ANTHONY Y. H. LU COMMEMORATIVE ISSUE
NITRIC OXIDE SYNTHASE STRUCTURE AND ELECTRON TRANSFER
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This paper is available online at http://www.dmd.org

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 biotoperin 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 tetrahydrobiotperin 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) 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.

The work in the author’s laboratory was supported by National Institutes of Health grant GM25515.

Abbreviations used are: NOS, nitric oxide synthase; CaM, Ca2+-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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0090-9556/98/2612-1185–1189$02.00/0
Printed in U.S.A.

D RUG M EtabOLISM AND D ISPOSITION Vol. 26, No. 12
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Published in U.S.A.
because CaM is bound to that isoform in an essentially irreversible manner (Cho et al., 1992).

Comparison of NOS Active Site Structures

In order to carry out structural and mechanistic studies, we have developed systems for expression of the NOS isoforms in *Escherichia coli* and have expressed and purified the recombinant proteins (Gerber and Ortiz de Montellano, 1995; Rodriguez-Crespo et al., 1996; Gerber et al., 1997a and 1997b). All of these proteins, including rat nNOS, bovine and human eNOS, and murine and human iNOS, are obtained in a correctly folded, heme-bound state. Successful expression of iNOS requires co-expression of a gene coding for CaM, as the protein does not fold correctly and is inactive in the absence of CaM co-expression (Gerber et al., 1997b; Fossetta et al., 1996). CaM co-expression is not required for the expression of nNOS or eNOS, but the yield and quality, at least of eNOS, is higher when it is co-expressed with CaM (Rodriguez-Crespo and Ortiz de Montellano, 1996). The recombinant proteins are soluble and are obtained free of H4B because there is no H4B, and no myristoylation or palmitoylation, in *E. coli*.

We have employed aryldiazanes as topological probes to explore the active sites of the NOS isoforms in much the same way that we previously used them to characterize the topologies of cytochrome P450 enzymes (Ortiz de Montellano, 1995). Aryldiazanes react with the heme group of hemoproteins to form stable s-bonded aryl-iron complexes (Fe-Ar). The absorption maxima of these aryl-iron complexes is therefore readily monitored by spectroscopic methods. Earlier studies with the P450 enzymes demonstrated that in situ oxidation of the aryl-iron complexes with ferricyanide causes the aryl group to migrate from the iron to one of the four nitrogens of the heme (Ortiz de Montellano, 1995). This migration only occurs within the intact active site if the iron is thiolate-ligated, as in the P450 and NOS enzymes, at approximately 480 nm. Formation and decay of the aryl-iron complexes is therefore readily monitored by spectroscopic methods. Earlier studies with the P450 enzymes demonstrated that in situ oxidation of the aryl-iron complexes with ferricyanide causes the aryl group to migrate from the iron to one of the four nitrogens of the heme (Ortiz de Montellano, 1995). This migration only occurs within the intact active site if the iron is thiolate-ligated. The four possible N-phenylprotoporphyrin IX isomers thus formed can be demetallated and individually quantitated by high-performance liquid chromatography (Swanson and Ortiz de Montellano, 1991). Earlier studies with the crystalline P450 enzymes show that the N-arylporphyrin isomer ratio is primarily determined by the degree to which each of the four pyrrole nitrogens is sterically protected by active site residues. Topological information is thus provided by the N-aryl porphyrin regioisomer ratios, using different aryldiazane probes; by the rates of aryl-iron complex formation and decay; and by the changes caused by cofactors, substrates, and inhibitors in these rates and isomer ratios (Ortiz de Montellano, 1991).

The three NOS isoforms react with phenylidyazene (PhN==NH) to give phenyl-iron complexes (Gerber and Ortiz de Montellano, 1995; Gerber et al., 1997b). In general, the binding of CaM stimulates the phenylidyazene reaction, whereas the binding of both L-Arg and H4B inhibits it, as expected if the bioppterin cofactor and the substrate obstruct the entry channel or the heme site itself. The phenylidyazene reaction is slower with eNOS than with the other two isoforms both in the presence and absence of L-Arg or H4B, which suggests that the eNOS active site is smaller than those of the other two isoforms. This conclusion is supported by the finding that 2-naphthylidyazene forms a 2-naphthyl-iron complex with nNOS and iNOS, but not eNOS, and that 2-biphenylidyazene only forms the p-biphenyl-iron complex with nNOS. The minimum height above the iron required to erected the phenyl complex is ~5.8 Å; the 2-naphthyl complex, 7.1 Å; and the p-biphenyl complex, ~9.9 Å. The ceiling height directly over the iron is therefore between ~6 and 10 Å, with eNOS closer to the lower and nNOS to the higher limit.

Shift of the phenyl group from the iron to the porphyrin nitrogens gives, with all three NOS isoforms, mixtures of the four N-phenylprotoporphyrin IX regioisomers in which the isomer with the N-phenyl on pyrrole ring D predominates (Gerber and Ortiz de Montellano, 1995; Gerber et al., 1997b). The regioisomer ratios for the CaM-bound proteins without H4B or L-Arg are (N_B:N_A:N_C:N_D, where the subscript indicates the N-phenyl pyrrole ring): nNOS, 11:12:03:74; eNOS, 04:04:11:81; and iNOS, 10:17:08:65 (Gerber et al., 1997b). Addition of H4B decreases the proportion of the ND regioisomer to 41%, 47%, and 40%, respectively, for nNOS, eNOS, and iNOS. These results indicate that, in all three isoforms, the most open region of the active site in the absence of L-Arg and H4B is above pyrrole ring D. Furthermore, they indicate that H4B binds close to pyrrole ring D and thereby decreases both the rate of the reaction with phenylidyazene and the extent to which the phenyl in the preformed complex shifts to the nitrogen of pyrrole ring D (fig. 1). These structural inferences are confirmed by the recent crystal structure of the L-Arg- and H4B-containing iNOS heme domain, which shows that H4B binds in the substrate access channel close to pyrrole ring D of the heme (Crate et al., 1998). A similar active site geometry is observed in the crystal structure of eNOS (T. Poulos, personal communication, 1998).

Role of H4B

The reason for the absolute catalytic requirement of NOS for H4B remains unclear. Many of the functions ascribed to H4B can be satisfied by dihydrobiopterin, a cofactor that binds to NOS without forming a catalytically active enzyme. The binding of H4B to H4B-free NOS causes a shift of the heme iron atom from the low- to the high-spin state (Rodriguez-Crespo et al., 1996), but a similar shift is observed with dihydrobiopterin (Presta et al., 1998). H4B promotes the dimerization of iNOS (Baek et al., 1993) and stabilizes the nNOS dimer (Klatt et al., 1995), but it is not required for the dimerization of eNOS (Rodriguez-Crespo et al., 1996; Rodriguez-Crespo and Ortiz de Montellano, 1996). Furthermore, dihydrobiopterin is able, at least for iNOS, to promote dimerization without conveying catalytic activity to the dimer (Presta et al., 1998). Dihydrobiopterin, like H4B, facilitates electron transfer from the flavoprotein domain to the iron to give the ferrous enzyme (Presta et al., 1998). Indeed, reduction of the ferric to the ferrous state can be observed in the complete absence of any bioppterin cofactor (fig. 2). Thus neither the allosteric effect of H4B on the iron coordination state, nor its dimer-stabilizing properties, nor its effect on reduction of the ferric to the ferrous enzyme, accounts for the absolute catalytic requirement for H4B. These results are consistent with the view that H4B plays a critical redox role not met by dihydrobiopterin, but efforts to identify a redox role for H4B have not
been successful. However, a recent low temperature study implies a redox role for the biotin cofactor and provides a possible explanation for the previous failures to detect such a role (Bec et al., 1998). At −30°C, Bec et al. observed a spectrum that they assigned to the nNOS ferrous dioxygen 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°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 dihydrobiotin 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 biotin cofactor radical thereby 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 dioxygen 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 (500–1500 nmol·min⁻¹·μmol⁻¹) and about twice that of eNOS (100–200 nmol·min⁻¹·μmol⁻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., 1997). The intrinsic ability of the flavin domain to deliver electrons to the heme is a major limiting step in 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 about 8000 min⁻¹, a value similar to that of CaM-bound nNOS (8700 min⁻¹) and iNOS (≈6000 min⁻¹) but much higher than that of CaM-bound eNOS (≈700 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 E/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 Montellano, 1998).
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 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 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$^{2+}$-concentration. The activity of wild-type iNOS remains essentially constant as the concentration of the Ca$^{2+}$-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 Ca$^{2+}$-concentration involve, at least partially, residues of the flavin domain. This finding agrees with the conclusions of Ruan and coworkers, 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$^{2+}$-independence of the enzyme (Ruan et al., 1996).

We have further explored very recently the role of flavin domain residues in controlling the Ca$^{2+}$/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$^{2+}$ 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, H4B 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 H4B 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.

**References**


