CaM-dependent even when both the AR and the C-terminal tail are deleted. Alternatively, CaM binding may be still required to properly align the two subunits of the dimer, such that the FMN of one subunit transfers electrons to the heme of the opposite subunit. It is interesting that in the absence of AR, the nNOS reductase domain can transfer electrons to the hemes of both subunits of the dimer (i.e. intra- as well as inter-molecular electron transfer), although intra-molecular heme reduction appears to be nonproductive for NO synthesis, whereas in the intact enzyme, electrons flow exclusively from the reductase of one subunit to the heme of the other subunit (i.e. inter-molecular transfer) (11). The proposed interaction of AR with CaM may thus be cross-subunit, i.e. the AR of one subunit interacts with CaM bound to the other subunit, and it is this specific interaction that properly aligns the FMN of one subunit for electron transfer to the heme of the opposite subunit.

Because iNOS does not contain an AR element, it is interesting to speculate that it does not require the AR element because CaM is always bound, and the FMN subdomain is always in the “locked” conformation. iNOS does require the C-terminal tail, however, to maintain control by NADPH/NADP⁺ so that electron transfer is not too fast for optimal heme reduction and NO formation.

The data presented provide support for a mechanistic model involving the concerted interaction of the C-terminal tail, the autoregulatory insert, and CaM in the regulation of the reductase domain of nNOS, which postulates that nNOS can exist in at least two conformational states as follows: a closed, NADPH-bound form, and a fully open form in which the FMN is separated from FAD via the flexible connecting domain, permitting its interaction with its oxygenase partner. A partially open or intermediate form may also exist. The reduction of NOS-bound heme in the oxygenase domain requires these concerted interactions to catalyze a fully coupled production of NO.

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Regulation of Electron Transfer in the nNOS Reductase Domain

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