Nitric Oxide Synthase: Aspects Concerning Structure and Catalysis

Review

Michael A. Marletta
Interdepartmental Program in Medicinal Chemistry
College of Pharmacy and Department of Biological
  Chemistry
School of Medicine
The University of Michigan
Ann Arbor, Michigan 48109-1065

The rapid development of our understanding of the biological actions of nitric oxide (NO) has, to a large degree, been paralleled by our understanding of the enzyme responsible for the synthesis of NO, nitric oxide synthase (NOS). The relatively fast pace of advance in NOS enzymology is primarily due to the fact that structure/function questions have crossed over several well-established enzymatic problems. As will be outlined below, NOS is a complex enzyme involving several tightly bound redox cofactors that are apparently organized into discrete domains that can be associated with a particular activity. First, the enzyme has significant homology to NADPH cytochrome P-450 reductase and has been shown to contain a cytochrome P-450-type heme and to carry out P-450 chemistry in the formation of NO. What then is the relationship of NOS to the large class of cytochrome P-450 isoenzymes? Second, constitutive NOS isoforms require Ca^{2+} and calmodulin (CaM) while inducible NOS (iNOS) shows no requirement, although these isoforms apparently have CaM as a tightly bound subunit. What is the nature of this important difference in the recognition and binding of CaM? NOS also has a tightly associated reduced pterin that is very important for an enzyme activity whose function is still not known. Third, the product of the reaction, NO, typically is a strong heme ligand. How is it that the enzyme escapes self-inactivation during turnover? These questions shall be the main the focus of this review.

General Characteristics of the Reaction

The reaction catalyzed by NOS is illustrated in Figure 1. As shown, the reaction requires molecular oxygen (O_2) and reducing equivalents in the form of NADPH (Marletta, 1993). Except for some minor structural modifications, all NOS isoforms specifically utilize L-arginine as the substrate. The products of the reaction are NO and citrulline (presumed to be the L-isomer). It is assumed, given the monooxygenase-like activity of NOS and the heavy isotope labeling studies, that H_2O is the ultimate fate of the other oxygen atom. The initial NOS cDNA isolated was that of the neuronal isoform from rat cerebellum, where sequence analysis showed a significant homology of NADPH cytochrome P-450 reductase to the C-terminal sequence of this NOS isoform (Bredt et al., 1991). All clones isolated subsequently have demonstrated the same homology. Given that the normal function of this reductase is to supply reducing equivalents to cytochrome P-450, it has been assumed that this domain in NOS serves the same function. The findings that NOS also con-

tains a P-450-type heme moiety (see references in Marletta, 1993) and that this heme functions in the chemistry of the reaction (White and Marletta, 1992; Pufahl and Marletta, 1993) strengthen this hypothesis.

Relationship to Cytochrome P-450

Several laboratories have now shown with both inducible and constitutive isoforms that upon reduction and treatment with carbon monoxide, NOS shows a λ_{max} of ~450 nm. This relatively rare spectral characteristic is relegated to the large family of cytochrome P-450 enzymes. Most of the P-450s fall into a well-described supergene family whose function involves oxidative metabolism (hydroxylation) of endogenous and xenobiotic compounds (Nelson et al., 1993). In addition, with one notable exception, the P-450s require a flavoprotein reductase and sometimes an iron–sulfur protein to transfer electrons into the heme prosthetic group that is responsible for the oxidative catalysis. The exception to this rule is a fatty acid monooxygenase, $P-450_{BM3}$, isolated from Bacillus megaterium, where the flavoprotein reductase and the heme are contained within a single polypeptide (Narhi and Fulco, 1986). This P-450 has been called a self-sufficient P-450 since the need for a separate reductase is no longer necessary.

NOS would appear to be the first self-sufficient mammalian P-450. However, comparison of NOS to the P-450s does not reveal any significant homology. This remains true even when the analyses are focused on the N-terminal domain where the heme site is located. The signature P-450 spectrum is derived from the ligation of a cysteine thiolate to the iron of the heme, and a 10-residue consensus sequence that includes this cysteine residue (FXXGXXXCXG) has been found in most of the P-450s characterized. As several groups have noted (McMillan et al., 1992; Renaud et al., 1993; White and Marletta, 1993), this 10-residue sequence is not present in NOS. However, several cysteines are conserved among the cloned NOS isoforms; alignment of some of those conserved cysteines with the P-450 consensus sequence is shown in Table 1. $P-450_{cam}$ is a camphor hydroxylase isolated from Pseudomonas putida, and $P-450_{BM3}$ is the self-sufficient P-450 fatty acid hydroxylase mentioned above. The conserved cysteine in NOS that provides the best alignment to this 10-residue P-450 sequence corresponds to neuronal NOS (nNOS) residues 409–417 and murine macrophage iNOS residues 188–196. In both cases, with a gap at position 7, the alignment of the amino acid side chains of known
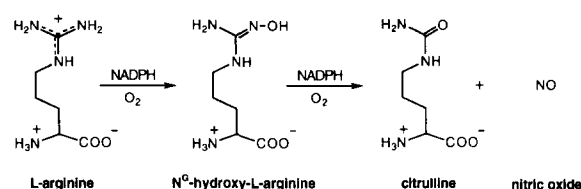

Figure 1. The Reaction Catalyzed by NOS

L-arginine   N⁰-hydroxy-L-arginine   citrulline   nitric oxide

Table 1. Comparison of the Putative Heme-Binding Regions of NOS to Cytochrome P-450

Relative Position	1	2	3	4	5	6	7	8	9	10
P-450 consensus	F	X	X	G	X	X	X	C	X	G
P-450$_{cam}$	F	G	H	G	S	H	L	C357	L	G
P-450$_{BM3}$	F	G	N	G	Q	R	A	C401	I	G
nNOS$_{(409-417)}$	W	R	N	A	S	R	—	C415	V	G
nNOS$_{(666-674)}$	Y	R	C	R	G	G	—	C672	P	A
iNOS$_{(188-196)}$	W	R	N	A	P	R	—	C194	I	G

function determined from the crystal structure of P-450$_{cam}$ (Poulos et al., 1987) is well conserved. Also shown in Table 1 is the alignment of nNOS residues 666–674. C672 is conserved in the other NOS isoforms, and when nNOS is aligned with P-450$_{BM3}$, C672 aligns with C401, the iron ligand in $_{BM3}$. However, the rest of the sequence alignment is relatively poor. Further studies will be necessary before the cysteine ligand to the iron heme is definitively known.

The fact that there is little other homology in the N-terminus when compared with the P-450s is not too surprising considering that the comparison is being made to enzymes whose substrates are very hydrophobic molecules. The NOS substrate L-arginine is very hydrophilic, and NOS isoforms must have the appropriate residues to accommodate this substrate and any intermediates formed during the course of the reaction. Therefore, it appears that NOS lies outside the P-450 superfamily and has converged on using the P-450 chromophore for the chemistry that it carries out. In this regard NOS joins chloroperoxidase, allene oxide synthase, and thromboxane synthase as proteins that have the optical properties of P-450s, but otherwise appear to be only distantly related.

Mechanism of the Reaction

As shown in Figure 1, the NOS reaction is known to involve an initial hydroxylation of L-arginine to generate N^G-hydroxy-L-arginine (NHA) (Stuehr et al., 1991; Pufahl et al., 1992). This step presumably involves the heme moiety of NOS (White and Marletta, 1992; Pufahl and Marletta, 1993). Although the chemical steps involved in the conversion of NHA to NO and citrulline are not known, speculation based on enzymatic and chemical precedent has led to the proposal that the heme is directly involved in the oxidation of NHA (Marletta, 1993). This proposed mechanism involves a heme ferric peroxide (Fe(III)–OO$^-$) nucleophile as a key reaction intermediate. In addition, the mechanism proposes that NHA reduces the ferrous oxy–heme complex (Fe(II)–O$_2$) to form Fe(III)–OO$^-$ generating a radical species on the substrate that would ultimately become the odd electron radical on NO. Based on their determined oxidation potential of NHA, Korth et al. (1994) have proposed a slight, but important, modification to this mechanism.

NOS Isoforms

A number of NOS isoforms have been purified from different tissues, and several more have been cloned and, in some cases, functionally expressed. Based on both sequence analyses and biochemical studies, these NOS isoforms are a quite closely related family of proteins, which are apparently the products of three distinct genes. In general, the synthases are grouped into two broad categories: a constitutive, Ca^{2+}/CaM-dependent type that is involved in cellular signaling and an inducible isoform characterized in macrophages and other cells that typically does not show a dependence on Ca^{2+}/CaM. The first constitutive NOS isoforms purified were from rat and porcine cerebellum (see references in Marletta, 1993; Nathan, 1992). These proteins, with reported monomeric M$_r$ values on SDS–polyacrylamide gels ranging from 150 to 160 kDa, are cytosolic and exist as homodimers under native conditions. A constitutive isoform (135 kDa on an SDS–polyacrylamide gel) isolated from bovine vascular endothelium is distinct in that it is membrane bound (Pollock et al., 1991). The cDNA-derived amino acid sequence of this endothelial cell NOS isoform shows a myristoylation consensus sequence at the N-terminus that is absent in the cytosolic isoforms isolated from murine macrophages and rat cerebellum (Lamas et al., 1992). In addition, the bovine endothelial NOS sequence showed no transmembrane domain regions, suggesting that myristoylation is responsible for the membrane anchor of this NOS isoform, and subsequent studies convincingly showed that myristoylation is critical for the membrane association of this isoform (Busconi and Michel, 1993).

The inducible isoform purified from murine macrophages is a cytosolic protein with a monomeric M$_r$ of 130 kDa and, again, found to be dimeric under native conditions (Marletta, 1993). A sequence comparison shows that this isoform is about 50%–60% identical to the cerebellar isoforms. In general, isoforms from the same tissue are essentially identical. For example, the sequence comparison of the bovine endothelial to human endothelial shows them to be 95% identical (Marletta, 1993; Nathan and Xie, 1994), whereas both of these isoforms show 50%–60% identity to the macrophage-inducible isoform. The rat hepatocyte-inducible isoform is essentially identical (96%) to the murine macrophage isoform. An interesting picture is emerging with regard to the comparison of inducible isoforms. Several human inducible isoforms have been cloned, including one from hepatocytes (Geller et al., 1993) and one from a colorectal adenocarcinoma cell line (Sherman et al., 1993). These isoforms are essentially identical to each other (99%) with only a few amino acid differences. However, comparison of these inducible isoforms to that from the murine macrophage shows about 80% identity. In general, while all human isoforms are essentially identical to each other regardless of the origin
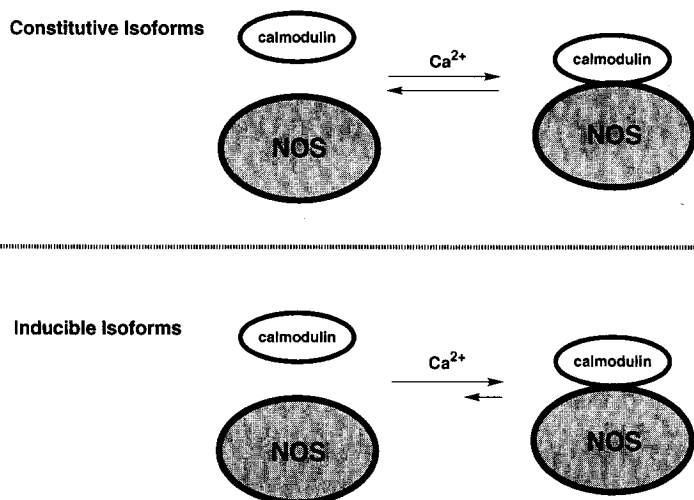
Constitutive Isoforms

Inducible Isoforms

Figure 2. Interactions of CaM with NOS

The constitutive isoforms show a typical interaction in which CaM–NOS complex formation is controlled by Ca^{2+}. The inducible isoforms, especially those from murine macrophages, form a very tight complex at low levels of Ca^{2+}.

of the tissue, significant differences exist when compared with inducible rodent isoforms. A truncated mRNA coding for the rat cerebellar NOS has been isolated; however, the significance of this finding is not clear, especially since the region missing comes about from an in-frame deletion of two exons. These exons are from a highly conserved region and likely represent an important part of the substrate or reduced pterin-binding site.

Ca^{2+}/CaM Dependence of NOS

The activity of the constitutive NOS isoforms is strictly controlled by Ca^{2+} and CaM, and while other mechanisms of activity regulation, particularly for the particulate endothelial form, are being investigated (Nathan and Xie, 1994), control of these isoforms by regulation of intracellular Ca^{2+} levels represents a critical point of control (see Figure 2). CaM recognition sequences have been found in all NOS isoforms (Lowenstein and Snyder, 1992). Since the constitutive forms showed a dependence on Ca^{2+}/CaM, it was expected that a sequence of this type would be present. However, it was not clear why this sequence would be present in the iNOS isoforms until it was shown that the iNOS isolated from murine macrophages appears to co-purify with a tightly bound CaM subunit (Nathan and Xie, 1994). In hindsight it appears that this particular inducible isoform from murine macrophages is at the extreme in the ability to bind CaM in that other inducible isoforms show some level of inhibition after treatment with Ca^{2+} chelating agents, CaM antagonists, or both (see references in Marletta, 1994). This unusual protein–CaM interaction and the role of Ca^{2+} in this binding clearly deserve further attention.

The potential role of Ca^{2+}/CaM in the NOS reaction has recently received some attention. Titration of the neuronal constitutive isoform with Ca^{2+}/CaM led to quenching of the intrinsic fluorescence of the protein, suggestive of a conformational change upon complex formation (Sheta et al., 1994). The implication is that this conformational change allows for efficient electron transfer and catalysis. That Ca^{2+}/CaM binding is critical for electron transfer is supported by the finding that showed that the rate of NADPH oxidation for the neuronal isoform is primarily influenced by Ca^{2+}/CaM and not L-arginine (Abu-Soud and Stuehr, 1993). However, the rate of NADPH oxidation by murine macrophage iNOS is measurable without substrate and increases in the presence of L-arginine (Abu-Soud and Stuehr, 1993). The results would suggest that the constitutive isoforms would reduce O_2 in the absence of substrate generating superoxide (O_2^-) and hydrogen peroxide (H_2O_2) coincident with Ca^{2+}/CaM–NOS complex formation, whereas the inducible isoform would generate O_2^- and H_2O_2 at some basal level, which would increase and become coupled to product formation in the presence of substrate. Given the dependence of reduction rates (NADPH oxidation) in P-450s on heme spin state and the known influence of substrate on this parameter, and the known effect of tetrahydrobiopterin on the spin state, the conclusions reached thus far about the role of Ca^{2+}/CaM in the NOS reaction are likely to be oversimplifications.

Potential for End Product Inhibition

NO has long been known and used, especially in inorganic chemistry, as a ligand in iron–heme complexes. These studies include heme–NO complexes of chemical model compounds and hemoprotein–NO complexes. The possibility has been raised that the NO derived from NOS could inhibit subsequent turnover of the enzyme (Rengasamy and Johns, 1993; Rogers and Ignarro, 1992). The ability of NO to form a relatively stable complex with both ferric and ferrous P-450 hemes has been established a number of years ago; however, the complexes were unstable in the presence of oxygen. Stable heme–NO complexes are typically formed with hemoproteins such as hemoglobin where the ligand to the heme is histidine and not cysteine, as in P-450 and NOS. The potential of NO to inhibit NOS under physiological conditions is still an open question; however, the chemistry of NO interaction with a P-450-type heme and the reactivity of NO with ferrous oxy–heme complexes such as hemoglobin argues against this potential mode of inhibition. Further characterization of the stability of NOS–NO complexes will be needed before this question can be answered.

Conclusions

The reaction catalyzed by NOS involves some unusual chemistry, and the enzyme seems to have evolved with pieces of previously characterized proteins that, when fitted together, allow for the efficient synthesis of a key molecular signaling agent on the one hand and an apparent piece of the chemical weaponry in immune system surveillance on the other. While many molecular details still need to be worked out, the story to date represents an interesting structure/function relationship.

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