The C-Terminus of Mouse Macrophage Inducible Nitric Oxide Synthase Attenuates Electron Flow Through the Flavin Domain*

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Running title: iNOS C-terminus attenuates electron flow.
SUMMARY

The sequences of nitric oxide synthase flavin domains closely resemble that of NADPH-cytochrome P450 reductase (CPR), with the exception of a few regions. One such region is the C-terminus; all NOS isoforms are 20-40 amino acids longer than CPR, forming a tail that is absent in CPR. To investigate its function, we removed the 21 amino acid C-terminal tail from iNOS holoenzyme and from a flavin domain construct. Both the truncated holoenzyme and reductase domain exhibited cytochrome c reductase activities that were 7-10-fold higher than the nontruncated forms. The truncated holoenzyme catalyzed NO formation approximately 20% faster than the intact form. Using stopped-flow spectrophotometry, we demonstrated that electron transfer into and between the two flavins and from the flavin to the heme domain is 2-5-fold faster in the absence of the C-terminal tail. The heme-nitrosyl-complex, formed in all NOS isoforms during NO catalysis, is 5-fold less stable in truncated iNOS. Although both CPR and intact NOS can exist in a stable, one-electron-reduced semiquinone form, neither the truncated holoenzyme nor the truncated flavin domain demonstrate such a form. We propose that this C-terminal tail curls back to interact with the flavin domain in such a way as to modulate the interaction between the two flavin moieties.
INTRODUCTION

Nitric oxide synthases (NOSs)\(^1\) produce NO from L-arginine through a series of monooxygenation reactions (for review, see 1-3). The three isoforms, nNOS, iNOS, and eNOS, produce NO by the same mechanism, but play very different physiological roles due to the type of cell in which they are located. nNOS, located in neurons in the brain and at neuromuscular junctions, is involved in neurotransmission (4,5); iNOS, located in macrophages, is involved in the immune response (6,7); and eNOS, located in endothelial cells, is involved in hemodynamic regulation (8,9). The NO produced by nNOS and eNOS exerts its effects through the stimulation of guanylate cyclase, while the NO produced by iNOS exerts its effects directly or by combining with superoxide to form peroxynitrite, both of which are free radicals that harm proteins and DNA.

The NOSs consist of two domains, a heme and H\(_4\)B-containing oxygenase (or heme) domain, which binds the substrate L-arginine, and a flavin-containing reductase (or flavoprotein) domain, which binds the flavins FAD and FMN, as prosthetic groups, and the cofactor NADPH. A calmodulin-binding region bisects the two domains. Calmodulin is required for NO production and its binding is dependent on cellular calcium levels. Two of the isoforms (nNOS and eNOS) are constitutive; induction of NO synthesis activity requires an influx of calcium to promote calmodulin binding (5,10). The iNOS enzyme is induced at the transcriptional level and calmodulin is bound even at basal calcium concentrations (11).

At the time Bredt and Snyder (5) reported the cloning of the first nitric oxide synthase isoform (nNOS), they noted the similarity of the sequence of the carboxy half
of the protein with cytochrome P450 reductase (CPR). Subsequently, a heme moiety was discovered to be the active site in the amino half of the enzyme and attention has focussed primarily on the elucidation of catalysis in that domain (for review, see 12). Although the three isoforms are about 50-60% identical at the amino acid level (13), recently published crystal structures of the heme domains of the iNOS, eNOS, and nNOS isoforms demonstrate very minor structural differences (14-17, footnote 2), yet these isoforms catalyze NO synthesis at vastly differing rates. The reductase domains of NOSs are structurally similar to the NADPH-cytochrome P450 reductase (CPR), with the nNOS flavoprotein domain sharing 36% identity and 58% close homology at the amino acid level (18). Like those of CPR, the flavins of NOSs can also transfer electrons to artificial electron acceptors, such as cytochrome c, 2,6-dichlorophenolindophenol, and ferricyanide (19,20); these rates of reduction also differ very greatly from CPR and between the NOS isoforms.

To account for such differences in what are otherwise very homologous structures, attention was focussed on the search for control mechanisms in several regions that exist in the flavin domains of NOSs that have no counterpart in CPR. A putative autoinhibitory domain described by Salerno et al. (21), consisting of about 45 amino acids located in the middle of the FMN binding region, is present in the constitutive NOSs (nNOS and eNOS), but is absent in iNOS and CPR. This autoinhibitory domain was proposed to control the calmodulin binding/activation of the constitutive isoforms. Also, nNOS\(\mu\), present in skeletal muscle myotendinous junctions (22), contains 34 additional amino acids in the FMN-containing subdomain of nNOS in
an alternatively processed form of the nNOS gene (23). The function of this additional segment remains unknown. Each of the NOS isoforms contains about 21-42 additional amino acids at the C-terminus (42, 33, and 21 amino acids in bovine eNOS, rat nNOS, and murine iNOS, respectively), forming a tail that is not present in CPR. Xie et al (24) showed that deletion of 22 or 23 residues from the C-terminus of iNOS, which removed the tail plus 1 or 2 additional residues, reduced the rate of NO synthesis 26% or 66%, respectively. To investigate the functional role of the additional 21 residues at the carboxy terminus of the NOS isoforms, we have removed them from the iNOS holoenzyme and from an expressed iNOS reductase domain and compared the resulting enzymes to the intact proteins. The mechanistic implications of this deletion are discussed.
EXPERIMENTAL PROCEDURES

**Chemicals.** (6R)-5,6,7,8-Tetrahydrobiopterin (H₄B) was from Research Biochemicals International (Natick, MA) and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade available.

**Enzymes.** Pfu turbo polymerase was from Stratagene (La Jolla, CA); ligase and restriction enzymes were purchased from either Promega (Cambridge, MA) or New England Biolabs (Boston, MA). Shrimp alkaline phosphatase was from United States Biochemical (Cleveland, OH).

**Plasmids.** The murine macrophage iNOS cDNA was provided by Drs. Solomon Snyder and David Bredt at Johns Hopkins Medical School, Baltimore, MD. CAMpACMIP, containing rat calmodulin cDNA, was from Dr. Anthony Persechini at the University of Rochester in Rochester, NY. PCW<sub>ori+</sub> was given by Dr. Michael Waterman at Vanderbilt University in Nashville, TN.

**Recombinant DNA manipulations.** iNOS<sub>CW-tr1</sub>, the plasmid for the expression of the truncated iNOS (residues 1-1123) in *E. coli*, and iNOS<sub>red-tr1</sub>, the plasmid for the expression of the truncated iNOS reductase domain in *E. coli* (residues 499-1123), were constructed as follows. iNOS-tr1: The initial 1123 codons of pmacNOS (from the ATG start codon to the final residue minus 21) were amplified by PCR to incorporate the recognition sequence for Ndel. Primer tr1-1 (upstream primer, with Ndel site) was 5'-TGCTTAGGAGGTCATATG 3' and primer tr1-2 (return primer) was 5'-CAGTCAAGCTTAACCGAAGATATCTTCATG 3'. iNOSred-tr1: The nucleotides
encoding residues 499 to 1123 of pmacNOS (from the codons of the beginning of the calmodulin binding site to the final residue minus 21) were amplified by PCR to incorporate the recognition sequence for NdeI and six histidine codons for ease of purification. Primer redtr1-1 (upstream primer, with NdeI site) was

5'-GGGAATTCCATATGGCTCACCACCACCACACCAAGCTGAGGCCCAGGAGG-3' and primer redtr1-2 (return primer) was 5'-CAGTCAAGCTTAACCGAAGATATCTTCATG-3'.

Primers were synthesized by the Center for Advanced DNA Technologies at the UT Health Science Center at San Antonio. Reaction mixtures included 50 pmol of each primer, 20 ng macNOS template, 200 µM dNTPs, 1 x Pfu turbo polymerase buffer, and 2.5 units Pfu turbo polymerase in 50 µl total volume. The mixture was preincubated for 1 minute at 94°C prior to the addition of Pfu turbo polymerase, followed by amplification for 18 cycles: 95°C for 45 s, 55°C for 60 s, and 68°C for 10 m. The PCR product was gel-purified using the GeneClean II kit (Bio101, Vista, CA) and digested with NdeI and HindIII. PCW_{ori+} DNA was digested with NdeI and HindIII and the ends were dephosphorylated. The two pieces were ligated and the resultant products were used to transform E. coli XL10-gold competent cells (Stratagene) using the manufacturers instructions. Positive colonies were identified by restriction digest analysis of plasmid DNA isolated from small (2 ml) cultures of select colonies.

CAMpACMIP was co-transformed with iNOSpCW-tr1 or iNOSred-tr1 into E. coli BL21 cells via electroporation using an Invitrogen Electroporator II (San Diego, CA)
according to the manufacturers instructions and plated on LB agar containing 50 µg/ml ampicillin and 35 µg/ml chloramphenicol.

Protein expression/purification. Protein expression and purification are as described in Roman et al. (25) for iNOS and iNOS-tr1 and in McMillan and Masters (26) for iNOSred and iNOSred-tr1. The iNOS-tr1 and iNOSred-tr1 proteins were purified in tandem with holo iNOS and iNOSred, respectively, to reduce differences resulting from preparation to preparation.

Spectrophotometric methods. Absolute spectra and CO difference spectra were performed as described (25,26). The molar protein concentrations for iNOS and iNOS-tr1 were determined based on heme content via reduced CO difference spectra, where ε = 100 mM⁻¹cm⁻¹ for ΔA₄₄₅-₄₇₀; those for iNOSred and iNOSred-tr1 were based on total flavin concentration and ε = 21 mM⁻¹ cm⁻¹ at 455 nm. All spectral analyses were performed using a Shimadzu Model 2101 UV/visible dual-beam spectrophotometer.

Stopped-flow spectroscopy. Stopped-flow reactions were performed aerobically under turnover conditions at 23 °C, as described in Miller et al. (19), using an Applied Photophysics SX.18MV diode array stopped-flow spectrophotometer, which had a dead time of 2 msec. Reactions contained 3 µM enzyme, 100 µM NADPH, 10 µM H₄B, and 100 µM L-arginine in 50 mM HEPES, pH 7.4. When the reductase domain constructs were used, L-arginine and H₄B were omitted. Heme reduction was monitored at 397 nm and heme-nitrosyl complex formation was monitored at 436 nm. Flavin reduction was monitored at 485 nm, rather than the absorption peak value at 455 nm, to avoid
spectral contamination from the heme.

*Measurement of Activity.* Nitric oxide formation (hemoglobin capture assay) and cytochrome c reduction were measured at 23° C as described by Sheta, et al. (20), with the exception that the cytochrome c reduction assays were performed in a buffer containing 50 mM HEPES, pH 7.4, in the absence of NaCl.

Oxidation of NADPH was monitored at 340 nm in the presence of 50 mM HEPES, pH 7.4, 100 µM NADPH, and 500 nM enzyme (iNOSred or iNOSred-tr1) at 23°. The rate was determined using an extinction coefficient of 6.2 mM⁻¹ at 340 nm for NADPH.

The reoxidation of reduced flavins was monitored at 485 nm for both holoenzymes and reductase domains in the presence of 50mM HEPES, pH 7.4, 20 µM NADPH, and 2 µM enzyme at 23°.
RESULTS AND DISCUSSION

The ability of the truncated enzymes to catalyze the reduction of cytochrome c was examined and the results are shown in Table 1. Both the wild type iNOS and its reductase domain catalyze the reduction of cytochrome c at the same rate, approximately 3000 min⁻¹; for comparison, CPR also reduces cytochrome c at the same rate, i.e., 3000 min⁻¹ (not shown). The C-terminally truncated enzymes, however, reduce cytochrome c at a rate 7-10-fold higher than the intact proteins. Thus, the removal of 21 residues from the C-terminus of the reductase domain greatly potentiates the ability of iNOS holoenzyme and iNOSred to reduce cytochrome c.

The rate of cytochrome c reduction by iNOS-tr1 was not attenuated in the presence of superoxide dismutase and catalase (Table 1), indicating that electrons were being transferred directly to the cytochrome c and not to oxygen to form superoxide, which can also reduce cytochrome c. This observation supports the contention that the increased rate of cytochrome c reduction of iNOS-tr1 is due to an increased rate of electron transfer from NADPH through the NOS flavins directly to the heme of cytochrome c and not a result of mediation by superoxide.

In the absence of a protein electron acceptor, CPR and the NOS flavin domains will shuttle electrons directly to molecular oxygen to form superoxide, albeit at a slower rate. As an indirect measurement of this process, NADPH oxidation by iNOSred and iNOSred-tr1 was monitored. iNOSred consumed NADPH at a rate of 2.1 min⁻¹, while
iNOSred-tr1 consumed NADPH at 9.0 min\(^{-1}\), an increase of 5-fold. Thus, the removal of 21 residues from the C-terminus also potentiates the ability of iNOSred to form superoxide. The rate of electron transfer to molecular oxygen is so slow in either case, however, that the formation of superoxide cannot be contributing significantly to cytochrome c reduction, in agreement with the inability of superoxide dismutase to change the reaction rate.

In view of the increased rate of electron transfer to the heme of cytochrome c, we examined whether iNOS-tr1 also had the ability to transfer electrons more rapidly from its flavins to its heme. Initially, the rate of NO production was measured. As shown in Table 1, a difference exists in the rate of NO production between holo iNOS and iNOS-tr1. Although the rate of NO production by iNOS-tr1 was slightly, but consistently, higher, the difference is not as dramatic as that of cytochrome c reduction. This indicates that, whereas the rate-limiting step for cytochrome c reduction by holo iNOS is determined by its flavin domain, that of NO production is not. That is, the heme of cytochrome c is able to accept electrons as fast as NOS can transfer them; this is supported by the observation that the ratio of cytochrome c reduced to NADPH oxidized is 1:1 (data not shown). The rate of electron transfer to the heme of NOS, however, is regulated differently; the flavin domain is able to transfer electrons much faster than the heme is able to accept them. This agrees with Miller et al. (19), who have concluded that the flavoprotein to heme electron transfer is rate-determining for initial NO production in all NOS isoforms.

To examine flavin and heme reduction of holo iNOS and iNOS-tr1 more directly,
we used stopped-flow spectrophotometry under turnover conditions. Spectral changes were monitored at 485 nm (flavin reduction), 397 nm (heme reduction), and 436 nm (heme-nitrosyl complex formation) following rapid mixing of NOS with NADPH. The spectral traces are shown in Figure 1 and the actual rate constants derived from these data are shown in Table 2. The rates for iNOS and iNOS-tr1 heme and flavin reduction are biphasic, consisting of an initial fast phase followed by a secondary slow phase. The biphasic nature of these curves and the derived rates for holo iNOS are similar to those reported by Miller et al. (19).

The data in Table 2 clearly show that flavin reduction is far faster than heme reduction (5-10-fold). Since the flow of electrons is from NADPH to FAD to FMN and finally to the heme (27), the rate of reduction of the flavins and intramolecular transfer between them are certainly not limiting the rate of heme reduction, similar to the mechanism displayed by CPR (28). The initial fast phase of flavin reduction most likely represents the conversion of the fully oxidized flavoprotein to the fully reduced and semiquinone forms. This process consists of the transfer of electrons from NADPH to the FAD moiety followed by the transfer of electrons to FMN at a rate as fast as, if not faster than, that of transfer from NADPH to the first flavin (29). Since the transfer of electrons from FAD to FMN is so fast, the rate of the fast phase most likely reflects the rate of electron transfer from NADPH to FAD. The slower, second phase seen in the stopped-flow measurement of flavin reduction is much slower than the rate of heme reduction, too slow to be involved in transfer of electrons to the heme domain, and thereby likely represents the comproportionation of electrons, including forward and
back reactions between the flavins. Table 2 shows that iNOS and iNOS-tr1 differ with respect to both phases of flavin reduction, with iNOS-tr1 exhibiting rates 2x and 5x those of iNOS for the fast and slow phases, respectively, consistent with the 10-fold increase observed in cytochrome c reduction. Similar data are obtained with the corresponding reductase domain enzymes (Table 2). The rates of the fast and slow phases are 1.5-fold and 5.4-fold higher, respectively, for iNOSred-tr1 over iNOSred. Thus the transfer of electrons to FAD (fast phase) and the shuffling of electrons between the flavin prosthetic groups (slow phase) are both faster in the absence of the C-terminal tail.

Once NADPH is exhausted and catalysis stops, the enzyme returns eventually to its resting, fully oxidized state, but only after a transient period in a partially reduced state. In studies with nNOS, Miller et al. (19) and Noble et al. (30) demonstrated the formation of an air stable, one-electron-reduced semiquinone, which cannot reduce either its own heme or that of cytochrome c, and which persists for about 20 minutes before reverting to the fully oxidized state (not shown). This redox behavior is reminiscent of that observed with CPR, the flavoprotein with which it shares substantial sequence homology in the flavin domain. This fact has led to speculation that the flavoprotein domain of NOS operates through a similar, if not identical, mechanism to that of CPR (2). To determine whether the C-terminal tail affects this process, the rate of flavin reoxidation was examined for the intact and truncated enzymes using the reductase domain constructs (iNOSred and iNOSred-tr1) to avoid signal contamination by the heme domain. Flavin reoxidation was monitored at 485 nm and appears as an
increase in flavin absorbance as the reaction begins with fully reduced flavins (Figure 2).

As shown in Figure 1, flavin reduction occurs very quickly, on the order of msec. Since these flavin reoxidation experiments were performed in a standard, rather than stopped-flow, spectrophotometer, flavin reduction was already complete at the start of the reaction, having occurred during the approximately 10 sec between mixing components and the start of absorbance monitoring. The initial, zero absorbance represents fully reduced flavins catalyzing the oxidation of NADPH. After the NADPH is exhausted, flavin reoxidation, seen as an absorbance increase, occurs. NADPH is utilized 5-fold faster by iNOSred-tr1 than iNOSred, as discussed above regarding superoxide production and as reflected by the 5-fold longer lag before the absorbance increase seen for iNOSred. The absorbance change plateaus, representing the air-stable form of the flavins. In Figure 2, the amplitude of the signal for iNOSred-tr1 is twice that of iNOSred, indicating that, while iNOSred initially forms the stable, one-electron-reduced semiquinone, iNOSred-tr1 does not, instead becoming fully oxidized. This behavior is also observed with the holo iNOS and iNOS-tr1 (not shown). The slope of the line represents the rate of flavin oxidation and it is clear that this rate for iNOSred-tr1 is twice that of iNOSred, which is consistent with both flavins being rapidly and fully oxidized, rather than just one as in the semiquinone form. To directly address this difference between holo and truncated enzymes, the same reaction was performed, but monitored at 585 nm, which specifically measures the neutral semiquinone form (28). For iNOSred, the semiquinone form accumulates steadily until it reaches a plateau at about 180 sec, which persists for the remaining 120 sec of the experiment.
(data not shown). This is entirely consistent with the data shown in Figure 2, where the change in absorbance reflecting reoxidation of one flavin occurs between 170 and 210 sec. With iNOSred-tr1, such an accumulation and persistence are not seen.

The reduction of heme in iNOS is also a biphasic reaction and, interestingly, is unique to this isoform. nNOS and eNOS heme reduction are both monophasic under the conditions of our experiments (19). It is evident from the data in Table 2 that there are only small differences in heme reduction between iNOS and iNOS-tr1, with the fast and slow phases of iNOS-tr1 being 1.2-fold and 2.4-fold higher, respectively, than those of iNOS. It is unclear what the two phases of the reaction represent, but perhaps a clue comes from the work of Abu-Soud et al. (31), who showed that nNOS becomes inactivated during catalysis due to the rapid formation of a heme-nitrosyl complex between the enzyme and the NO it generates. This NO can also bind to and inactivate the ferric form of the enzyme and, while in nNOS the ferrous form is primarily produced, it is the ferric-NO complex that predominates in an aerobic environment with iNOS (32). As much as 70-90% of the enzyme may be present in the inactive form during the steady state. The heme-nitrosyl complex is also formed with eNOS, which forms the complex the most slowly, while iNOS forms it the most rapidly of all the isoforms (19). Thus, heme reduction during catalysis would demonstrate an initial fast phase, because no heme-nitrosyl complex is present, followed by a slower phase, reflecting both an equilibrium between free and nitrosyl-complexed heme and the inability of this nitrosyl-complexed heme to bind oxygen until it dissociates. Comparing the data in Figure 1 for wavelengths 397 nm (heme reduction) and 436 nm (nitrosyl-complex formation) shows
that there is a lag in the onset of nitrosyl complex formation of almost 20 msec, in the case of iNOS-tr1, which corresponds with the onset of the second phase of heme reduction. This behavior is less clear for the wt enzyme, where nitrosyl complex formation is faster and heme reduction is slower; in this case, the lag is about 8 msec. This biphasic behavior in heme reduction thus represents a switch in the rate-limiting step from heme reduction to the dissociation of the heme-nitrosyl complex (33). Abu-Soud et al. (34) demonstrated that the off-rate of NO from the ferric-NO complex of an iNOS heme domain construct is $13 \pm 3 \text{ sec}^{-1}$ (at $10^\circ C$), which is similar to our value for the slow phase of heme reduction in the holo-enzyme at $25^\circ C$.

As shown in Table 2, iNOS forms the heme-nitrosyl complex at a rate of $105 \text{ sec}^{-1}$, while iNOS-tr1 forms this complex four-fold slower ($26 \text{ sec}^{-1}$). Perhaps the more rapid flux of electrons into the heme, reflected by the 20% increase in the fast phase, affects either the association or dissociation of the heme-nitrosyl complex formation. Thus, these two factors, the increase in the rate of heme reduction (fast phase) and the decreased formation and/or destabilization of the heme-nitrosyl complex, which results in an apparent increase in the slow phase of heme reduction, account for the slightly higher rate of NO formation of iNOS-tr1 over iNOS.

Although the removal of the C-terminal tail residues has some effect on the function of the heme domain in this enzyme, the majority of the effect is obviously in the function of the flavin domain. iNOS-tr1 transfers electrons faster into the FAD from NADPH (fast phase of flavin reduction) and from the flavin domain to the heme domain (fast phase of heme reduction) than intact iNOS. The steady-state level of flavin
semiquinone, i.e., a form containing a one-electron-reduced FMN moiety, in the truncated enzymes is very low. Based on the flow of electrons depicted in Scheme 1, the rate constant of electron transfer either to the heme ($k_4$) or to FAD ($k_3$) is necessarily faster in the truncated enzymes than in the intact enzymes. Since the rate of cytochrome c reduction is greatly potentiated in the truncated forms, the increase must be in $k_4$. This is also reflected in the higher rate of heme reduction and NO and superoxide formation by iNOS-tr1. This accelerated electron transfer to the heme domain also results in a decreased rate of formation or increased rate of dissociation of the heme-nitrosyl complex.

Comproportionation of the electrons between the FMN and FAD, described by the rate constants $k_2$ and $k_3$ and reflected by the slow phase of flavin reduction in the stopped-flow experiments, also varies significantly between the intact and truncated enzymes. Since $k_2$ is extremely fast and $k_4$ is faster in the truncated enzyme than in the intact enzyme, $k_3$ must be decreased in iNOS-tr1.

Interestingly, although the C-terminus is also involved in the stabilization of the one-electron-reduced semiquinone form of iNOS, CPR, which does not have the C-terminal tail and which iNOSred-tr1 was created to mimic, does form the air stable one-electron-reduced semiquinone. Thus, whatever role the C-terminal tail plays in the stabilization of the semiquinone form of iNOS is served differently in CPR. Although the reductase domains of the NOSs are thought to be very similar in structure to CPR, there must be differences in the way the two flavins interact with each other. The FAD-
binding portion of the nNOS flavin domain has been crystallized and was shown to be identical to the FAD domain of CPR (12,35). However, the C-terminal tail does not show up in the crystal structure, indicating that it is not an ordered, but rather a flexible, part of the molecule. It is easy to imagine that this tail curls back to interact with the flavin domain in such a way as to modulate the interaction between the two flavin moieties, and perhaps interactions between FMN and the heme in cytochrome c, a situation which does not occur in CPR. In the structure of CPR (36), the two flavin (FAD and FMN) rings are juxtaposed to each other at their dimethyl benzene rings and form a wall with an angle of about 150°. Both the N- and C-termini of the CPR polypeptide lie near the junction of the two flavins; the C-terminus lies on one side of the flavin wall (re-face of the FAD ring), whereas the N-terminus lies on the other side of the flavin plane. Therefore, it is conceivable that the C-terminal tail of the intact iNOS protein covers the concave side of the flavin wall, and modulates both the distance and angle of the two flavin rings. These, in turn, regulate the electron flow between the two flavins and also between FMN and cytochrome c or the heme domain of the NOS protein. It is also possible that the interface of the two flavin domains is more open in the NOSs than in CPR, and that the C-terminal tail is protecting the "opening" of this interface. Without this protective C-terminal overhang, the air-stable semiquinone, found in both CPR and wild type iNOS, is no longer stable in the truncated form of iNOS. It should be noted that, in iNOS-red, the calmodulin-binding domain is included at its N-terminus, and that the protein contains a stoichiometric amount of Ca++/calmodulin tightly bound to the protein. Thus, this bound Ca++/calmodulin lies at the other side of the flavin-flavin
wall (the convex side), and together with the C-terminal tail protects the flavin interface. It remains to be seen whether the C-terminal tails of the other two NOSs, nNOS and eNOS, which unlike iNOS are modulated by Ca\textsuperscript{2+}/calmodulin, would have a similar effect on their electron transfer rates. It is also entirely conceivable that the redox potentials of the flavins in the truncated form of the enzyme are different from those of the intact proteins. This work is another of several indications that important modulatory regions exist in the flavoprotein domains of the NOSs.
REFERENCES


FOOTNOTES

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1 Abbreviations: NOS, nitric oxide synthase; nNOS, rat neuronal nitric oxide synthase; eNOS, bovine endothelial nitric oxide synthase; iNOS, murine macrophage inducible nitric oxide synthase; iNOS-tr1, iNOS with a 21 amino acid truncation at the C-terminus; iNOSred, the reductase domain of iNOS; iNOSred-tr1, the reductase domain of iNOSred with a 21 amino acid truncation at the C-terminus; NO, nitric oxide; CPR, NADPH-cytochrome P450 reductase; H4B, (6R)-5,6,7,8-tetrahydrobiopterin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

FIGURE LEGENDS

Figure 1. Stopped-flow kinetic spectrophotometry of iNOS and iNOS C-terminally truncated enzymes under turnover conditions. Reaction contained 3 µM enzyme, 100 µM L-arginine, 10 µM H4B, and 100 µM NADPH and 50 mM HEPES, pH 7.4. The curves shown are the average of 4-5 experiments. The experimental fits for heme reduction and heme-nitrosyl complex formation excluded the initial decrease in absorbance (5-20 msec, indicated by the vertical line in each graph), which is a spectral contribution by the flavins. The curves for flavin and heme reduction were best fit by a double-exponential curve, while that of heme-nitrosyl complex formation was best fit by a single exponential curve. The smooth line is the theoretical fit. Note that the time scale for all the curves is 0-100 msec, except for iNOS heme-nitrosyl complex formation (436 nm), which is 0-70 msec. The experiments were performed as described Experimental Procedures.

Figure 2. Flavin reoxidation of iNOSred and iNOSred C-terminally truncated enzymes. The solid line represents reoxidation by iNOSred-tr1 and the dashed line is that by iNOSred. Reaction contained 2 µM enzyme, 20 µM NADPH, and 50 mM HEPES, pH 7.4. The experiments were performed as described in Experimental Procedures.
Table 1  Catalytic Activities of iNOS and iNOS C-terminally Truncated Enzymes*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cytochrome c Reduction (min$^{-1}$)</th>
<th>NO Production (min$^{-1}$)</th>
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<tr>
<td>Holo iNOS wild type</td>
<td>3000 ± 20</td>
<td>185 ± 21</td>
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<tr>
<td>Holo iNOS-tr1</td>
<td>31,467 ± 278</td>
<td>216 ± 19</td>
</tr>
<tr>
<td>Holo iNOS-tr1 + SOD + catalase$^a$</td>
<td>31,850</td>
<td></td>
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<tr>
<td>iNOS reductase wild type</td>
<td>3260 ± 500</td>
<td>n/a$^b$</td>
</tr>
<tr>
<td>iNOS reductase-tr1</td>
<td>22,700 ± 1370</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*The values reported for cytochrome c reduction and NO production are the means of at least 3 different experiments and are presented as ± SEM. Experiments were performed as described in Experimental Procedures.

$^a$ Reaction mixtures contained 1000 units each SOD and catalase.

$^b$ not applicable
Table 2. Rates of Electron Transfer of iNOS and iNOS C-terminally Truncated Enzymes Under Turnover Conditions*

<table>
<thead>
<tr>
<th></th>
<th>Holo iNOS Wild Type</th>
<th>Holo iNOS-tr1</th>
<th>iNOSred</th>
<th>iNOSred-tr1</th>
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<tr>
<td><strong>Flavin reduction</strong></td>
<td></td>
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<tr>
<td>(485 nm)</td>
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<tr>
<td>Fast phase</td>
<td>564 ± 6.44</td>
<td>965 ± 31</td>
<td>326 ± 30.9</td>
<td>472 ± 15.2</td>
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<tr>
<td>Slow phase</td>
<td>19.5 ± 0.1</td>
<td>95 ± 0.22</td>
<td>14.2 ± 0.76</td>
<td>75.6 ± 1.4</td>
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<td><strong>Heme reduction</strong></td>
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<tr>
<td>(397 nm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast phase</td>
<td>83.9 ± 1.46</td>
<td>102 ± 1.56</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Slow phase</td>
<td>11.1 ± 0.06</td>
<td>26.6 ± 0.44</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Heme-nitrosyl complex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>formation (436 nm)</td>
<td>105 ± 2.27</td>
<td>25.6 ± 0.15</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* All numbers are reported in the unit of sec⁻¹. The values reported for the holo wild type and truncated enzymes are derived from the traces shown in Figure 1. All numbers are the average of 4-5 experiments and are the mean ± SEM. The curves for flavin and heme reduction were best fit by a double-exponential curve, while those of heme-nitrosyl complex formation were best fit by a single exponential curve. The experiments were performed under turnover conditions as described in Experimental Procedures.

a not applicable
ACKNOWLEDGEMENTS

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NADPH $\xrightarrow{k_1} \text{FAD} \overset{k_2}{\leftrightarrow} \text{FMN} \xrightarrow{k_4} \text{HEME}$
The C-terminus of mouse macrophage inducible nitric oxide synthase attenuates electron flow through the flavin domain
Linda J. Roman, R. Timothy Miller, Melissa A. de la Garza, Jung-Ja P. Kim and Bettie Sue Siler Masters

J. Biol. Chem. published online April 25, 2000

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