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## NITRIC OXIDE SYNTHASE STRUCTURE AND ELECTRON TRANSFER

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### ABSTRACT:

The nitric oxide synthases (NOS), although unrelated to the cytochromes P450 in terms of sequence, exhibit spectroscopic and catalytic properties strongly reminiscent of those of the P450 system. One important difference is the requirement of the NOS enzymes for tetrahydrobiopterin. The biopterin cofactor is shown by chemical studies to bind close to pyrrole ring D of the prosthetic heme group, a position confirmed recently for inducible NOS and endothelial NOS by crystal structures. The only plausible role so far for the tetrahydrobiopterin is as a transient electron donor for the

activation of molecular oxygen. NADPH-derived electrons are provided to the heme by the NOS flavin domain, but the biopterin may be required to provide an electron at a faster rate than that supported by the flavin groups. Chimeras in which the reductase domains of the isoforms have been exchanged indicate that the overall rate of catalytic turnover is directly governed by the ability of the flavin domain to deliver electrons. Electron transfer from the flavin to the heme domain, and within the flavin and heme domains, is thus a critical determinant of the catalytic turnover of NOS.

The nitric oxide synthase (NOS)<sup>1</sup> isoforms consist of a heme domain linked to a flavoprotein by a CaM-binding peptide. The flavoprotein domain exhibits strong sequence and cofactor resemblance to cytochrome P450 (P450) reductase, but the heme domain has virtually no structural similarity to P450 other than the fact that a thiolate is coordinated to the heme iron atom. Nevertheless, the heme domain is similar to P450 in terms of spectroscopic, biochemical, and catalytic properties, and much of our understanding of the function of NOS is based on our comparatively advanced understanding of the structure and mechanism of P450. A discussion of NOS, a member of the heme-thiolate family of proteins that includes P450, is therefore appropriate within the context of a P450 symposium.

NOS catalyzes the oxidation of L-Arg to NO and citrulline (Stuehr, 1997; Marletta, 1988; Knowles and Moncada, 1994). Three major NOS isoforms have been identified: NOS-I (nNOS), a form initially associated with the brain (Bredt and Snyder, 1990); NOS-II (iNOS), a form most closely associated with macrophages (Xie *et al.*, 1992); and NOS-III (eNOS), an isoform that is localized in epithelial cells

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<sup>1</sup> Abbreviations used are: NOS, nitric oxide synthase; CaM, Ca<sup>2+</sup>-dependent calmodulin; P450, cytochrome P450; L-Arg, L-arginine; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; H4B, 5,6,7,8-tetrahydrobiopterin; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; heme, iron protoporphyrin IX regardless of iron coordination and oxidation state; PDZ, domains of ~80 amino acids found in structural proteins of the cytoskeleton and in enzymes that associate with the cytoskeleton, and therefore thought to be involved in protein-protein interaction (also called GLGF repeats or DHRs); E/N, chimera consisting of the eNOS heme and CaM-binding domains and the nNOS flavin domain; I/N, chimera consisting of the iNOS heme and CaM-binding domains and the nNOS flavin domain.

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(Pollock *et al.*, 1991). All three isoforms require heme, FAD, FMN, H4B, NADPH, O<sub>2</sub>, and CaM to be functional (Stuehr, 1997; Marletta, 1988; Knowles and Moncada, 1994; Marletta, 1993; Masters, 1994). All three are also homodimers of a polypeptide in which the heme- and H4B-binding domain is linked via a consensus CaM-binding sequence to a flavoprotein with binding sites for one FAD, one FMN, and NADPH (McMillan *et al.*, 1992; White and Marletta, 1992; Klatt *et al.*, 1992; Bredt *et al.*, 1991; Tayeh and Marletta, 1989; Kwon *et al.*, 1989). The isoforms differ, however, in their tissue localization, regulation, and function. nNOS is longer than the other isoforms due to the presence of a PDZ region at the amino terminus that is involved in subcellular targeting of the protein (Brenman *et al.*, 1995), and eNOS is distinguished by the presence of myristoylation and palmitoylation sites at the amino terminus that serve a similar function (Busconi and Michel, 1993; Garcia-Cardena *et al.*, 1996). Furthermore, nNOS and eNOS are constitutive enzymes that are regulated by the Ca<sup>2+</sup>-dependent binding of CaM to the CaM-binding sequence. Their activity is thus physiologically controlled by local changes in the Ca<sup>2+</sup> concentration (Moncada and Higgs, 1993; Nathan and Xie, 1994). In contrast, iNOS binds CaM in essentially a Ca<sup>2+</sup>-independent, irreversible manner, and its activity is transcriptionally regulated by cytokines rather than by changes in the Ca<sup>2+</sup> concentration (Moncada and Higgs, 1993; Nathan and Xie, 1994).

The three NOS isoforms catalyze the same two-step reaction sequence and appear to have the same catalytic mechanism. They oxidize L-Arg to the stable intermediate N-hydroxy-L-arginine, and subsequently oxidize this intermediate to NO and citrulline. Both steps in this sequence are NADPH- and O<sub>2</sub>-dependent (Stuehr, 1997; Marletta, 1988; Knowles and Moncada, 1994). The electrons required for the reaction flow from NADPH to the FAD, then to the FMN, and finally to the heme iron atom. The flow of electrons from the FMN to the heme iron is gated by the binding of CaM to the CaM-binding sequence and is therefore the locus of the Ca<sup>2+</sup>-dependent regulation of the activities of nNOS and eNOS (Abou-Soud and Stuehr, 1993). Of course, the electron flow is permanently turned on in iNOS



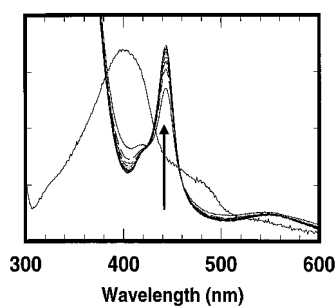


FIG. 2. Formation of the ferrous-CO complex with an absorption maximum at 450 nm in an incubation of H4B-free eNOS with NADPH under an atmosphere of CO. eNOS obtained by expression in *E. coli* was used without any exogenously added biopterin. The broad spectrum is that of the protein prior to the addition of NADPH.

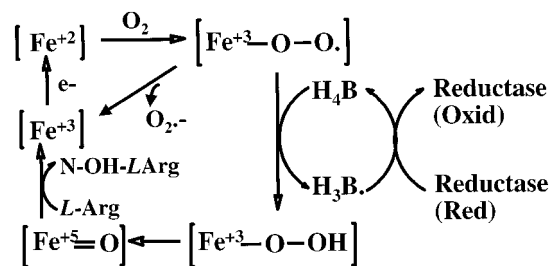


FIG. 3. Reaction scheme proposed by Bec et al. (1998) invoking a role for H4B in the first hydroxylation of L-Arg by the NOS enzymes. The iron in brackets represents the NOS heme iron atom. The reductase represents the flavins in the enzyme

been successful. However, a recent low temperature study implies a redox role for the biopterin cofactor and provides a possible explanation for the previous failures to detect such a role (Bec *et al.*, 1998). At  $-30^{\circ}\text{C}$ , Bec *et al.* observed a spectrum that they assigned to the nNOS ferrous dioxy complex. This spectrum, in the presence of H4B and L-Arg, was converted to a new species with an absorbance maximum at 428 nm. This reaction was much faster at  $-30^{\circ}\text{C}$  than oxidation of the flavin groups, which was only observed spectroscopically as the temperature was raised. Furthermore, this sequence of reaction steps resulted in the production of *N*-hydroxy-L-arginine. The reaction sequence is not observed when dihydrobiopterin is used instead of H4B. These results led the authors to propose that H4B provides the electron required to activate the ferrous dioxygen complex and that the biopterin radical thus formed is rapidly reduced, under normal turnover conditions, by electron transfer from the flavin groups (fig. 3). There are ambiguities in this study, notably the following: (a) the ferrous dioxy spectrum does not agree with that reported earlier by stopped-flow studies (Abu-Soud *et al.*, 1997), (b) the spectroscopically detected intermediate is not observed with a heme domain dimer that lacks the flavin groups, and (c) artifacts can be introduced at low temperature that are not pertinent to turnover at physiological temperatures. Nevertheless, the low temperature studies provide a paradigm that may explain the unique requirement for H4B in the catalytic function of NOS.

### Electron Transfer

The maximum rates of NO-synthesis by the three CaM-bound NOS isoforms differ, the activities of nNOS and iNOS being comparable ( $500\text{--}1500\text{ nmol}\cdot\text{nmol}^{-1}\cdot\text{min}^{-1}$ ) but severalfold higher than that for eNOS ( $100\text{--}200\text{ nmol}\cdot\text{nmol}^{-1}\cdot\text{min}^{-1}$ ) (table 1) (Nishida and Ortiz de Montellano, 1998). The intrinsic ability of the flavin domain to deliver electrons can be independently evaluated by measuring the rate at which it reduces cytochrome c, an alternative electron acceptor (Klatt *et al.*, 1992). All three isoforms reduce cytochrome c, but in the absence of  $\text{Ca}^{2+}/\text{CaM}$  the activities of the constitutive nNOS and eNOS isoforms are much lower than that of the permanently CaM-bound iNOS (table 1). When CaM binds to the constitutive isoforms, their ability to reduce cytochrome c is greatly increased (Klatt *et al.*, 1992; Heinzel *et al.*, 1992). However, whereas the enhanced cytochrome c reductase activity of CaM-bound nNOS is comparable to that of iNOS, that of CaM-bound eNOS remains tenfold lower (table 1). It is to be noted that the rates of cytochrome c reduction in all cases are much higher than the corresponding rates of NO synthesis.

To examine the link between the intrinsic reductase activity of the flavin domain and the overall activity of the NOS isoforms, we constructed and characterized chimeras in which the isoform flavin

TABLE 1

Activities of wild-type and chimeric NOS<sup>a</sup>

Protein	$\text{Ca}^{2+}/\text{CaM}$	Reductase Activity	NOS Activity
nNOS	—	$630\text{ min}^{-1}$	$0\text{ min}^{-1}$
	+	7060	96
eNOS	—	67	0
	+	670	16
iNOS <sup>b</sup>	+	5880	105
E/N	—	98	0
	+	6010	63
I/N <sup>b</sup>	+	4590	83

<sup>a</sup> Source: Nishida and Ortiz de Montellano, 1998.

<sup>b</sup> CaM-coexpressed.

domains were interchanged. All six of the possible chimeras of this type have been assembled and examined, but the results have only been published for two of them: E/N, in which the eNOS heme and CaM domains are fused to the nNOS reductase domain, and I/N, in which the iNOS heme and CaM domains are fused to the neuronal reductase domain (Nishida and Ortiz de Montellano, 1998). Comparison of the NO-synthesizing and cytochrome c-reducing activities of the chimeras with those of the parent wild-type enzymes indicate that the intrinsic activity of the reductase is a major determinant of the overall activity of the enzyme. This is well-illustrated by the E/N chimera, which has a CaM-bound cytochrome c reductase activity of  $\sim 6000\text{ min}^{-1}$ , a value similar to that of CaM-bound nNOS ( $\sim 7000\text{ min}^{-1}$ ) and iNOS ( $\sim 6000\text{ min}^{-1}$ ) but much higher than that of CaM-bound eNOS ( $\sim 700\text{ min}^{-1}$ ) (table 1). This result indicates that the activity of the nNOS reductase domain is not attenuated when it is placed in the context of the eNOS heme and CaM-binding domains. More importantly, the NO-synthesizing activity of the E/N chimera is fourfold higher than that of wild-type eNOS and approaches the activities of wild-type nNOS and iNOS (table 1). One clear conclusion from this study is that the ability of the reductase domain to deliver electrons to the heme is a major limiting step in the overall activity of the enzyme. Thus the activity of the chimeras parallels that of the protein that provides the reductase domain (fig. 4).

Analysis of the effects of L-Arg and H4B on the rate of NADPH consumption by the three wild-type isoforms and the two chimeras establishes, furthermore, that modulation of the enzyme activity by the substrate and cofactor are exclusively mediated through interactions with the heme domain. Thus the consumption of NADPH is greatly stimulated by H4B and L-Arg in iNOS but not nNOS or eNOS (fig. 5). This is also observed with the I/N but not E/N chimera. The consumption of NADPH by H4B- and L-Arg-free eNOS is slightly stimulated by the addition of L-Arg and slightly depressed by the addition of H4B, in a manner similar to the activity observed when both the cofactor and substrate are present (Nishida and Ortiz de

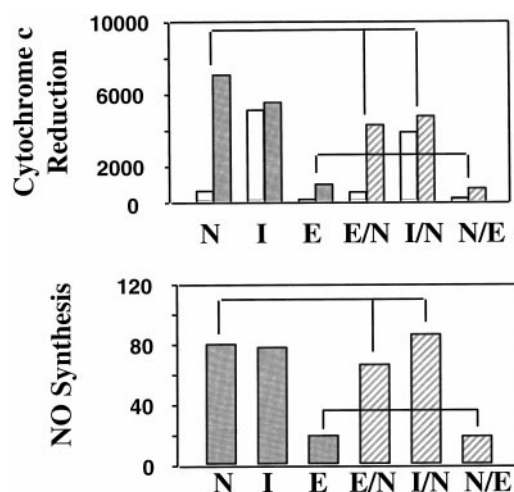


FIG. 4. The NO-synthesizing and cytochrome *c*-reducing activities of three wild-type NOS isoforms and three NOS chimeras. N, I, and E indicate, respectively, nNOS, iNOS, and eNOS. The open bars in the cytochrome *c* reductase panel indicate activities in the absence of added CaM. Closed and hatched bars indicate, respectively, the activities in the presence of CaM of the wild-type and chimeric proteins. Lines are shown that connect the forms of the enzyme having the same reductase domain. eNOS and the N/E chimera, in which the eNOS reductase replaces the reductase of nNOS, have relatively low activities in both assays. In contrast, nNOS, iNOS, and chimeras with the nNOS reductase domain, including E/N, have high activities.

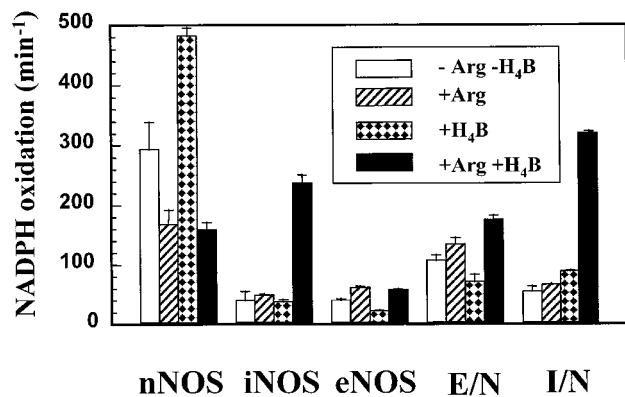


FIG. 5. Heme-domain specificity of the effects of L-Arg and H<sub>4</sub>B on the catalytic activity of NOS.

Montellano, 1998). This same pattern, which differs from those observed with nNOS and iNOS, is observed for the E/N chimera. Analysis of the dimerization properties of the chimeras shows that this property also resembles that of the parent heme domain. The effects of the substrate and cofactor on NADPH consumption are a composite of their effects on the rate of electron transfer to the heme, the rate of autooxidation of the ferrous dioxy complex, and the amount of NADPH consumed by uncoupled reduction of molecular oxygen.

A third important feature that is affected by exchanging the reductase domains is the insensitivity of the activity of iNOS to the Ca<sup>2+</sup>-concentration. The activity of wild-type iNOS remains essentially constant as the concentration of the Ca<sup>2+</sup>-chelating agent EGTA is increased from 0 to 2.5 mM. However, the activity of the I/N chimera is no longer insensitive to the presence of the chelating agent and exhibits a decreased NO synthesizing activity in the presence of 100 mM EGTA, although it is still active at the highest concentration of EGTA examined (fig. 6) (Nishida and Ortiz de Montellano, 1998). Thus the protein contacts that govern the tight association of CaM to iNOS and the apparent independence of CaM-bound iNOS to the

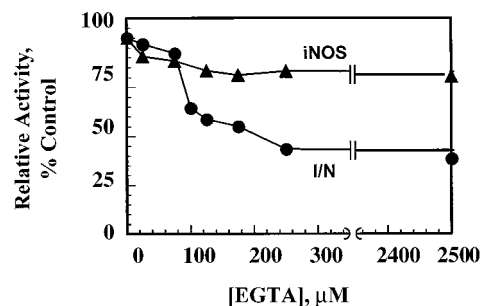


FIG. 6. EDTA-dependence of the activity of wild-type iNOS and the I/N chimera.

Ca<sup>2+</sup>-concentration involve, at least partially, residues of the flavin domain. This finding agrees with the conclusions of Ruan and co-workers, based on studies of nNOS and iNOS chimeras, that sequences of iNOS in addition to those in the consensus CaM-binding sequence are required in order to produce the Ca<sup>2+</sup>-independence of the enzyme (Ruan *et al.*, 1996).

We have further explored very recently the role of flavin domain residues in controlling the Ca<sup>2+</sup>/CaM-dependent activation of eNOS. The peptide insert identified by Salerno *et al.* (Salerno *et al.*, 1997) in the eNOS FMN domain has been deleted from the cDNA and the protein has been expressed and purified. A comparison of the wild-type and insert-deleted proteins shows that removal of the insert (a) decreases the concentration of Ca<sup>2+</sup> required to activate the protein in the presence of CaM and (b) elevates the total activity of the reductase domain as measured by its ability to reduce cytochrome *c* (C. Nishida and P. Ortiz de Montellano, unpublished results, 1998).

## Conclusions

Electron transfer, and control of the rate of electron transfer, are critical in determining the activities of the NOS isoforms. The NOS isoforms resemble P450 enzymes in this, as in many other, respects because electron transfer to the ferrous dioxy complex is also a key rate-determining step in the P450 systems. Indeed, H<sub>4</sub>B may be essential for the formation of NO because it functions as a rapid—but transient—source of electrons in the activation of oxygen by the NOS enzymes. Although the absolute rate of catalytic turnover is determined by the ability of the reductase domain to provide electrons, the effects of H<sub>4</sub>B and L-Arg on electron transfer are determined exclusively by interactions of the substrate and cofactor with the heme domain of the protein. Control of electron transfer from the flavin to the heme domain, and within the flavin and heme domains, is a complex but critical aspect of the function of the NOS enzymes.

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