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Focused review

Dissecting regulation mechanism of the FMN to heme interdomain electron transfer in nitric oxide synthases

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ABSTRACT

Nitric oxide synthase (NOS), a flavo-hemoprotein, is responsible for biosynthesis of nitric oxide (NO) in mammals. Three NOS isoforms, iNOS, eNOS and nNOS (inducible, endothelial, and neuronal NOS), achieve their biological functions by tight control of interdomain electron transfer (IET) process through interdomain interactions. In particular, the FMN—heme IET is essential in coupling electron transfer in the reductase domain with NO synthesis in the heme domain by delivery of electrons required for O_2 activation at the catalytic heme site. Emerging evidence indicates that calmodulin (CaM) activates NO synthesis in eNOS and nNOS by a conformational change of the FMN domain from its shielded electron-accepting (input) state to a new electron-donating (output) state, and that CaM is also required for proper alignment of the FMN and heme domains in the three NOS isoforms. In the absence of a structure of full-length NOS, an integrated approach of spectroscopic, rapid kinetic and mutagenesis methods is required to unravel regulation mechanism of the FMN—heme IET process. This is to investigate the roles of the FMN domain motions and the docking between the primary functional FMN and heme domains in regulating NOS activity. The recent developments in this area that are driven by the combined approach are the focuses of this review. A better understanding of the roles of interdomain FMN/heme interactions and CaM binding may serve as a basis for the rational design of new selective modulators of the NOS enzymes.

1. Introduction

1.1. Biomedical importance of nitric oxide synthases (NOSs)

NOSs (EC 1.14.13.39) are a family of enzymes catalyzing the synthesis of nitric oxide (NO) from L-Arg. There are three mammalian NOS isoforms: neuronal, endothelial and inducible NOSs (nNOS, eNOS and iNOS, respectively). NO is a ubiquitous signaling molecule for vasodilatation and neurotransmission at nanomolar concentrations and a defensive cytotoxin at higher concentrations [1,2]. For signaling, NO is produced by eNOS in the endothelial cells that line the inner surface of arteries (for controlling blood pressure), or by nNOS in the brain for nerve signal transduction [3] (nNOS is also present in the muscle tissue and peripheral nervous system). In addition, NO is produced in macrophages by iNOS as an immune defense mechanism [4–8]. The two constitutively expressed NOS isoforms (cNOS), eNOS and nNOS, are Ca^{2+}/calmodulin (CaM) responsive, whereas the Ca^{2+}-insensitive iNOS binds CaM at basal calcium concentrations and is expressed in response to cytokines or bacterial products. NO generated from the NOS enzymes is the primary source of NO, while an alternative pathway for NO generation in vivo is the reduction of inorganic anions nitrate [9] and nitrite [10]. Recent studies indicate that hemoglobin reacting with nitrite can be a source of NO bioactivity [11]. This can transport NO bioactivity over much longer distances and time periods.

NO's availability is tightly regulated at the production level by NOS. Aberrant NO synthesis by NOS is implicated in an increasing number of human pathologies, including cancer and ischemic injury caused by stroke [2,12]. Selective NOS modulators are required for therapeutic intervention because eNOS is crucial for maintaining proper blood pressure. Non-selective NOS inhibitors will lead to hypertension. It was challenging to design selective NOS inhibitors due to the high active site conservation among the NOS isoforms [13]; significant advances in developing nNOS-selective inhibitors have been made in recent years [14–16]. Clinical agents that selectively modulate NOS isoform activity remain elusive. Clearly, molecular mechanisms of NOS isoform regulation, once fully understood, could identify key targets for rational development of new selective drug entities for treating wide range of diseases that currently lack effective treatments.

1.2. NOS enzymology—overview

NOS enzymes are monooxygenases, generating NO and citrulline from L-arginine (L-Arg), NADPH and O_2:

$$\text{L}-\text{Arg} + 1.5\text{NADPH} + 1.5\text{H}^{+} + 2\text{O}_2 \rightarrow 1.5\text{NADP}^{+} + \text{Citrulline} + \text{NO} + 1.5\text{H}_2\text{O}$$

Under optimal fully coupled conditions, NO production requires 1.5 NADPH molecules per NO molecule. Deviation from this optimum

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indicates that reactive oxygen species such as superoxide are being formed at the expense of NADPH (i.e., uncoupling). As such, this stoichiometry is not generally observed.

NOS catalysis is a two-step process: the substrate, L-Arg, is first converted to N-hydroxy-L-arginine (NOHA), which in turn is converted to NO and citrulline [17,18] (Scheme 1). The oxidation mechanism of the substrate by NOS heme is intricate, and many details of the chemistry mechanism remain to be fully elucidated [18–24].

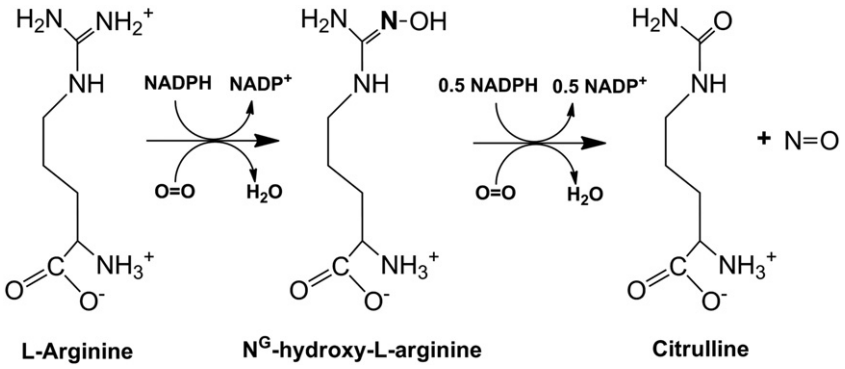
Eukaryotic NOS evolved via a series of gene fusion events, resulting in a modular heme- and flavin-containing enzyme [25]. In contrast to the cytochrome (cyt) P450-mediated systems, the flavins and heme cofactors of the NOS are bound to the same polypeptide chain, and the existence of (6R)-5,6,7,8-tetrahydrobiopterin (H_4B) and a coordinated zinc ion set NOS apart from these multicomponent P450 systems. Structurally, mammalian NOS enzyme is a homodimeric flavo-hemoprotein, and each subunit is comprised of two major domains (Fig. 1): an N-terminal catalytic heme-containing oxygenase domain (NOSoxy), which belongs to class of heme-thiolate proteins and is not structurally related to cyt P450s, and a C-terminal flavin-containing reductase domain (NOSred), which is structurally similar to P450 reductase. The L-Arg substrate and an essential cofactor, H_4B, both bind near the heme center in the oxygenase domain [26], where the catalytic production of NO takes place. NOSred contains ferredoxin–$NADP^+$-reductase (FNR) and FMN modules [27] and is similar to other NADPH-utilizing dual flavin oxidoreductases [28,29]. NOSred catalyzes the transfer of the reducing equivalents from the two-electron donor NADPH to the heme iron, a one-electron acceptor, where dioxygen molecule can bind to Fe^{2+} and be activated. Eukaryotic NOS isozymes are thus catalytically self-sufficient. On the other hand, NOSred differs from P450 reductase primarily because of insertions and extensions related to control of NO synthesis (see below). The NOS FMN and heme domains are connected by a CaM binding linker (Fig. 1), and its occupation by CaM is required for electron transfer from the reductase domain to the oxygenase domain. In the Ca^{2+}-independent iNOS isoform, the linker is bound to CaM tightly (but reversibly [30]). In contrast, in nNOS and eNOS the CaM binding is [Ca^{2+}] dependent [25]. The binding of CaM to NOS is fast and competent for efficient electron transfer [31].

The NOS enzyme is active in NO production only as dimer [32] (that forms through the heme domains). The heme does bind to the monomer as there is an iNOS monomer crystal structure [33], while the heme binding is necessary for dimerization [34] (heme insertion into apo-NOS is important in NOS maturation to active dimeric enzyme [35,36]). On the other hand, the dimer is required for H_4B site formation and other changes that enable substrate binding [37,38] and allow intersubunit electron transfer from the reductase-domain FMN to the oxygenase-domain heme [39]. Isolated NOS heme domain, but not the flavin domain, dimerizes [40]. A tetra-coordinated zinc ion is important for structural integrity of the dimer; each subunit provides two of the cysteine ligands for zinc ion.

NOS holoenzymes and truncated constructs (Fig. 1) can be expressed in bacterial and baculovirus systems [41] and yeast [42]. The separated domains of the modular NOS protein are active [40,43], and studies on the catalytic and electron transfer mechanisms by using the homologous NOS constructs are well-accepted in the field [25,44]. For example, NOSoxy construct has been used to study the oxidative pathways [24], and a bi-domain oxygenase/FMN (oxyFMN) construct is a minimal electron transfer complex designed to favor the interactions between the FMN and heme domains [45]. Activity assay of truncated NOS domain requires additional treatments (e.g. H_2O_2-supported oxidation of NOHA [45], pre-reduction of NOSoxy construct). This is due to lack of endogenous electron transfer partners in the truncated constructs.

The first X-ray crystal structures of NOS domain were reported in 1997 [33]. The structures are of monomeric murine iNOSoxy construct lacking NH_2-terminal residues 77 to 114 and the H_4B cofactor, and the heme pocket is more exposed in these truncated monomeric structures [33] than in the iNOSoxy dimer structures reported later [26]. Since then, crystal structures have been reported for the NOS domains (the oxygenase domains of each of the NOS isoforms [26,37,46], rat nNOS reductase constructs [47,48], CaM-bound human iNOS FMN domain [49]), and CaM bound to peptides corresponding to the CaM-binding region in eNOS [50], nNOS (PDB 2O60), and iNOS (PDB 3GOF). A majority of the structural work has been on the NOS heme domains co-crystallized with inhibitors/ligands [51–55]. These structures give considerable insights into the NOS mechanism, and provide a basis for studying structural–functional relationship of the NOS isoforms. However, the long-standing quest for the structure of a full-length mammalian NOS protein has eluded many investigators, probably because of significant NOS domain motions (see below). Strategies to decrease overall conformational flexibility may help crystallization of the NOS holoenzyme.

In contrast to P450 enzymes (where the heme active site undergoes large open/close motions to enable substrate binding and product release during catalysis) [56], the NOS heme active site is rigid [57] and remains relatively exposed [37]. The open NOS active site favors release of the NO product, but the Fe-oxy intermediate could be exposed to bulk solvent, resulting in nonspecific protonation that short-circuits the normal reaction path [56]. To circumvent this problem, it was proposed that the H_4B cofactor near the NOS heme center is recruited to provide a rapid and coupled electron/proton transfer system, which avoids the indiscriminate reaction with bulk solvent [56]. The unprecedented involvement of the H_4B cofactor as an electron donor is unique among P450s and pterin containing proteins, and the roles of the H_4B cofactor in iNOS [22,58,59], nNOS [60] and eNOS [61] isoforms were extensively studied. H_4B promotes dimerization and transiently donates electrons during NOS catalysis [62]. H_4B is also proposed to provide proton(s) during the oxidative chemistry and product release (in the second step of NOS catalysis [22]) processes. Here the timing and source of proton donation are the key to deciding the pathways of substrate oxidative reactions.



Scheme 1. Production of NO by NOS enzyme.