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Regulation of Interdomain Electron Transfer in the NOS Output State for NO Production

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Abstract

There is still much that is unknown about how nitric oxide (NO) biosynthesis by NO synthase (NOS) isoform is tightly regulated at the molecular level. This is remarkable because impaired NO production *in vivo* has been implicated in an increasing number of diseases that currently lack effective treatments, including stroke and cancer. Given the significant public health burden of these diseases, the NOS enzyme family is a key target for development of new pharmaceuticals. Three NOS isoforms, inducible, endothelial and neuronal NOS (iNOS, eNOS and nNOS, respectively), achieve their key biological functions via intriguing regulations of interdomain electron transfer (IET) processes. Unlike iNOS, eNOS and nNOS isoforms are controlled by calmodulin (CaM) through facilitating catalytically significant IET processes. It is proposed that CaM activates NO synthesis in eNOS and nNOS through a conformational change of the flavin mononucleotide (FMN) domain from its shielded electron-accepting (input) state to a new electron-donating (output) state. The FMN–heme IET within the NOS output state is essential for NO synthesis at the catalytic heme. Due to lack of reliable techniques for specifically determining the inter-domain FMN–heme interactions and their direct effects on the catalytic heme center, the molecular mechanism that underlies the output state formation remains elusive. The recent developments in our understanding of mechanisms of the NOS output state formation that are driven by a combination of molecular biology, laser flash photolysis, and spectroscopic techniques are the subject of this perspective.

Nitric oxide synthase in disease biology and treatment

NOS is the enzyme responsible for the oxidation of L-arginine (Arg) to NO, a signal for vasodilatation and neurotransmission at low concentrations and a defensive cytotoxin at higher concentrations ^{1, 2}. NO's availability is tightly regulated at the synthesis level by NOS. Aberrant NO synthesis by NOS is associated with an increasing number of human pathologies, including stroke, inflammation, arthritis, and cancer ^{2, 3}. Selective NOS modulators are required for therapeutic intervention because of the ubiquitous nature of NO in mammalian physiology, and the fact that multiple NOS isoforms are each capable of producing NO *in vivo*. Three NOS isoforms function differently in human health and disease. For example, in acute ischemic stroke, NO produced by the neuronal NOS or inducible NOS (nNOS or iNOS) is detrimental, whereas NO derived from the endothelial NOS (eNOS) and the accompanying dilation are beneficial ⁴. Previous advances in

understanding the structural and functional mechanisms of this enzyme class led to identification of agents that are designed to modulate the various NOS enzymes⁵. However, agents that selectively modulate NOS isoform activity remain elusive for clinical usages because of incomplete understanding of NOS regulation at the molecular level. Clearly, molecular mechanisms of NOS regulation, once fully understood, are potentially key targets for development of selective new pharmaceuticals for treating a wide range of diseases that currently lack effective treatments. For example, an exogenous synthetic peptide was designed based upon an internal fragment of eNOS proteins important in controlling electron transfer processes in NOS⁶. This peptide enhances pulmonary artery vasorelaxation through directly activating NOS activity, and thus has significant potential therapeutic applications in the regulation of pulmonary vascular function.

NOS enzymology

There are three mammalian NOS isoforms: eNOS, nNOS, and iNOS. Mammalian NOS is a homodimeric flavo-hemoprotein that catalyzes the oxidation of L-Arg to NO and L-citrulline with NADPH and O₂ as co-substrates (Scheme 1)⁷.

Each subunit contains a C-terminal electron-supplying reductase domain with binding sites for NADPH (the electron source), flavin adenine dinucleotide (FAD) and FMN, and an N-terminal catalytic heme-containing oxygenase domain. The oxygenase and reductase domains are connected through a CaM-binding region, which is irreversibly bound to CaM in iNOS at all physiological Ca²⁺ concentrations, whereas reversible CaM binding to the region of nNOS and eNOS requires an increase in intracellular Ca²⁺⁸. The major difference between the CaM-regulated isoforms of nNOS/eNOS and the Ca²⁺-insensitive isoform iNOS is the presence of intrinsic control elements⁸, including a unique autoregulatory (AR) insert within the FMN domain of eNOS/nNOS⁹ and the C-terminal tail¹⁰.

There are crystal structures available for the oxygenase domains of each of the NOS isoforms^{11–14}, the reductase constructs^{15, 16} and NOS homologues¹⁷, as well as CaM bound to peptides corresponding to the CaM-binding sequence in human eNOS¹⁸ and nNOS (PDB 2O60). The substrate, L-arginine, and a cofactor, (6R)-5,6,7,8 tetrahydrobiopterin, both bind near the heme center in the oxygenase domain^{11–13}. These crystal structures provide important information on the mechanism for controlling NO synthesis by NOS. Based on the crystal structures, a combination of kinetic methods and redox titrations, along with site-directed mutagenesis, have provided useful information about the roles of specific NOS amino acid residues within the reductase domain^{19–24}, oxygenase domain^{25, 26}, and CaM-binding region²⁷ in modulating NOS function.

However, no structures of the complete NOS holoenzyme are available; this is presumably due to dynamic structural rearrangements that occur within the reductase domain and CaM-binding domains of the NOS enzymes (see below). As a consequence, the structural basis for the assembly of NOS domains and CaM during catalysis remains unknown. It is thus of current interest to obtain crystal structures of CaM-bound NOS constructs/domains.

Interdomain electron transfer processes controlled by CaM binding are key steps in eNOS/nNOS catalysis

Although knowledge of the NOS catalytic mechanism is incomplete^{28–32}, it is well established that interdomain electron transfer (IET) processes are key steps in NO synthesis by coupling reactions between domains (Fig. 1)^{7, 33, 34}. Unlike iNOS, eNOS and nNOS synthesize NO in a Ca²⁺/CaM dependent manner. Here CaM-binding facilitates the IET reaction from the FAD hydroquinone (FADH₂) to FMN semiquinone (FMNH[•]) within

subunit A (reaction 1)³⁵, and enables IET from the FMN hydroquinone (FMNH₂) to the catalytic heme iron in the oxygenase domain of subunit B (reaction 2)³⁶. It is generally accepted that CaM binding has little or no effect on the thermodynamics of redox processes in NOS^{37–39}, which indicates that the regulation by CaM is accomplished dynamically by controlling redox-linked conformational changes required for effective IET.

Importantly, the CaM-controlled FMN–heme IET (reaction 2, Fig. 1) is essential in coupling electron transfer in the reductase domain with NO synthesis in the oxygenase domain by the delivery of electrons required for O₂ activation in the catalytic heme domain, and is thus under extraordinarily tight control.

Intrinsic control elements contribute to the CaM dependence of IET

Control of the IET processes in eNOS/nNOS by CaM has been shown to involve intrinsic control elements in NOS: the CaM binding site, the AR in the FMN binding domain, and the C-terminal tail, which regulate the IET in a concerted manner^{9, 10, 40–42}. A unique AR (~40 amino acids) within the FMN domain of eNOS/nNOS^{9, 40} pins the FMN domain to the reductase complex via a network of hydrogen bonds in the CaM-free state¹⁶. Electron transfer modulation appears also to involve a much smaller insertion present in the hinge subdomain⁴³ as well as C-terminal tail differences¹⁰. Control element deletion studies indicate that the control elements repress electron flow into and out of the NOS reductase domain in the CaM-free state, and CaM-binding relieves the repression^{10, 42, 44–47}. A recent crystal structure of an intact nNOS reductase domain has revealed key information about how the control elements may repress electron transfer in the CaM-free state (see below)¹⁶.

Tethered shuttle model and the NOS output state

An “FMN-domain tethered shuttle” model was originally proposed by Salerno and Ghosh^{48, 49}. This model involves the swinging of the FMN domain from its original electron-accepting (input) state to a new electron-donating (output) state (Fig. 2). This molecular rearrangement facilitates efficient IET between the FMN and the catalytic heme in the oxygenase domain (reaction 2 in Fig. 1). CaM binding unlocks the input state, thereby enabling the FMN domain to shuttle between the two enzyme states. The tethered shuttle model thus involves an input state that corresponds to the structure of a nNOS reductase domain in the CaM-free state (Fig. 3, see below)¹⁶, and an output state in which the FMN binding domain associates with the oxygenase domain.

Crystal structure studies on an intact nNOS reductase domain construct in the CaM-free state¹⁶ reveal a structure in which FMN is in close proximity with FAD, as required for electron transfer in the input state of FMN from NADPH through FAD (Fig. 3). This structure is not as it exists in the output state; FMN cannot be located to within tunneling distance of the heme, and thus electrons cannot be transferred to the heme in this state. This constrained state of FMN is locked through a concerted interplay of control elements such as the C-terminal tail and the AR (Fig. 3)^{16, 50}.

In order for electrons to be transferred from the FMN to heme for activating NO production, the FMN domain must be unlocked via CaM binding, and must move to another position such that it is accessible to the heme. The formation of the output state involves two primary steps: (1) dissociation of the FMN domain from its reductase binding site, triggered by CaM binding, and (2) the subsequent re-association of FMN with the oxygenase domain (Fig. 2). Mechanistic information suggests that the rate-limiting step in NOS catalysis is the formation of the output state, because NADPH reduction is 3 orders of magnitude faster than NO production, and cytochrome (cyt) *c* reduction is intermediate between the two⁵¹. The

NOS state formed at step (1) is competent to reduce cyt *c*, which requires accessibility of FMN to cyt *c*. This is necessary but not sufficient for NO production by eNOS/nNOS, which in addition requires CaM dependent promotion of the oxygenase-FMN domain interactions (step 2).

The structure of the functional output state has not yet been determined. This makes studies that focus on crucial interaction sites in regulating the formation of the output state very important. A promising strategy is to use a combined kinetics (in particular kinetics of discrete IET steps), spectroscopic and site-directed mutagenesis approach to probe the interdomain FMN-heme interactions within the NOS output state.

Our laboratories have been using laser flash photolysis to determine the kinetics of the FMN-heme IET in the output state for NO production. In this Perspective, we will first briefly review the laser flash photolysis approach that has allowed the attainment of new levels of insight into the IET between the FMN and Fe centers in NOS enzymes. We will review IET studies on NOS bi-domain oxygenase/FMN constructs that are validated models of the NOS output state. The IET kinetic studies on full-length NOS enzymes and the roles of control elements on the FMN-heme IET in the nNOS holoenzyme will also be discussed. Finally, we will discuss recent kinetics and thermodynamics studies by other researchers that are closely relevant to the NOS output state of the tethered shuttle mechanism.

Laser flash photolysis for directly determining the FMN-heme IET kinetics in NOS

Laser flash photolysis is a powerful technique to study rapid IET kinetics in proteins^{52, 53}. We have successfully developed a new CO photolysis approach (Scheme 2) to directly measure the FMN-heme IET in the NOS enzymes^{49, 54–57}.

Scheme 2 summarizes the processes that take place after photolysis of the Fe(II)–CO complex in a partially reduced form (*i.e.* [Fe(II)–CO][FMNH[•]]) of the nNOS isoform. In the Scheme, species in dashed boxes are CO-bound forms, whereas those in solid boxes are CO-free and participate in the FMN-heme IET. The laser-induced CO dissociation results in a drop of the midpoint potential of the heme and rapidly converts a good electron acceptor (the Fe(II)–CO complex) into an electron donor (the free Fe(II) species), favoring the IET from Fe(II) to FMNH[•]. In the absence of added CaM, CO rebinding to Fe(II) (reaction 2) competes well with the slow IET between the heme and FMN domains (reaction 3), whereas in the presence of CaM, CO rebinding is a poor competitor for efficient IET (reaction 4). A CO/Ar mixture is used to retard the CO rebinding rate (reaction 2), and thus favor the IET from Fe(II) to FMNH[•] in the CaM-bound nNOS (reaction 4). This makes the loss of FMNH[•] observable as a bleaching at 580–600 nm (Fig. 4). Briefly, the NOS solution in the presence of deazariboflavin is illuminated for an appropriate period of time (~90 seconds) to obtain [Fe(II)–CO][FMNH[•]], and the partially reduced protein is subsequently flashed with 450 nm laser excitation to dissociate CO from Fe(II)–CO, and generate a transient Fe(II) species that is able to transfer one electron to the FMNH[•] intramolecularly to produce FMNH₂ and Fe(III).

It is important to note that we can individually time-resolve the consumption of FMNH[•] (due to the IET to Fe(II)) and CO rebinding, and thereby reliably measure the rapid FMN-heme IET (Fig. 4) followed by a much slower CO rebinding process (Fig. 5). Heme reduction in the NOS isoforms was indirectly probed by formation of the Fe(II)–CO complex using a stopped-flow approach, which is unable to distinguish these two reactions as only formation of the Fe(II)–CO complex was observed^{36, 58}. Thus our CO photolysis approach offers clear advantages since it allows us to observe both processes directly. Most importantly, our recent results⁵⁵ demonstrate that the FMN-heme IET kinetics can be used as a direct measure for formation of the NOS output state, and the CO photolysis method is

thus a powerful approach in studying the roles of specific regions in formation of the output state (see below).

Our experiments do not involve any step other than the IET between the FMN and heme domains, and therefore provide the first direct determination of the kinetics of the following IET process between catalytically significant redox couples of the FMN and heme centers (eq 1):

Note that the FMN–heme IET is reversible because the redox couples of Fe(III)/Fe(II) and FMNH₂/FMNH[•] are nearly iso-potential⁵⁹, and therefore the IET rate constant of the backward reaction (*i.e.* heme reduction) is about one-half of the observed rate constant, which is the sum of the forward and backward rate constants.

We have also conducted similar CO photolysis experiments on the NOS oxygenase construct, which does not contain FMN. Therefore by carefully comparing the absorption changes of NOS oxyFMN and oxygenase constructs occurring upon a 450 nm laser pulse, we can distinguish the process of FMN–heme IET from rebinding of CO to Fe(II). For example, in Fig. 4, the 600 nm trace of nNOS oxyFMN is a bleaching (*i.e.* loss of absorbance), whereas the trace of nNOS oxygenase stays above the baseline. This comparison gives us definite evidence that the bleaching is due to consumption of FMNH[•] triggered by formation of free Fe(II), *i.e.* the FMN–heme IET (eq 1).

Another important control experiment is that we always collect traces with different amplitude of signals, *i.e.* at different intermediate protein concentrations generated by the flash. We thus confirm that this process is protein concentration independent, which indicates an intra-protein process. This is important because other inter-protein processes may confound the absorbance changes, and if so, the observed rate would depend upon protein concentration. Therefore, the kinetics must be determined at various protein concentrations to be sure that we are studying the intra-protein IET.

NOS oxygenase/FMN domain construct as a model of the output state

To favor observation of the output state of the shuttle mechanism, Ghosh and Salerno designed two-domain NOS oxyFMN constructs in which only the oxygenase and FMN domains along with the CaM-binding region are expressed (Fig. 6) to favor the interaction of the FMN binding domain with the oxygenase domain over interactions within the reductase unit⁵⁹. The absence of the NADPH-FAD binding domain removes the dominant input state complex from the conformational repertoire of the construct. This provides us with a greatly enhanced opportunity to observe the putative output state, in which the FMN binding site is closely associated with the oxygenase active site rather than with the NADPH-FAD binding domain. The homologous dimeric oxyFMN construct is active and stable, binds cofactors nearly stoichiometrically, and has characteristic absorption and EPR spectra similar to the holoenzyme⁵⁹. Potentiometric titration studies have shown that the redox potentials of the oxyFMN construct are comparable to the holoenzyme⁵⁹. Therefore the truncated construct appears to be a reasonable approximation of the holoenzyme. Our IET kinetics data have further demonstrated that these oxyFMN constructs are well-validated models of the NOS output state (see below).

The kinetics of FMN–heme IET in a rat nNOS oxyFMN construct in the presence and absence of added CaM⁴⁹, in a murine iNOS oxyFMN⁵⁷ and in a human iNOS oxyFMN⁵⁴ were directly determined using laser flash photolysis of CO dissociation in comparative

studies on partially reduced oxyFMN and single domain heme-containing oxygenase constructs. For nNOS oxyFMN, the IET rate constant in the presence of CaM (262 s^{-1}) was increased approximately ten-fold compared to that in the absence of CaM (22 s^{-1})⁴⁹. We believe that the slow but still substantial rate of IET in the absence of CaM is real and may reflect the different conformations of the CaM-bound and CaM-free proteins.

The effect of CaM on inter-domain interactions was further evidenced by EPR spectra; upon adding CaM, changes in the line shape and g values of the high spin heme EPR signal have been observed⁴⁹. This distortion is probably due to the CaM-driven association of the FMN and heme domains in the nNOS oxyFMN construct. Such distortion effects have not yet been reported in the holoenzyme, probably because most of the holoenzyme is in the input state. Collectively, these kinetic and EPR results provide the first direct evidence of CaM control of electron transfer between FMN and heme domains through facilitation of the interdomain FMN–heme interactions in the output state. Therefore, CaM controls IET between heme and FMN domains by a conformational gated mechanism. This is essential in coupling electron transfer in the reductase domain in NOS with NO synthesis in the oxygenase domain.

Previously, CaM control of NOS electron transfer was believed to be primarily localized within the reductase portion of the enzyme⁶⁰. Evidence for multiple steps of CaM control was attributed to separate CaM modulation of FMN reduction and heme reduction (32, 33). Our recent experiments with the oxyFMN constructs 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 in the oxygenase domain⁴⁹. This may be the original role of CaM in the evolution of NOS proteins, and could predate the evolution of the modern control elements.

IET kinetics measured utilizing full-length NOS proteins support the tethered shuttle model

We have further extended our CO photolysis approach to measure the FMN–heme IET kinetics in a rat nNOS holoenzyme⁵⁶, and in murine and human iNOS holoenzymes⁵⁴. The IET kinetics for the NOS holoenzymes are approximately an order of magnitude slower than the corresponding NOS oxyFMN constructs. This fact suggests that in the holoenzyme the rate-limiting step in the IET reaction is the conversion of the input state to the output state (*i.e.*, the first step in the following scheme, in which the FMN becomes accessible to the heme), and that the role of CaM is to allow this conversion to occur. The IET reaction scheme in the holoenzyme can thus be represented as follows:

where sq represents “semiquinone”, hq represents “hydroquinone”, red represents “reduced”, and ox represents “oxidized”.

Note that in the iNOS oxyFMN and nNOS oxyFMN (with added CaM) constructs, the IET rate constants are much larger and not equal in the oxyFMN constructs, although they are similar in order of magnitude (850 ± 50 , 320 ± 45 and $262\pm 40\text{ s}^{-1}$, for oxyFMN constructs of murine iNOS⁵⁷, human iNOS, and rat nNOS⁵⁶, respectively). Therefore the rate-limiting factor in the nNOS and iNOS holoenzyme is not the FMN–heme IET *per se*. As a preliminary interpretation, we propose that at least some of the interactions that constrain the holoenzyme to be in the input state are lost in these oxyFMN constructs, making it easier for the truncated protein to achieve the output state, thereby increasing the IET rate constant. Structural studies will be required in order to better understand this difference.

The fact that there is no rapid IET component in the traces obtained with the iNOS holoenzyme implies that the holoenzyme is mainly in the input state, despite having CaM bound to it; the iNOS holoenzyme is still constrained by the contacts that exist which control the rate constant for the IET ⁵⁴.

CaM unlocks the input state, thereby enabling the FMN domain to shuttle between the two enzyme states, and thus make contacts with the heme domain (Fig. 2); the CaM effect is primarily kinetic, although there may be a thermodynamic component as well ⁶¹. CaM does not affect the kinetics of the rate-limiting step (*i.e.* conversion of the input state to output state, see above); the evidence for this is that the IET rate constant for the holoenzyme is smaller than that for the oxyFMN construct. Thus in the holoenzyme, the IET rate constant is still controlled by the conversion step, even when CaM is present. The IET in the oxyFMN constructs are faster because it has lost contacts with the FAD domain that constrain this motion.

It is interesting that the reduction of oxygenase heme and of cyt *c* both require release of the FMN domain from the input state, but that V_{max} for cyt *c* reduction ¹⁰ is an order of magnitude faster than the reduction of heme measured by laser flash photolysis. For cyt *c* reduction one can postulate a reaction first order in ferric cyt *c* and in accessible FMN domains; the rate of formation of the complex with cyt *c* is then the second order rate constant k_{cf} (cyt *c* complex formation) times the concentration of enzyme molecules with accessible FMN binding domains. The rapid rate of cyt *c* reduction could be accounted for by a large value of k_{cf} , but it is more informative to consider the 'free' FMN binding domain as partitioning into a range of conformational states. If a much large number of these states are accessible to cyt *c* than to the oxygenase domain, one would expect much more rapid reduction of cyt *c* under saturated cyt *c* concentration. This is a reasonable expectation given the small size of cyt *c* and the conformational constraints placed on the oxygenase/FMN complex by the dimeric structure of NOS and the connecting polypeptides linking the domains.

Deletion of the AR insert modulates the FMN–heme IET in rat nNOS holoenzyme

Increasing evidence shows that the AR exerts its regulatory function by stabilizing certain NOS states via interdomain interactions. Identified originally from sequence alignments and modeling and postulated to restrict alignment of the FMN binding domain with the oxygenase and/or FAD binding domains ⁹, this AR insert within the FMN domain, in the absence of CaM, locks the FMN binding domain to the reductase complex via a network of hydrogen bonds so as to obstruct CaM binding and enzyme activation (Fig. 3) ¹⁶. When CaM binds to the linker between the FMN and oxygenase domains at high $[Ca^{2+}]$, the AR insert is proposed to be displaced so that the enzyme can be activated. Evidence for a conformational change in this AR region upon CaM binding came from both proteolysis and fluorescence experiments ⁹. Importantly, recent studies by Roman and Masters suggest that the AR insert may also be involved in stabilizing the output state of the FMN domain for NO synthesis in the presence of CaM ²³. Indeed, in the crystal structure of the nNOS reductase ¹⁶, the AR insertion is well positioned to interact with the oxygenase or FMN domain; 28 of the 42 residues in the AR insert are not observable, suggesting flexibility of the AR insert.

We have conducted comparative CO photolysis kinetic studies on wild type and the AR insert-deletion mutant of full-length rat nNOS proteins, to directly investigate the role of the unique AR insert in the FMN–heme IET ⁵⁵. Although the amplitude of the IET kinetic traces was decreased two- to three-fold, the AR deletion did not change the rate constant for the calmodulin-controlled IET. This suggests that the rate-limiting conversion of the electron-accepting state to a new electron-donating (output) state does not involve interactions with

the AR insert, but that the AR may stabilize the output state once it is formed. Our results indicate an important role of the AR insert in stabilizing interdomain FMN–heme interactions in the output state, rather than simply playing the role of an inhibitory element⁹. This provides new information on the role of the AR insert in regulating electron transfer in the nNOS isoform, and further demonstrates that the IET kinetics measured by our CO photolysis approach can be used as a direct measure for the output state formation in full-length NOS proteins, *i.e.* the efficiency of the FMN domain docking onto the heme domain for effective IET.

In the iNOS holoenzyme the rate constant for the IET between heme and FMN is indistinguishable from our previously reported rate for CaM activated nNOS holoenzyme⁵⁶. This is a remarkable observation considering that in the nNOS holoenzyme the IET in the absence of CaM is too slow to measure⁵⁶. Control of the IET processes in eNOS/nNOS by CaM has been shown to mainly involve the CaM binding site⁶², and the unique AR insert within the FMN binding domain^{9, 40}. The AR insert within the FMN domain, in the absence of CaM, locks the FMN binding domain to the reductase complex via a network of hydrogen bonds so as to obstruct CaM binding and enzyme activation¹⁶. When CaM binds to the linker between the FMN and oxygenase domains at high $[Ca^{2+}]$, the AR insert is proposed to be displaced so that the enzyme can be activated. Indeed, our results suggest that in the nNOS holoenzyme CaM activation effectively removes the restraints imposed by the nNOS unique AR insert on the release of the FMN binding domain, at least in single turnover.

Kinetic studies by others support the tethered shuttle model

The tethered shuttle model is also strongly supported by recent kinetic studies by other researchers^{19, 63–66}. Interdomain FAD/FMN interactions have been shown to be also important in modulating electron transfer from FAD to the heme via FMN motions^{19, 66}. Moreover, it has been suggested that regulation of the FMN conformational equilibrium differs markedly in reductase domain constructs of eNOS and nNOS, and this difference can explain the lower electron transfer activity of the eNOS reductase domain construct⁶⁴. Interestingly, a single mutation in the FMN-binding loop of nNOS leads to a FMN conformation that resembles the output state of the CaM-bound nNOS with respect to the cyt *c* reduction activity²¹.

Another recent interesting study showed that the hinge region, which tethers the FMN domain to the connecting domain between the FMN and FAD domains, is important in restricting the activity of eNOS relative to other NOS enzymes.⁶⁵ This information strongly supports the tethered shuttle model in which FMN motion controlled by CaM binding is critical in regulating NO production in NOS isoforms. More importantly, the fact that the hinge substitution did not completely convert eNOS to nNOS (regarding the NO synthesis activity) implies that other structural elements of the eNOS reductase domain must also help restrict electron flux to the heme.⁶⁵ These results support a synergistic control mechanism of formation of the NOS output state through CaM binding, and intrinsic control elements such as the AR insert, and C-terminal tail.

Perspectives

The aim of the present review is to highlight current mechanistic information about the NOS output state. It has become clear from these studies that our understanding of NOS enzymes will be moved forward by increasing applications of a combined approach of molecular biology, rapid kinetics (laser flash photolysis and stopped flow), redox titrations, advanced spectroscopy, protein crystallography, and computational modeling. This is clearly a fertile area for future study. Although considerable progress has been obtained, some outstanding

questions remain unanswered, and certainly deserve further investigation at the molecular level. These can be summarized as follows.

1. Due to lack of a crystal structure of the FMN–heme complex, there is a clear need to probe the effects of FMN–heme interactions at the molecular level using alternative methods. EPR studies have suggested the presence of magnetic interactions between the paramagnetic centers of the FMN semiquinone radical and heme iron in NOS holoenzymes^{67–69}. The narrow line width of the NOS flavin radical signal suggests that the radical is spin-spin coupled with the heme iron in the purified nNOS holoenzyme⁶⁷. This work was further supported by progressive microwave power saturation and EPR saturation recovery studies, and the data indicated that the flavin and heme centers are positioned near each other in nNOS, consistent with their participating in an IET⁶⁸. An independent EPR study of an eNOS holoenzyme also showed that the FMN radical possesses efficient electron spin relaxation as a consequence of dipolar interaction with the heme center⁶⁹. However, another EPR study on an nNOS holoenzyme argued that the flavin and heme centers are not magnetically coupled⁷⁰. These paramagnetic resonance studies strongly indicate the need to develop new and complementary spectroscopic approaches aimed at determining the nature of interdomain FMN–heme interactions with molecular-level resolution. Interestingly, a recent low-temperature MCD study by us provides the first direct paramagnetic spectroscopic evidence to indicate that the docked FMN domain affects the nature of interactions between the L-Arg substrate and the catalytic heme center located in an adjacent domain in iNOS.⁷¹ This work has shown that a combination of low-temperature MCD and EPR spectroscopies can serve as a promising site-selective probe of key interdomain FMN–heme interactions that modulate the formation of the NOS output state.
2. Currently, only the oxygenase and reductase domain structures, *per se*, are available. It is unclear when the holoenzyme structure will emerge and whether it will be in complex with CaM. An added complication is the fact that the NOS reductase crystallizes as a head-to-head dimer¹⁶. Trying to piece together (*in silico*) the heme and reductase domain structures can provide some insights, but alternative models will be difficult to distinguish when compared to the favored ones. Therefore, the best approach is to identify a method that can also provide distance constraints within the FMN–heme IET complex. In particular, pulsed EPR such as ELDOR⁷² is a promising approach towards measuring the precise distance between the heme iron and FMN centers, and this approach has the potential to take us to the next level in attempting to understand the nature of the IET complex before the crystallographic results are in place.
3. Emerging evidence indicates the importance of appropriate docking of the FMN domain in efficient IET. Efforts to carry out extensive mutagenesis studies on conserved surface residues in NOS enzymes, especially of the strictly conserved charged residues near the heme and FMN centers in human NOS enzymes, should prove fruitful. Further laser flash photolysis and spectroscopic studies of such mutants should provide additional insights into the role of surface charge in the docking of the FMN domain and IET in NOS. A potential additional strategy would be to label the domain(s) with fluorescence probe(s), and then examine the interdomain interaction with the fluorescence resonance energy transfer technique.
4. A wider variety of experiments need to be proposed that would begin to provide new insights as to the underlying mechanism by which association of CaM with the AR region promotes interactions between the FMN and heme domains. For example, one can ask: what conformational space is explored by the AR region and

how is this changed by CaM binding; also how is it that CaM binds, and how does it do so without sterically blocking the interaction between the FMN and heme domains? These are challenging questions to address, and require more structural information than is presently available. It is certainly important to expand our study in future experiments to include the dynamic role of the AR in CaM controlled NOS function.

5. There are two tethers for the FMN domain motion in the tethered shuttle model (Fig. 2): the hinge region, which is between the FMN and connecting domains (Fig. 3), and the CaM binding region between the FMN and oxygenase domains. An important question is how the tethers act in ways to give the FMN domain both an appropriate freedom (to move closer to the heme or FAD domain) while simultaneously providing the necessary restriction to dock precisely. It will be interesting to study the roles of the tethers in regulating formation of the NOS output state and NOS catalysis, by either shortening their length or by altering their flexibility via mutagenesis.
6. Emerging evidence indicates that the requirement of CaM for iNOS is associated with proper alignment of the FMN and heme domains in the output state⁵¹. Importantly, our recent kinetic studies implied that the iNOS holoenzyme remains mainly in its input state, and that the basic control mechanisms acting in the NOS isoforms are similar⁵⁴. A fluorescence resonance energy transfer study demonstrated that CaM does not bind to iNOS in a sequential manner as proposed for nNOS⁷³. The mechanism of CaM binding and activation to the iNOS enzyme is poorly understood, and it is of significant importance to investigate the detailed molecular mechanism of CaM-dependent promotion of the FMN–heme interactions in iNOS, in order to understand how CaM specifically regulates NOS isoforms by key CaM/NOS interactions. It is exceedingly difficult to study CaM/iNOS interactions because CaM binds to iNOS very tightly, and iNOS protein needs to be co-expressed with wild type CaM because of its propensity to aggregate when residues of the highly hydrophobic CaM-binding domain are exposed to an aqueous environment⁷⁴. An attractive strategy is to use a “CaM-free” iNOS⁷⁴ along with CaM mutants.
7. Since the IET reaction is an equilibrium process (eq 1), we are in fact measuring it in both directions; the CO photolysis technique triggers the IET process in the reverse direction of the enzymatic turnover. The steady state rate of reduction of ferriheme is unlikely to correspond exactly to the single turnover rate measured by CO photolysis. More precisely, the steady state rate of heme reduction will be determined by the interaction of multiple processes in the catalytic cycle of the reductase unit. The single turnover IET rates measured in our experiments represent important features of this cycle and provide upper limits of the steady state rates, but in addition assessment of the steady state rates require partitioning of the system (*e.g.*, by King-Altman kinetics). Understanding the kinetics and control of NOS will require an assessment of the interaction of kinetics models of the oxygenase unit with kinetics models of the reductase unit.
8. It will be interesting to study the dynamics of NOS domain re-arrangement during catalysis by using NMR spectroscopy at room temperature. The size of the NOS enzymes (~120–160 kDa per subunit) prevents application of NMR spectroscopy with methods available to date. Nonetheless, such techniques may be applicable to NOS problems in the future with the development of new NMR approaches and preparation of labeled NOS/CaM proteins.

Conclusions

The NOS output state is a complex between the FMN domain and the catalytic heme domain, and it thus facilitates the catalytically significant IET between the FMN and heme centers. Recent kinetic studies have strongly supported the important role of FMN motion in interdomain electron transfer processes during NOS catalysis. Evidence is rapidly accumulating to show that CaM controls formation of the output state through facilitation of the interdomain FMN–heme interactions. Nonetheless, the molecular mechanism that underlies the CaM-modulated output state formation remains elusive. There is a clear need to understand how the FMN–heme IET is modulated at the molecular level, and how this IET step specifically regulates catalytic activity of the various NOS isoforms. A combined kinetic, spectroscopic and site-directed mutagenesis approach will permit detailed investigation of molecular mechanisms that underly formation of the NOS output state for NO production.

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Fig. 1.

IET in NOS from NADPH through FAD and FMN to heme (as shown in red arrows). CaM binding activates the FMN–heme IET (*reaction 2*) in eNOS and nNOS, and is also required for proper alignment of the FMN and heme domains in iNOS.

Fig. 2.

Tethered shuttle model: FMN-binding domain shuttles between the FAD domain and heme-containing oxygenase domain. Top: input state; bottom: output state. The two tethers are the hinge region between the FMN and connecting domains, and the CaM-binding linker between the FMN and oxygenase domains. CaM binding unlocks the input state, which thereby enables the FMN domain to shuttle between the two states.

Fig. 3.

Structure of an intact CaM-free nNOS reductase construct (PDB entry 1TLL) ¹⁶; the ribbon diagram shows the FMN module (yellow) docked against the NADPH/FAD domain (blue) in the FMN-shielded conformation, *i.e.* the input state of FMN for electrons from NADPH through FAD, but not the output state. From the N to C terminus: yellow, FMN domain; pink, hinge; green, connecting domain; blue, NADPH-FAD-domain; red, C-terminal tail (CT). Cofactors FAD (orange) and FMN (gray) are shown as stick representations. Because of their highly flexible nature, the AR, hinge and CaM-binding regions were not well resolved in this structure.

Fig. 4.

Transient trace at 600 nm of the $[\text{Fe(II)-CO}][\text{FMNH}^\bullet]$ form of a nNOS oxyFMN with added CaM flashed by 450 nm laser⁴⁹. Note that FMNH[•] dominates the absorption in the range of 580–600 nm. As a control, the 600 nm absorption of the nNOS oxygenase construct (which does not contain FMN) stays above the baseline (inset). Solid lines correspond to exponential fits to the data.

Fig. 5. Transient trace at 600 nm at longer time scale obtained for a nNOS oxyFMN with added CaM flashed by 450 nm laser⁴⁹. Note that rapid FMN–heme IET (as shown in Fig. 4) occurs prior to the slow CO rebinding process. Solid line is an exponential fit to the data.

Fig. 6.

(a) Full-length eNOS, nNOS and iNOS, and (b) truncated oxyFMN constructs consisting of the heme-containing oxygenase domain, CaM-binding site, and the FMN domain. The oxyFMN constructs are designed to preclude the FAD/FMN interactions, and favor interactions between the FMN-binding domain and the heme domain⁵⁹. Note that the eNOS, nNOS and iNOS oxyFMN constructs consist of residues 66–715, 290–947, and 71–723 of the corresponding holoenzymes; these truncating sites are chosen to minimize proteolysis during purification. The eNOS and nNOS proteins possess a unique AR insert (represented as a magenta arc) *within* the FMN domain; this AR element is absent in iNOS.

Scheme 1.

Scheme 2.