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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 O₂ 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 absence of a structure of full-length NOS, an integrated approach of spectroscopic, rapid kinetics 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.

Keywords

Nitric oxide synthase; Calmodulin; FMN; Heme; Electron transfer

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 NOS (nNOS, eNOS and iNOS). 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

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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 reduction of inorganic anions nitrate [9] and nitrite [10]. Recent studies indicate 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 :

Under optimal fully coupled conditions, NO production requires 1.5 NADPH molecules per NO molecule. Deviation from this optimum 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 (Figure 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 transfer of the reducing equivalents from the two-electron donor NADPH to the heme iron, a one-electron acceptor, where dioxygen molecule can bind 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 (Figure 1), occupation of which 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 $[\text{Ca}^{2+}]$ 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 bind [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 (Figure 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 H₄B cofactor in iNOS [22, 58, 59], nNOS [60] and eNOS [61] isoforms were extensively studied. H₄B promotes dimerization and transiently donates electrons during NOS catalysis [62]. H₄B 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 is the key to deciding the pathways of substrate oxidative reactions.

1.3. The interdomain electron transfer (IET) processes in NOS catalysis

It is well accepted that the IET processes from NADPH to heme through FAD and FMN cofactors are key steps in NO synthesis through coupling reactions in the domains [17, 25, 63]. Tight coupling of the IET processes in NOS enzyme is important for normal physiological function and prevention of disease, because uncoupled or partially coupled NOS leaks electrons and results in deleterious effects (via production of excessive reactive oxygen species) [61, 64, 65]. Therefore, it is of current interest to study the NOS IET control mechanism.

The catalytic cycle starts with the air stable 1-electron reduced FAD-FMNH[•] form (Figure 2). The electron source, NADPH, donates two electrons to FAD (through a hydride transfer). This is followed by sequential one-electron transfers from FAD to FMN. After reduction of the FAD-FMNH[•] form by NADPH, interflavin electron transfer occurs from FAD_{hq}[−]-FMNH[•] to produce FADH[•]-FMN_{hq} (FMN hydroquinone). The fully reduced FMN (*i.e.*, FMN_{hq}) is the species that donates electrons to the heme catalytic center in NOS_{oxy}. The FAD-FMN pair cycles between the 1-electron and 3-electron reduced forms in the course of catalysis (Figure 2). At high concentrations of NADPH, the intermediate FAD-FMN_{hq} is reduced to a four-electron reduced form FAD_{hq}[−]-FMN_{hq} by NADPH, and a 2-electron/4-electron cycle is also possible (partially shown in dark grey in Figure 2); this has been discussed in detail in a recent excellent review [66]. The NOS_{red} domain thus can cycle between the 1-electron and 3-electron reduced or 2-electron and 4-electron reduced forms in the course of catalysis depending on the NADPH/NADP⁺ ratio, while the 1e/3e cycle is likely the major mechanism *in vivo* [66].

The FMN domain containing FMN_{hq} is proposed to move away from the NADPH/FAD module to transfer electrons to the ferric heme in the oxygenase domain. Unlike iNOS, eNOS and nNOS synthesize NO in a Ca²⁺/CaM dependent manner: CaM-binding (induced by an influx of Ca²⁺) enhances the IET reaction from FAD_{hq} to FMNH[•] [67], and triggers the IET from the FMN_{hq} to the catalytic heme iron in the oxygenase domain of another subunit [39, 68–70]. CaM binding has little effect on the thermodynamics of redox processes in NOS [71–73]. It should be noted that CaM binding was also reported to notably shift redox potentials of the flavin cofactors in an NOS reductase construct [74]. Nonetheless, it is generally accepted that the regulation by CaM is primarily accomplished by controlling redox-dependent conformational changes required for effective IET.

The repression of electron transfer in CaM-free cNOS, and its relief by CaM binding, involves intrinsic control elements in the reductase domain [25]. Analysis of protein sequences and related mutagenesis studies show that the cNOS reductase domain has at least three control elements which are absent in P450 reductase [25]: an autoregulatory (AR) insert (~ 40 amino acids) in the FMN domain [75, 76], a short insert in the connecting domain between the FAD and FMN domains (CD2A loop) [77], and a C-terminal tail (CT) [78, 79]. The cNOS-unique AR insert only exists in nNOS and eNOS, but not in iNOS. The length of the CT is different in the three NOS isoforms: shortest in iNOS and relatively longer in eNOS and nNOS. Control elements deletion studies indicated that these control elements repress electron flow into and out of the NOS_{red} domain in the CaM-free state,

and may help to relieve the repression upon CaM binding [78–83]. It has been also demonstrated that the control elements regulate the IET in a concerted manner [75–79]. It is also of note that extrinsic posttranslational modifications to the control elements, including phosphorylation at the AR and CT sites, as well as inter-protein interactions, are important in NOS regulation in cellular environment (reviewed in ref. [25]).

The crystal structure of an intact, CaM-free nNOSred construct has revealed key information about how certain control elements may synergistically repress electron transfer and NOS activity by locking the FMN binding domain in an electron-accepting position [48]. The position and conformation of the NOS-specific regulatory elements, including the AR insert within the FMN subdomain and the CT, were revealed [48] (Figure 3). Specifically, part of the AR insert forms a helix connecting the FMN and NADPH/FAD binding domains via a network of hydrogen bonds, and locks the FMN domain to the reductase complex. About half the AR is disordered in this structure. CT lies at the interface between the FMN and FAD subdomains, and its position indicates that the CT helix restrains the FMN module from moving away from the FNR module, thus stabilizing the FMN-shielded conformation. The terminal 16 residues of CT are not observable, suggesting flexibility in this region. Conformational diversity in NOSred is also evident from weakly defined electron density for parts of the connecting domain (CD), including the hinge linking the FMN domain to the CD, and the α -finger containing the CD2A regulatory element (which contributes to the observed cNOS Ca^{2+} /CaM dependence by interacting with the CaM-binding linker [77]). Rotational flexibility of the FMN domains is also observed [48].

1.4. Catalytically essential FMN–heme IET

The intersubunit 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 [39]. This IET step is also proposed to be important for reduction of the pterin radical following hydroxylation of L-Arg substrate [84]. However, the molecular mechanism underlying regulation of the FMN–heme IET remains unclear, which constitutes a critical barrier for understanding the CaM controlled NOS catalytic mechanisms. In collaboration with Prof. Gordon Tollin and Dr. Andrei Astashkin, we have recently developed an integrated approach (Figure 4) of laser flash photolysis [23, 31] (for determining the FMN–heme IET kinetics as a measure of the IET complex formation; a typical transient trace is shown in Figure 5), pulsed EPR (for determining the FMN–Fe distance in the interdomain FMN/heme complex [29], and probing structure of the heme active site [38]) and site-directed mutagenesis (for modifying the key interacting sites). All the data collectively can be used to directly test working model on critical structural determinants in modulating the FMN–heme IET in NOS (Table 1); a case study is given below. The technical details of this new approach have been discussed in recent reviews [63, 85]. The scope of this review is primarily on control mechanisms of the FMN–heme IET process. We will also discuss future directions addressing important mechanistic questions on formation of the NOS output state for NO production.

2. Mechanism of the FMN–heme IET in NOS

2.1. FMN-domain tethered shuttle model

A tethered shuttle model (Figure 6) was proposed by Ghosh and Salerno [86], and supported by IET kinetics [44, 87–95] and thermodynamic [96] studies. This model is similar to that of NADPH-cytochrome P450 reductase and P450 BM3, which came out earlier and derives from the crystal structures of P450 reductase [29, 97] and P450 BM3 [98]. The novel aspect of NOS is the involvement of CaM. Here CaM activates NO synthesis in eNOS and nNOS through a conformational change of the FMN domain from its shielded electron-accepting

(input) state to a new electron-donating (output) state [89, 94, 95, 99, 100]. In the two states, the FMN domain is engaged in distinctly different interdomain interactions, and the module swings back and forth to contact the FNR module and the NOS heme domain (Figure 6). The process involves a swinging mechanism in which the same interacting surface of the FMN domain accepts and provides electrons during catalysis [101]: after the FMN domain has moved away from the FNR unit, it is rotated so that the same face is able to interact with the electron transfer acceptor, heme domain (Figure 6). The movement of the FMN domain likely occurs by pivoting on a conserved Arg(NOS)/Glu47(CaM) pair [49] (which is predicted to regulate the electron transfer from FAD to FMN and from FMN to heme by adjusting the relative orientation and distance among the three cofactors). Indeed, four conformations in the crystal structure of the CaM-bound iNOS FMN domain indicate the FMN domain movements in electron transfer [49]. The tethered shuttle model (Figure 6) thus involves an input state corresponding to the existing intact reductase structure (Figure 3) [48] (*i.e.* docked FAD/FMN state), and an output state in which the FMN binding domain associates with the oxygenase domain (*i.e.* docked FMN/heme state). Free FMN domain conformations also exist in between these two docked states [102]. Based on the crystal structure of an intact reductase domain, a similar model was proposed by Garcin *et al.* [48].

Hypotheses have been proposed regarding the roles of redox state [44, 93] and NADPH binding/NADP⁺ release [103, 104] in regulation of the conformational change whereby the FMN domain switches from the FAD/NADPH domain docking to the heme domain docking. CaM binding to nNOS activates electron transfer to electron acceptors, which is caused by a conversion from a locked form of the enzyme to an open form, improving FMN accessibility [104]. NADPH binding only stabilized the input state conformation of NOSred, whereas CaM binding shifted the conformational equilibrium A toward the output state conformation [93]. Haque *et al.* recently suggested that the rates of interflavin electron transfer and FMN domain conformational motion are balanced so that both processes may limit electron flux through the enzymes [105]. Control of the flavin reduction is important because it ensures that the rate of heme reduction is sufficiently fast to enable NO synthesis by NOS.

The structure of the functional output state has not yet been determined. To favor observation of the output state, bi-domain NOS oxyFMN constructs, in which only the oxygenase and FMN domains along with the CaM binding region are expressed (Figure 1), were recently designed and constructed [44, 45, 106]. Biochemical, kinetics and spectroscopic results have shown that these homologous dimeric oxyFMN constructs are validated models of the NOS output state for NO production [45, 90, 91, 107]. One may argue that the results of the truncated oxyFMN system may not be fully applicable to the holo-NOS. Nonetheless, utilization of a similar bi-domain construct of P450 BM3 yielded useful results in understanding the full-length enzyme mechanism [98, 108].

The FMN–heme IET kinetics in a rat nNOS holoenzyme [89], and in murine and human iNOS holoenzymes [87] have been directly measured by laser flash photolysis. The IET kinetics for the NOS holoenzymes are approximately an order of magnitude slower than the corresponding NOS oxyFMN constructs. This suggests that in the holoenzyme the rate-limiting step in the IET reaction is the conversion of the input state to the output state (in which the FMN becomes accessible to the heme), and that the role of CaM is to allow this conversion to occur. The observed IET rate in the holoenzyme may thus be the same as the conformation conversion rate. Interestingly, the IET rate constant of eNOS holoenzyme is about ten times smaller than those of both nNOS and iNOS holoenzymes, and a slower conformational change is likely the cause of the slower IET in eNOS [109].

2.2. Conformational sampling mechanism for the FMN–heme IET

An appreciable decrease in the FMN–heme IET rate constant value of a human iNOS oxyFMN construct was observed with an increase in the solution viscosity [110]. The normalized values of the IET rate constants, determined in buffered mixtures of water with two different viscosogens, ethylene glycol and sucrose, fall on the same line when plotted versus the viscosity. These results showed that it is solution viscosity, and not other properties of the added viscosogens, that is responsible for the remarkable dependence of IET rates on the solvent concentration. The IET rate constant is inversely proportional to the viscosity for both viscosogens, which can be explained by the following mechanism:

The model shows two conformationally different precursors, being designated NR (for electron transfer non-reactive) and R (for electron transfer reactive), respectively. Based on steady-state approximation, one can derive eq 1 (assuming FMN_{hq} is FMNH[−]) or eq 2 (assuming FMN_{hq} is FMNH₂).

$$k_{\text{et(obsd)}} = (k_1 k_2 + k_1 k_{-2} + k_{-1} k_{-2}) / (k_{-1} + k_2 + k_{-2}) \quad \text{eq 1}$$

$$k_{\text{et(obsd)}} = (k_1 k_2 [\text{H}^+] + k_1 k_{-2} + k_{-1} k_{-2}) / (k_{-1} + k_2 [\text{H}^+] + k_{-2}) \quad \text{eq 2}$$

In either case, of the four microscopic rate constants, only k_1 and k_{-1} are expected to depend on viscosity, giving $1/k_{\text{et(obsd)}}$. Thus the model is in agreement with the experimental results. The IET kinetics and NOS flavin fluorescence results indicate that the observed IET rate is not due to the intrinsic electron transfer process, but rather is gated by a large conformational change [110].

The FMN–heme IET kinetics over the temperature range from 283 to 304 K in wild type (wt) full-length, wt oxyFMN [111] and E546N mutant oxyFMN [112] of human iNOS were determined. An appreciable increase in the rate constant value was observed with an increase in the temperature. The Eyring equation was used to analyze the temperature dependence data. The Eyring parameters and the IET rates at 21 °C are listed in Table 2. The obtained magnitude of activation entropy for the IET in the wt oxyFMN construct is only one-fifth of that for the wt holoenzyme. It has been shown that the NADPH/FAD domain has a much greater capability to interact with the FMN domain than does the oxygenase domain [44]. In the NOS holoenzyme, even in the presence of bound CaM, its dominant conformational state is thus in the docked FAD/FMN state (because of the stronger intra-domain FAD/FMN interactions) [113], which must undergo a transformation to an IET-active docked FMN/heme conformation before the FMN–heme IET can take place. On the other hand, the oxyFMN construct does not contain the NADPH/FAD domain, and is thus better poised for the FMN–heme IET (while in the E546N oxyFMN mutant, the docking process is perturbed). The substantially larger negative activation entropy of the holoenzyme is due to the fact that the FMN domain in the holoenzyme must “test/sample” more conformations than in the oxyFMN constructs prior to the IET. The entropy of the “free” state of the holoenzyme should be larger because there are a larger number of undocked

microscopic states (due to the larger size of the holoenzyme). It is also of note that analyses of the data with Marcus theory [114] and the redox potential data of the oxyFMN and full-length iNOS proteins [45] did not give unreasonable values of H_{AB} and IET distance [111]. Nonetheless, in combination of the viscosity dependence data [110], a gated-IET model represents a more reasonable explanation.

Interestingly, the order of the IET rate constant (wt oxyFMN > E546N oxyFMN > holoenzyme) is in agreement with the order of the magnitude of the activation entropy (Table 2). This can be explained by a conformational sampling model, in which sampling of a continuum of conformational states gives a range of transient donor-acceptor complexes, only a subset of which are IET-competent [44, 102, 115]. This presents an important mechanism for regulation in NOS enzyme, which avoids the necessity for tight binding of the FMN domain to achieve efficient IET. Interdomain FMN/heme interactions in the NOS enzymes are rather weak, and the docked IET complexes are short-lived [44, 110]. The observed rate of heme reduction for a population of NOS conformations is thus limited by the relatively infrequent formation of the docked IET-competent complexes. More conformations to sample (as in the holoenzyme and E546N mutant) give higher magnitude of activation entropy, resulting in less frequent formation of the FMN/heme complex and slower subsequent IET (Table 2). This supports a mechanism by which the FMN domain can modify the IET by altering probability of the docked state formation.

Three types of electron transfer (ET) mechanisms between proteins were proposed [116]: “true” electron transfer, coupled (the ET step is combined with a fast inter-conversion equilibrium in the protein), and gated (the ET step is combined with a slower and thus rate-limiting structural rearrangement). In the cases of true and coupled ET, the observed ET rates are expected to be independent of viscosity [117]. A recent electron transfer kinetics study at various temperatures suggested that the FMN–heme IET in nNOS holoenzyme may be coupled to the pre-requisite conformational movement of the FMN domain [118]. However, no viscosity effect data were reported in their work [118]. Detailed thermodynamic studies, coupled with structural and spectroscopic information, are required to further investigate the role of FMN domain conformational change in the FMN–heme IET process in NOS proteins.

2.3. CaM binding is a primary factor in controlling the IET

In the absence of CaM, no FMN–heme IET was observed in the laser flash photolysis experiments for the nNOS and eNOS holoenzymes [89, 109] and nNOS oxyFMN construct [90], showing that CaM is a primary factor in controlling the FMN–heme IET. The formation of the NOS output state involves two primary steps: (i) CaM-binding triggered dissociation of the FMN domain from the FNR domain, and (ii) subsequent docking of FMN to the heme domain [90] (Figure 6). Calcium-dependent CaM binding to eNOS/nNOS unlocks the input state, thereby enabling the FMN domain to shuttle between the FAD and heme domains [89, 90]. The NOS state formed at step (i) is competent to reduce cyt *c*, which requires accessibility of FMN to cyt *c*. This is a necessary but not a sufficient condition for NO production by eNOS/nNOS, which in addition requires additional CaM-dependent facilitation of alignment of the FMN and heme domains. This is strongly supported by the results of a recent study showing that NADPH reduction is three orders of magnitude faster than NO production, and cyt *c* reduction is intermediate between the two [119]. This can be explained by the small size of cyt *c* and the extra conformational constraints placed on the oxygenase/FMN complex by the dimeric structure of NOS and the connecting polypeptides linking the domains [87].

Previously, CaM control of NOS electron transfer was believed to be primarily localized within the reductase portion of the enzyme [120]. Evidence for multiple steps of CaM

control was attributed to separate CaM modulation of the FMN reduction and the heme reduction. Recent experiments have clearly demonstrated CaM dependence outside the reductase complex, *i.e.* CaM-dependent formation of the output state, through the FMN domain interacting with the heme domain [90]. An elegant approach was used to separately measure Ca^{2+} concentration required for CaM to bind and activate eNOS [121, 122]. Fluorescence and Förster resonance energy transfer were recently applied to monitor the orientation and conformation of CaM when bound to the NOS peptides and enzymes [123, 124]. Based on these kinetics and spectroscopic results, a sequential CaM binding model was proposed, in which CaM binds to the cNOS enzymes with the Ca^{2+} -replete C-lobe binding first followed by the Ca^{2+} -replete N-lobe [124, 125]. On the other hand, the iNOS isoform has a distinctly different mechanism: both the N- and C-terminal EF hand pairs bind to the iNOS peptide in a Ca^{2+} -independent manner, but only the C-terminal domain showed notable Ca^{2+} -dependent conformational changes when associated with the target sequence [123]. Interestingly, a recent cryo-electron micrograph study suggested a model in which CaM binding and docking position the reductase domains near the oxygenase domains and promote docking of the FMN-binding modules required for electron transfer [126].

Emerging evidence indicates that CaM is also important for proper alignment of the FMN and heme domains in iNOS [87, 119]. A recent IET kinetics study of a human iNOS oxyFMN protein co-expressed with the N-terminal Ca^{2+} -binding domains of CaM (residues 1–75) indicated that CaM, in particular its N-terminal half, is important in proper alignment of the FMN and heme domains in iNOS [127]. Another mutational and kinetics study indicated a new role of an isoform-specific NOS serine residue of the key CaM/FMN(NOS) bridging site in regulating the FMN–heme IET in iNOS [128].

2.4. Role of interdomain interactions

Biological ET systems depend on proper interactions between proteins/domains that shuttle electron(s) [115, 129–131]. Electrostatic interactions between protein/domain surfaces are important in molecular recognition, making the distribution of charged surface amino acids an active determinant of ET kinetics [130, 132]. Pronounced isoform-specific effects of salt concentration on NOS activities have been reported [133], suggesting importance of electrostatic interactions in the NOS electron transfer processes. The contacts between the heme and FMN domains of NOS is specific because if the FMN domain of nNOS is replaced by its counterpart from the cytochrome P450 reductase, the resultant chimeric enzymes no longer support electron transfer from FMN to heme, despite their similar ET functions [134]. What may be specific in the NOS FMN–heme interactions is complementary electrostatic surfaces but not just specific ion pairing. Indeed, recent work on rat nNOS mutants showed that heme reduction and NO synthesis in nNOS is facilitated by electrostatic interactions involving Lys423, Lys620, and Lys660, which form a triad of positive charges on the NOS heme domain surface [135]. The fact that the IET in a human iNOS E546N oxyFMN mutant is not completely suppressed also suggests that other residues participate in facilitating the interdomain FMN/heme alignment [112]. Interestingly, the same three charge-pairing residues on the FMN domain also govern electron flux through nNOSred by stabilizing its closed (FMN-shielded) conformational state and by retarding the rate of conformational switching between its open and closed conformations [136].

ET protein complexes are somewhat different from other types of protein-protein interactions. The ET complexes must form, pass electrons, and then split apart. The FMN/heme complex is transient, and very little complex is present at equilibrium. In other words, the FMN domain only docks to the heme domain as needed, and leaves after the IET is completed. This is supported by IET kinetics [110], fluorescence lifetime [102, 113], and stopped flow [44] results. For example, the observed monophasic kinetics in the viscosity experiment [110] indicate that the percentile of IET-competent docked FMN/heme

conformation is small because one would expect to see biphasic kinetics if the output state population is large (greater than 10 % of the population).

Besides electrostatic interactions, complementary hydrophobic interactions between aromatic amino acids may also be involved in the FMN-heme interdomain interactions. For example, steady state kinetic studies suggest that Trp421 in the oxygenase domain of rat nNOS may face towards the reductase domain, playing an important role in the IET [137].

2.5. Roles of other intrinsic elements

There are two tethers for the FMN domain motion in the tethered shuttle model (Figure 6): the hinge region, which is between the FMN and connecting domains (Figure 3), and the CaM binding region between the FMN and oxygenase domains. The native tethers should act in ways to give the FMN domain both an appropriate freedom (to move closer to the heme or FAD domain) while concurrently providing the necessary restriction to dock precisely. Indeed, the hinge connecting the FMN domain and the FNR unit was shown to control electron transfer and catalysis of nNOS and the native hinge yields a best balance among the FMN domain interactions and the electron transfer processes (to maximize NO production activity and minimize uncoupled NADPH consumption) [138]. The hinge region has been shown to constrain the activity of eNOS relative to other NOS enzymes [95]. Moreover, the fact that the hinge substitution did not completely convert eNOS to nNOS (in terms of the NO synthesis activity) implies that other structural elements of the eNOS reductase domain must also help restrict electron flux to the heme [95]. These results support a synergistic control mechanism of formation of the NOS output state through CaM binding and intrinsic control elements including the AR insert and CT.

The cNOS-unique AR insert within the FMN domain was originally identified from sequence alignments and modeling, and postulated to restrict alignment of the FMN binding domain with the oxygenase and/or FAD binding domains [75]. The AR insert exerts its regulatory function not only through competing with CaM binding (thus reducing the apparent affinity of the enzyme for CaM) [25], but also through stabilizing certain NOS state via interdomain interactions. The AR-deletion mutant of nNOS in the presence of CaM has only 30 – 50 % NO synthesis activity, indicating disruption of precise FMN/heme interdomain interactions in the mutants [76]. In other words, the AR insert is important for realignment of the FMN and heme domains. Indeed, our recent FMN–heme IET kinetics studies on the AR-deletion mutant of nNOS holoenzyme indicate a role of the AR insert in stabilizing interdomain FMN–heme interactions in the output state, rather than only playing the role of an inhibitory element [75]. In the crystal structure of the nNOS reductase domain [48], the AR insert is well positioned to interact with the oxygenase or FMN domain; 28 of the 42 residues in the AR region are not observable (Figure 3), suggesting flexibility of this region. Interestingly, the AR insert is shown to control the conformational equilibrium of the nNOS reductase domain [99]. This was originally proposed by Roman and Masters [139].

2.6. A case study- Regulatory role of human iNOS Glu546 in the FMN–heme IET

Our recent study of human iNOS oxyFMN E546N mutant [112] nicely demonstrated how one can employ the combined laser flash photolysis and pulsed EPR approach to directly examine role of specific residue in the FMN–heme IET (Figure 4). Glu546, located at the edge of the FMN domain surface of human iNOS, is a conserved charged residue, and is proposed to participate in the interdomain FMN/heme interactions [107, 140]. Extensive studies were also conducted on the analogous residue in nNOS [101, 141]. These two earlier papers showed that neutralization at the equivalent residue of rat nNOS (E762) induced the largest effect on heme reduction (decreasing the heme reduction rate by about 60 %) [101], and the slower electron transfer may be caused by a more positive FMN sq/hq midpoint

potential [141]. Their findings were used primarily to understand how the mutational effect increased the activity of the nNOS enzyme. However, the underlying mechanism for the slower electron transfer in the nNOS E762N mutant remained unclear.

We investigated the mechanistic role of the equivalent human iNOS E546 residue in modulating the FMN-heme IET rate, which in turn may provide insights about the interdomain FMN/heme alignment. Moreover, because the distance between redox centers is a key factor in controlling the electron transfer in proteins [142], pulsed EPR was employed to determine the FMN-Fe distance in the mutant [112]. The E546N mutation was found to retard the IET by significantly raising the activation entropic barrier (Table 2). Moreover, pulsed EPR data showed that the geometry of the docked FMN/heme complex in the mutant is basically the same as in the wt construct, whereas the probability of formation of such a complex is about twice lower [112]. The IET kinetics and pulsed EPR data showed conclusively that the E546N mutation retards the IET process by significantly raising the activation entropic barrier, without notably disturbing the FMN/heme alignment in the docked complex [112]. This work thus reveals an important mechanism by which the FMN subdomain (or, *vide infra*, other proteins) can modify the IET, by altering the number of conformations to sample. Now, we can work toward the next question that arises from this current work: what is the molecular mechanism by which the FMN domain motion influences NOS catalysis? This is an interesting question that should be addressed in future studies.

To investigate if the mutation at E546 affects the structural properties of the heme center, resonance Raman (rR) spectra of ferric and ferrous-CO derivatives of wt and E546N mutant proteins were also obtained [112]. The rR data show that the E546N mutation basically does not perturb the spectra. These results indicate that the docking of the FMN domain to the heme domain does not notably affect electronic and structural properties of the ground state of the heme active center. On the other hand, our previous data showed notable substrate-specific effects of the mutation in the FMN domain on the magnetic circular dichroism (MCD) spectra of the ferric centers in an adjacent domain [107, 140]. The apparent discrepancy between the MCD and rR results is due to the fact that the two techniques detect different structural variations at the NOS heme site: MCD probes the electronic transition from the ground to excited states, while resonance Raman provides information about the vibrational modes of the ground state. Interestingly, a recent kinetics study showed that a similar mutation at the equivalent FMN domain site of nNOS notably increases activity by enhancing ferrous heme-NO oxidation reactivity [141]. It is still unclear how the mutation in the FMN domain affects MCD spectroscopic properties and reactivity of the heme site in an adjacent domain. The mutation may lead to various changes (e.g., docking pathways, conformational equilibrium etc.). At the current stage, it is difficult to envisage how the local structural change (induced by the FMN domain mutation) may propagate down to the heme site. We emphasize, however, that the rR data not only probe the structure of the heme site, but the Raman data are also useful and significant in terms of directing future studies to address how the FMN domain may influence the NOS function. In addition, the data support the concept above that the mutation does not affect the docked structure, but only the population of the active conformation [112].

3. Perspective

Mechanistic roles of FMN/heme interdomain interaction and calmodulin binding, once fully understood, may serve as a basis for the rational design of new selective inhibitors of the NOS isoforms. A critical question in NOS catalysis mechanism is how interdomain electron transfer is coupled to substrate oxidation and NO production. This question has not been fully answered yet because of the large number of variables to deal with, such as domain

motions and multiple interdomain and interprotein interactions. To tackle this question, we and other investigators in the field have been employing a combined approach of rapid kinetics (laser flash photolysis and stopped flow), advanced spectroscopy (including pulsed EPR), freeze-quench, protein crystallography, and computational modeling. Although considerable progress has been obtained in recent years, some important questions on the CaM-controlled interdomain FMN/heme alignment remain unanswered, and certainly merit further investigation at the molecular level. These are summarized as follows.

1. The molecular mechanism, by which the interaction of CaM with the FMN and heme domains and the CaM-binding region activates the NO biosynthesis, is poorly understood. There is an urgent need to investigate the *dynamic* structural effects of CaM on the NOS enzyme in solution. Two aspects of the CaM-NOS interaction that are of fundamental interest are: how CaM (i) binds, and (ii) activates the three isoforms of NOS. The information about the geometry of the bound CaM (relative to the NOS domains) is essential for understanding details of NOS activation by CaM binding, but very little have been reported. The solution structure of CaM bound to the iNOS and eNOS CaM binding region peptides have been determined by NMR (to investigate the dynamic properties of CaM-NOS interactions) [143]. The size of the NOS holoenzymes (~120–160 kDa per subunit) precludes application of NMR spectroscopy with methods available to date. Nonetheless, such powerful techniques may be applicable to the labelled NOS/CaM samples in the future with the development of new NMR approaches.
2. The knowledge of the docking geometry of the oxygenase and reductase domains, including the distance and orientation between the redox centers, is required for better understanding the FMN–heme IET process and catalytic mechanism. The data will be complementary to the kinetics and functional studies. It will be also interesting to study other NOS forms/intermediates besides the [Fe(III)][FMNH[•]] form [144]. In particular, the pterin radical is a key player in NOS catalysis, and it is important to determine the distances between the radical and other cofactors (FMN, heme, FAD) in NOS. Here the generation and trap of the EPR-active intermediates in sufficient concentration and choice of appropriate pulsed EPR techniques are critical.
3. Compelling evidence showed that FMN domain docking to the heme domain modulates the reactivity [141] and structure [107, 140] of the heme active site. The key question here is how the docked FMN domain modulates the reactivity of heme active site in an adjacent domain. It is of note that ENDOR and ESEEM can probe 1st and 2nd sphere coordination environment of the heme site (type of magnetic nuclei, distance and direction) [145], and thus may shed light into how the FMN domain docking tunes the heme site structure, particularly of axial ligand (Table 1). It is important to note that the axial NO ligand is a useful ENDOR/ESEEM probe of the heme active site, particularly of the H-bonding network near the axial ligand. This is based on the facts that: (i) the unpaired electron primarily resides on the NO ligand, which enhances the pulsed EPR sensitivity to the molecular environment on the distal side of the heme, specifically, to the interactions with the substrate and water molecules; and (ii) H-bonding capability of NO is similar to that of the dioxygen ligand because both N and O can form H-bonds. Moreover, the NO complex has intrinsic biological significance in NOS catalysis [146]. Importantly, our recent ENDOR data of ferrous-NO samples of nNOS showed that in spite of its closer position, the guanidino nitrogen does not form a hydrogen bond with the NO ligand [145]. This is consistent with the previous reports that it is not the L-Arg substrate itself that would most likely serve as a direct proton donor to the diatomic ligands (NO and O₂) bound to the heme [106].

4. There is a clear correlation between the conformational equilibria (Figure 6) and overall NOS activity in the isoforms [100, 105]. A global catalytic model that highlights the role of NO as an intrinsic regulator was proposed by Stuehr and coworkers in 2001 [147]. The interplay of rates of three heme related processes (heme reduction, NO dissociation from the ferric-NO complex and oxidation of the ferrous-NO complex) determine isoform-specific steady state NO production activity, O₂ concentration response [146], and pulsatile NO generation [148]. It is time to further develop the global kinetics model to include the FMN domain conformational equilibria. The equilibrium A in Figure 6 (between the free and docked FAD/FMN states) can be probed by single turn-over stopped flow technique [44, 93]. However, measurement of the equilibrium between the free and docked FMN/heme states (Equilibrium B) remains elusive to date. Independent determination of the equilibrium is also necessary for interpretation of the pulsed EPR and IET kinetics data. A promising approach to probe the equilibrium is pulsed EPR techniques. Interestingly, ELDOR has been recently used to study di-radical form of P450 reductase, which shed new insight into energy landscapes for the flavin domain motion mediated IET [149].

4. Conclusions

Structurally, mammalian NOS protein contains the FMN and heme domains that are connected by a CaM-binding linker. The interdomain FMN/heme docking regulates the NOS enzyme function by controlling the physiologically essential FMN-heme IET and modulating reactivity and structure of the heme active site [85]. However, the molecular mechanism by which the FMN domain functions in the NOS catalysis is unclear. The structure of mammalian NOS holoenzyme has eluded investigators in the field and remains a significant and important challenge. In the absence of a structure of full-length NOS enzyme, a combined approach of spectroscopic, rapid kinetics and mutagenesis methods is required to unravel the molecular details of the interdomain FMN/heme interactions. 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 bioactivity. This is clearly a fruitful area for future studies. Such work will bring in important new insights on how the reactivity at the heme active site is modulated, which is an unresolved important question in NOS regulation mechanism.

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ABBREVIATIONS

NO	nitric oxide
NOS	nitric oxide synthase
CaM	calmodulin

eNOS	endothelial NOS
nNOS	neuronal NOS
cNOS	constitutive NOS
iNOS	inducible NOS
NOS_{oxy}	oxygenase domain of NOS
NOS_{red}	reductase domain of NOS
oxyFMN	bi-domain NOS construct in which only the oxygenase and FMN domains along with the CaM-binding region are present
FMNH[•]	FMN semiquinone
FMN_{hq}	FMN hydroquinone
FADH[•]	FAD semiquinone
FAD_{hq}	FAD hydroquinone
AR	cNOS unique autoregulatory insert in the FMN domain
CT	C-terminal tail
cyt	cytochrome
NOHA	N-hydroxy-L-arginine
H₄B	(6R)-5,6,7,8-tetrahydrobiopterin
dRF	5-deazariboflavin
IET	interdomain electron transfer
ET	electron transfer
FNR	ferredoxin-NADP ⁺ -reductase
wt	wild type protein
ENDOR	electron-nuclear double resonance
ESEEM	electron spin echo envelope modulation
RIDME	relaxation-induced dipolar modulation enhancement
ELDOR	electron-electron double resonance
rR	resonance Raman
MCD	magnetic circular dichroism

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Highlights

- Mammalian NOS enzyme is a flavo-hemoprotein, and its NO biosynthesis is tightly regulated under physiological conditions.
- It is of current interest to study how the interdomain electron transfer is coupled to NO production by NOS.
- The FMN-heme electron transfer is catalytically essential, but its control mechanism remains unclear.
- A combined spectroscopic and kinetics approach has been developed to tackle the key NOS problems.
- Advances in understanding of regulation mechanism of the FMN-heme interdomain electron transfer is reviewed.

Figure 1.

Schematic illustration of the full-length NOS protein showing its oxygenase and reductase domains, and CaM-binding region that joins the two domains. The FMN domain, connecting domain, FAD/NADPH domain, and a C-terminal tail (CT) in the reductase domain are depicted. The NOS enzyme is a modular protein, and homologous truncated NOS constructs have been utilized to study the catalytic mechanism. Two truncated NOS constructs are shown: (i) NOSoxy and (ii) bi-domain oxyFMN construct (which is a minimal electron transfer complex designed to favor the interactions between the FMN and heme domains [45]).

Figure 3.

Structure of an intact CaM-free rat nNOS reductase construct (PDB entry 1TLL) [48]; the ribbon diagram shows the FMN domain (yellow) docked onto the NADPH/FAD domain (blue) in the FMN-shielded conformation (input state). From the C to N terminus: C-terminal tail (CT, red), NADPH-FAD domain (blue), connecting domain (green) with a β -finger (cyan) and a flexible hinge (magenta), and FMN domain (yellow). Because of their highly flexible nature, part of the AR insert (gold) within the FMN domain and the hinge (magenta) regions were not well resolved in this structure. NADP⁺ (magenta) and cofactors FAD (light blue) and FMN (gold) are shown as stick representations. The graph was created using Discovery Studio Visualizer 3.5.

Figure 4. Combined approach of laser flash photolysis, pulsed EPR, and site-directed mutagenesis. This approach is designed to investigate molecular mechanism of NOS regulation by formation of the interdomain FMN/heme complex. Adapted from ref. [112] with permission.

Figure 5.

Transient traces at 460 nm obtained for the $[\text{Fe(II)-CO}][\text{FMNH}^*]$ form of nNOS holoenzyme flashed by a 446 nm laser. The graph is a combined plot of two individual traces at 0 – 0.2 s and 0 – 4 s using a logarithmic timescale. Solid lines correspond to the best single-exponential fit to the data in the different parts (the rate constants for the two phases are indicated): upon a laser excitation, the absorption at 460 nm decays below the pre-flash baseline (black solid line, 0 – 0.2 s), which is due to the FMN–heme IET resulting in heme re-oxidation, followed by a much slower recovery toward the baseline (grey solid line, 0.2 – 4 s), which is due to the CO rebinding to Fe(II). This is to show the spectral “transit n” *i.e.*, a reversal in direction of absorption changes over time in the traces. Such transition feature is a signature of successful observation of the FMN–heme IET process (*i.e.*, $[\text{Fe}^{\text{II}}][\text{FMNH}^*]$ $\rightarrow$ $[\text{Fe}^{\text{III}}][\text{FMN}_{\text{hq}}]$), and can only be detected in the transient traces at 580–600 nm (that is due to FMNH * reduction), and 465 or 460 nm (that is due to heme re-oxidation in iNOS and nNOS respectively), if the IET takes place. Anaerobic solution contained ~ 10 μM NOS protein, ~ 20 μM dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H $_4$ B, 1 mM Ca $^{2+}$ and 10 % glycerol).

Figure 6.

Tethered shuttle model. The FMN domain (grey) shuttles between the flavodoxin-NADPH reductase (FNR)-type two-domain reductase unit and the heme-containing oxygenase domain. CaM binding to eNOS/nNOS unlocks the FNR-FMN domain interacting state (*i.e.*, input state), thereby enabling the FMN domain to shuttle between the FAD and heme domains. The input state and FMN-heme domain interacting state (*i.e.*, output state) are well defined, while free FMN domain conformations also exist in between these two docked states. Equilibrium A of the FMN domain indicates the transition between its input state and conformations where the FMN domain is deshielded/free and available for interaction with electron acceptors cytochrome c (but not the heme domain). Equilibrium B indicates the change from the free conformations to the output state. Conformational equilibria A and B have their intrinsic set points and individual control in the NOS isoforms [93, 100, 136]. CaM is also required for proper alignment of the FMN and heme domains in the three NOS isoforms.

Scheme 1.
Production of NO by NOS enzyme.

Table 1

An integrated approach of pulsed EPR, laser flash photolysis and mutagenesis methods in studying the FMN-heme IET mechanism. Adapted from ref. [85].

Technique		Results	Insight from the data
Pulsed EPR ^a	RIDME/ELDOR	FMN-Fe distance, distance distribution and relative orientation of the paramagnetic FMN and heme centers in NOS	Structural information of the interdomain complex and a key constraint for choosing the computational IET complexes
	ENDOR/ESEEM	1 st and 2 nd sphere coordination environment of the heme site (type of magnetic nuclei, distance and direction)	Detailed structure of the heme active site structure, particularly of the axial ligand
Laser flash photolysis		Kinetics of the FMN-heme IET process	A measure of the interdomain FMN/heme complex formation
Site-directed mutagenesis		Mutation-dependent changes in the spectroscopic and IET kinetics properties	Roles of specific residues in regulating NOS function by the key interdomain interactions

^a Pulsed EPR techniques that can be used are: (i) relaxation-induced dipolar modulation enhancement (RIDME), (ii) electron-electron double resonance (ELDOR), (iii) electron-nuclear double resonance (ENDOR), and (iv) electron spin echo envelope modulation (ESEEM); they represent powerful tools for studying the structure and function of paramagnetic centers (such as metal ions) in biological systems [153].

Table 2

The FMN-heme IET rate k_{et} at 21 °C and Eyring parameters from temperature dependence analysis of the observed rates in wild type and E546N human iNOS oxyFMN proteins, along with those of wild type iNOS holoenzyme. Adapted from ref. [112].

	k_{et} (s ⁻¹)	ΔH (kJ/mol)	ΔS J/mol/K)
wild type human iNOS oxyFMN	343 ± 11	52.6 ± 3.7	-17.1 ± 1.2
E546N human iNOS oxyFMN	139 ± 3	45.7 ± 3.3	-47.0 ± 3.4
wild type human iNOS holoenzyme	34 ± 1	36.9 ± 1.6	-89.7 ± 4.5