[Frontiers in Bioscience 8, d193-209, January 1, 2003]

NITRIC OXIDE SYNTHASES: DOMAIN STRUCTURE AND ALIGNMENT IN ENZYME FUNCTION AND CONTROL

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1. ABSTRACT

Nitric Oxide Synthases are a family of enzymes that produce NO from arginine, oxygen and reducing power in the form of NADPH; they function as signal generators and as producers of cytotoxic levels of NO (e.g., in immune defense). Evolution of eukaryotic NOS from prokaryotic antecedents involved a series of gene fusion events, producing a modular enzyme, and the concomitant development of sophisticated control mechanisms that are isoform specific and tailored to the role of enzymes in signal transduction or immune response. Recent information on the structures of NOS isoforms at all levels from primary amino acid sequence to high resolution crystallography allows a deepening understanding of many aspects of these important proteins including interdomain interactions, dimerization, cofactor, substrate, and isoform specific inhibitor binding as well catalysis and control. The details of the NOS reaction mechanism and its control through the regulation of electron transfer by CaM binding and other mechanisms are still being elucidated and are well worth further examination.. The alignment of the molecular surfaces of the independently folded domains is a central feature of structure, catalysis and control in these important enzymes, and will be the focus of the present review.

2. INTRODUCTION

Over the last decade, nitric oxide generated from arginine by a novel family of enzymes has emerged as both a signal molecule of wide importance and a component of immune response. Endothelial nitric oxide synthase (eNOS) regulates vascular tone and smooth muscle tension (1-3). NO produced by neuronal nitric oxide synthase (nNOS) functions as a neurotransmitter (4, 5). These constitutive isoforms (cNOSs) are regulated by intracellular calcium/calmodulin (Ca^{+2}/CaM), and generate NO as an intercellular messenger (6-8). NO produced by cNOS activates soluble guanylate cyclase in target cells (9-12) (e.g., smooth muscle). This signal channel is the best studied, but NO affects many sites in the cell. In contrast, inducible nitric oxide synthase (iNOS) binds CaM irreversibly under all conditions and is instead transcriptionally regulated in response to cytokines (13-15). The primary sequence difference between mammalian cNOSs and iNOSs is a sequence insertion, present only in cNOSs, which functions as an autoinhibitory control element (16).

Since the discovery of nitric oxide synthases in mammals, homologs have been described in vertebrates including birds, fish and amphibians, e.g. (17, 18). More recently nitric oxide synthases have been describes in invertebrates including insects, molluscs and corals (19-23), and more primitive eukaryotes such as slime molds (24). These include both cNOSs and iNOSs by the criterion of the existence of an autoinhibitory element. Nitric oxide functions in these systems as a signal controlling aspects of development and behavior.

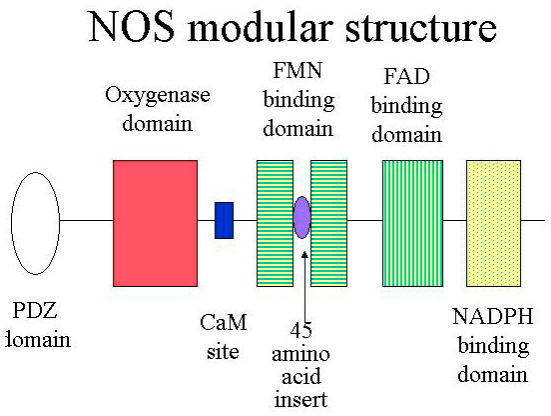

Figure 1. Schematic of the modular structure of eukaryotic NOS isoforms. Prokaryotic homologs have independently expressed NOS oxygenase domains.

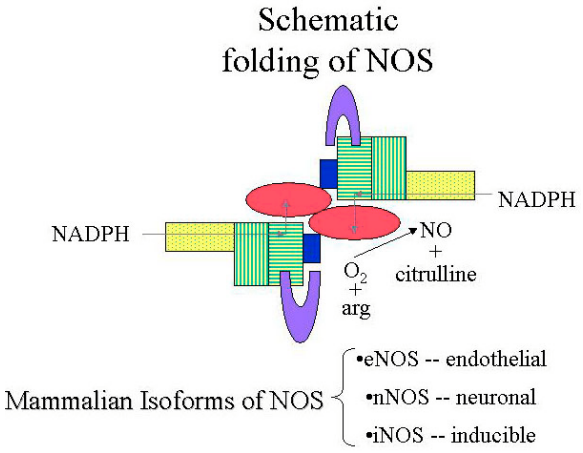

Figure 2. Schematic of NOS folding showing head to head arrangement of NOS oxygenase domains and intermonomer electron transfer.

Prokaryotic NOS genes have been sequenced, and some of the enzymes they code for have been studied (25-29). These enzymes contain only the oxygenase domains of the eukaryotic proteins. Presumably reducing equivalents are supplied by independently expressed proteins, such as flavodoxins and ferredoxins, with functions analogous to the NOS reductase domains.

All NOS isoforms function as unique double monooxygenases, generating NO and citrulline from L-arginine, two moles of O_2, and 1.5 mol of NADPH (30-32); N-hydroxyarginine is an intermediate. Oxygen chemistry takes place at a thiolate heme, spectroscopically resembling cytochrome P450 (33-35). Tetrahydrobiopterin (BH_4) is required for NO synthesis, and promotes dimerization (36-40). BH_4 is not consumed stoichiometrically during turnover; recent reports (41, 42) suggest pterin radical formation during the first step of NO synthesis. Electron transfer from NADPH is catalyzed by bound FMN and FAD (37, 43-45). CaM binding has been reported to affect electron transfer within the reductase domains in addition to FMN/heme electron transfer (46).

An extensive listing of review articles on nitric oxide in biology is available at: http://metallo.scripps.edu/PROMISE/NOS_REV.html

3. DOMAIN ORGANIZATION

Nitric oxide synthase monomers are very large (~133-161 kD per monomer) modular enzymes produced by a series of gene fusion events during evolution. Their overall structure is summarized in figure 1. Bredt, Snyder and coworkers (44) showed that the C terminal regions of NOS vertebrate isoforms are homologous to P450 reductase, and identified FMN, FAD, and NADPH binding regions. Limited proteolysis at the CaM binding site cleaves NOS into C terminal reductase and N terminal oxygenase fragments (47). The oxygenase fragment contains the arginine binding site, BH4, and heme. The Masters group pioneered expression and study of corresponding constructs in nNOS, iNOS and eNOS, as well as nNOS holoenzyme, in E. coli (48-52). Work in other laboratories using analogous constructs has resulted in significant improvements in understanding the structure and function of individual domains and how they work together (53-56).

The oxygenase domains of eNOS and iNOS extend from the N terminal to the CaM binding site. An N terminal sequence of about 200 amino acid residues, homologous to PDZ domains, functions as a protein recognition site in nNOS (57, 58). The N terminal leader sequence of nNOS contains a binding site for a highly conserved and widely expressed 89 amino acid protein PIN (Protein Inhibitor of Nitric Oxide synthase) which when bound to nNOS has been reported to destabilize thedimeric structure, thereby inhibiting catalytic activity (59). Subsequent evidence has placed PIN associated monomerization and inhibition in some doubt (60, 61).

Cys 415 was identified as the axial heme ligand of nNOS (50); corresponding Cys residues serve as axial ligands in other isoforms (e.g., (62)) as previously proposed (33). The heme domain has virtually no structural similarity to P450 other than the fact that a conserved thiolate is coordinated to the heme iron atom along with some similar surrounding residues (the so-called conserved ten amino acid peptide motif or heme binding peptide). Although it evolved independently of the P450 enzymes, because of its common ligation pattern NOS heme domain shows many similarity to P450 in terms of spectrocopic, biochemical and catalytic properties, and much of our knowledge to-date about the function of NOS is based on our comparatively advanced understanding of the structure and mechanism of P450.

NOS is active only as a homodimer (63) in which the subunits align in a head to head orientation with the oxygenase domains in contact and the reductase domains in contact only with the oxygenase domains as shown in figure 2 (47, 54, 64). Heme domains, either independently expressed or generated by proteolysis, dimerize, but flavoproteins do not; intermonomer interactions are primarily between the oxygenase regions (50, 55, 56). The reductase unit of one monomer reduces the oxygenase unit

of the other (65). This assignment of domain alignment is similar to sulfite reductase and nitrate reductase where the catalytic domain is dimeric while the domain involved in electron transfer is monomeric ((54) and references therein). All catalytically active oxygenase domain crystal structure solved to date are dimeric and involve a large interface involving both N-terminal and C-terminal residues contributed from both subunits.

4. OXYGENASE DOMAINS

4.1. Spectroscopic studies

Masters' laboratory performed the first optically monitored titrations of NOS with substrate analogs (66, 67). McMillan and Masters (66) used the strong heme Soret band to monitor substrate difference titrations of nNOS, reporting Kd values and demonstrating competition between imidazole and arginine analogs. Frey, *et al* (67) extended this work to the inhibitor thiocitrulline. Salerno suggested the use of competition between imidazole and arginine analogs to overcome the problem of measuring low Kds; this was first used by collaborators (67) and is now widely used by NOS investigators. We have recently described the use of spectroscopically monitored titrations to determine the Kd values of tight binding ligands and to assess interactions between ligands binding at adjacent sites (68). Kd values have been measured for three isoforms for imidazoles and other axial ligands and for arginine and a range of arginine analogs e.g., (69). In addition, potentiometric titrations have been used to assess coupling of ligand binding to redox reactions by us and by others (70).

Subsequent work using ferriheme electron paramagnetic resonance spectra demonstrated that the nNOS catalytic site is perturbed by the binding of substrate (L-arginine), intermediate (L-N-OH arginine; NHA) and arginine analog inhibitors, including thiocitrulline (TC), homothiocitrulline (HTC), N-methyl arginine (NMA), and N-nitro arginine (NNA) (71, 72). The ferriheme of nNOS holoenzyme exhibited epr spectra characteristic of four high spin states in response to the binding of arginine site ligands. These states differ in ligation geometry, and represent local conformations of the active site in adopted in response to specific interactions of substituent groups with the heme pocket. This work demonstrated that essentially all the enzyme was involved in substrate binding and showed that citrulline bound weakly to NOS, forming a low spin complex. The observed changes in the rhombicity of the NOS heme are not subtle; they span 75% of the rhombicity observed in all high spin thiolate heme proteins and model complexes. The only way to get more pronounced effect is to dissociate the axial thiolate ligand, or to convert the complex to low spin by binding a second axial ligand. Both these effects can also be observed in NOS.

In later studies with iNOS and eNOS holoenzyme and hemeprotein (52, 73), spectral effects were noted which are related to the isoform specificity of arginine analogs. All three isoforms make characteristic complexes with these compounds. The NMA state requires a hydrophobic substituent of modest size, while the NHA state is elicited by a more polar group. The NNA state is elicited by ligands posing a significant steric challenge, and appears to involve a significant conformational change. INOS, which binds NNA with lower affinity than other isoforms, makes an NNA complex with higher rhombicity. ENOS, which binds NMA less tightly, makes an NMA complex with lower rhombicity.

Interactions between the pterin binding site and heme were also observed. BH_4 binding affects the epr spectrum of high spin ferriheme in the presence and absence of arginine analog ligands, although these effects are more obvious in the presence of strong arginine analog inhibitors. BH_4 also decreases the accessibility of the heme to some axial ligands; BH_4 replete holoenzyme, for example, does not bind exogenous thiols (DTT or mercaptoethanol). Further work showed that independently expressed oxygenase domains of nNOS formed analogous high spin complexes with most arginine analogs, and analogous low spin complexes with heme axial ligands. In general, a greater number of complexes could be formed with 'hemeprotein' than with holoenzyme, suggesting that the loss of the reductase domains had conferred additional conformational flexibility. One example of such an 'extra state' is a unique high rhombicity species observed by epr after reconstitution with BH_4 in the absence of arginine or its analogs. This may be the result of BH_4 binding in the arginine site, which does not occur in holoenzyme.

Migata, *et al*, obtained epr results describing the FeII-NO complexes of nNOS (74). The hexacoordinate complex was stabilized by substrate L-arginine; in its absence the pentacoordinate NO complex formed due to the loss of the axial thiolate ligand. Differences in the orientation of NO with respect to the heme can be observed with the arginine site empty or when arginine or NHA are bound; the change in NO orientation may be related to oxygen redirection for the first and second oxygenation reactions. FTIR studies of the iNOS oxygenase domain in the absence and presence of BH_4 and L-arg by Jung *et al* (75) complement this observation. These studies revealed that the CO ligand must be H-bonded to the arginine substrate bound in the active site. Although CO does not represent the true dioxygen binding geometry, this study shows strong coupling of a diatomic axial heme ligand to L-arg and supports the idea that substrate assisted proton delivery is relevant for oxygen activation. Recent IR with eNOS and nNOS holoenzyme used labeled arginine to study specific interactions with groups within the binding site (76).

First spectral characterization of the dioxygen complex has been performed on the neuronal NO synthase oxygenase domain in stopped-flow-experiments with rapid-scanning technique (77) indicating a fast autoxidation rate which is affected by arginine and H_4B binding. The O_2 complex can be stabilized at lower temperatures (-30°C) for a time period long enough (~ 10 min) to characterize it with UV-visible spectroscopy (78, 79). The spectrum of the NOS-O_2 complex with its Soret band at ~ 419 nm is very

similar to that observed for cytochrome P450. It is assumed that O_2 binds to the NOS heme iron in a bend-on geometry as it was recently found in the crystal structure of the cryo-stabilized dioxygen complex of cytochrome P450cam (80). A recent ENDOR study (81) indicated that the relative orientation and position of the active nitrogen atom of arginine to the heme iron is similar in the three mammalian NOS isoforms, with subtle differences. Wang, *et al*, (82, 83) used resonance raman to describe the heme site, confirming the ligation of the heme by thiolate, observing perturbation of metal-axial ligand stretches by arginine analog and effects on heme site stability of tetrahydrobiopterin.

Couture *et al.*(84), using Resonance Raman to study the nNOS oxy complex, observed a O-O stretch mode at 1135 cm^{-1} which shifts to 1068 cm^{-1} upon $^{18}O_2$ isotope substitution but was not perturbed by arginine. The ferroheme-CO Resonance Raman results of Fan *et al.* (85) indicated different CO stretch modes for iNOS and nNOS. Small ferroheme axial ligands can be photodissociated and the rebinding kinetics reflect the dynamic behavior of the protein. This property has been extensively employed in heme proteins including NOS using the UV-visible Soret band as monitor (86-88).

4.2. Arginine analog inhibitors

An extensive literature, recently summarized by Griffiths and Gross (89), describes the effects of arginine analog inhibitors on NOS isoforms. Until recently, almost all of the available data was kinetic in nature. Unfortunately, much of the data from screening projects is in the form of $I.C._{50}$ values that are difficult to compare because they depend strongly on the assay conditions. True Ki values cannot be calculated from many of these experiments. Even at this level, a picture has emerged concerning the requirements for strong binding. The guanidinium moiety of arginine is critical, with smaller contributions from carboxylic acid and peptide nitrogen groups. Some variability in side chain length is tolerated; bulky substituents can only be tolerated on one epsilon-amino group, suggesting the presence of a small 'pocket' and a large 'pocket' in the binding site.

Existing Km and Kd data (e.g., (66, 67, 89, 90) reveal interesting trends. Substrate binding site homology works against isoform selectivity, but partial specificity has been observed. N-methyl arginine is a potent nNOS and iNOS inhibitor, but inhibits eNOS less effectively. N-nitroarginine strongly inhibits eNOS and nNOS, but is less potent with iNOS. More selective inhibitors are now available.

Inhibitors of NOS that have been described in the literature can interact with NOS enzymes in a variety of ways, most importantly with the heme pocket. Inhibitors that directly bind as ligands to the heme iron (e.g. imidazoles and their derivatives) can derive potency but not specificity from this interaction because the free energy contribution to the binding is similar in all NOS isoforms. The most common inhibitors are arginine analogs containing the guanidinium structural motif; these bind in the heme pocket adjacent to but not in contact with the iron and block the substrate-binding site. The conserved glutamate (Glu377 and Glu371 for human and mouse iNOS, respectively) hydrogen bonds with the substrate L-arginine. Recent studies with arginine analogs indicate that the binding site for the reactive guanidium nitrogen can accommodate a variety of alternative groups including those much larger than $–NH_2$ and =NOH (91).

In contrast, the binding site for the non-reactive, distal guanidium nitrogen cannot accommodate larger groups. This study suggests some differences between iNOS/eNOS and nNOS in terms of binding. iNOS is less able to bind N-alkyl groups, which are larger than ethyl near the heme, and cannot accommodate –S in the distal guanidinium pocket while nNOS does. This suggests that the L-arginine binding site of nNOS is more open than that of iNOS and eNOS, which awaits further investigation.

4.3. Crystal Structures

Recent important crystal structures contributed to our understanding of NOS structure, catalysis and control. The P450 reductase structure was solved, allowing modeling of homologous NOS reductase domains (92); this has been followed by a partial structure of the nNOS reductase domains in which the FMN binding domain is missing. High-resolution X-ray crystallographic structures for the oxygenase domains of all three mammalian NOS isoforms have been reported (42, 93-98).

This initial structure of delta 114iNOS oxygenase domain (94) was followed by improved dimeric NOS oxygenase domain structures including complexes with substrate L-arginine, the product analog thiocitrulline, and s-ethyl-isothiourea (42, 93, 95-98). The structure reveals an extended winged beta sheet unique to the NOS family. The NOS distal pocket, primarily constructed from beta sheet structures, differs fundamentally from the distal pockets of other heme based oxygenase proteins including P450s, oxidases, peroxidases, and catalases, all of which are primarily alpha helical. The helical cage pattern is in fact a general feature of heme proteins ranging from the hemoglobins/myoglobins family to electron carriers such as c type cytochromes.

All NOS oxygenase domain structures show an extensive dimer interface that buries approximately 2,800 A^2 of surface. BH_4 binding was observed in a pocket at the dimer interface. BH_4 H bonds directly to a heme propionic acid group; the substrate, L-arginine, interacts with the same propionate. The eNOS structure revealed Zn^{+2} tetrahedrally coordinated to pairs of symmetry-related Cys residues at the dimer interface. Zn^{+2} is present in human iNOSox, eNOSox and murine iNOS ox (95, 98,99). Differences in the conformations of regions forming the interface may be directly related to steps in dimer formation.

In recent papers Crane *et al* (99) reported both 'unswapped' structures, in which the N-terminal β-hairpin hook primarily interacts with its own subunit, and

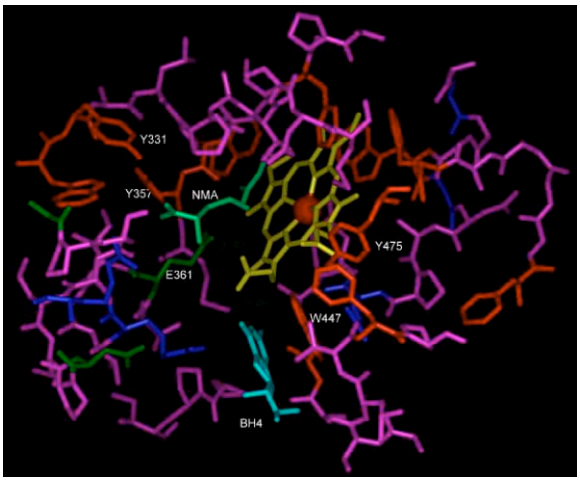

Figure 3. ENOS active site structure showing key features including the heme and pterin cofactors, an arginine analog inhibitor, and selected active site residues.

'swapped' structures, in which the N-terminal hook exchanges across the dimer interface to interact with the adjacent subunit. Absence of this N-terminal hook swapping in eNOS suggests differences in the conformation and composition of regions forming the interface that may influence steps in active dimer formation and H4B/substrate binding (93, 99, 100).

The crystal structure of the N-hydroxy-L-arginine (NHA) complex was recently solved (42); in the absence of axial heme ligands NHA binds in the active site with the same conformation as L-arginine itself. Modeling of the active site indicates that neither the arginine nor the NHA complex has a structure consistent with the simultaneous binding of a heme axial ligand; a bulky axial ligand has too much binding domain overlap to allow formation of a ternary complex, while a diatomic axial ligand requires displacement of arginine analogs to different extents. The flexibility of the side chains in the pocket can allow some of the H bond partners to be retained, but the free energy of rearrangement will decrease the binding energy along with any decrease in H bond efficacy.

CO and O_2 have been modelled into the crystal structure of the N-hydroxy-L-arginine (NHA) complex. The O_2 modeled structure rules out that proton transfer occurs from the hydroxy group of NHA because the distance between the NHA-OH group and the terminal oxygen atom of the bound dioxygen is too long (42). Recently the first crystal structure of eNOS bound with a NO iron-ligand has been reported (100) indicating a distance of only 2.7-2.8 Å between the NO oxygen and the nitrogen of the guanidinium group of arginine which is consistent with a hydrogen bond between both entities.

4.4. Oxygenase domain structure and active site residues

As described in the previous section, there have been significant advances in available structural information about NOS isoforms, with several different crystal structures of the oxygenase domains of iNOS and eNOS both with substrate and variety of inhibitors bound. A structure of nNOS is still to come. In contrast to the multi domain modular structure of the NOS reductase region, the NOS oxygenase domain is a single uniquely folded domain (93,95,97). We remarked in the previous section on the unusual association of a heme binding site in a structure predominantly of beta character; the entire catalytic site is dominated by the complex beta surfaces of the oxygenase domain, which form the substrate binding pocket as well as the cofactor binding sites.

A cutaway view of the eNOS active site is shown in figure 3. The heme iron is pentacoordinate and axially linked to the proximal cys (Cys 200 for human iNOS, cys 194 for mouse iNOS and cys 184 for bovine eNOS). The heme is buried in the protein interior and makes extensive van der waals interactions with hydrophobic and aliphatic side chains. Dimerization is accompanied by creation of a ~30 A deep, funnel shaped active center channel made by residues from the loop and helical elements from both N- and C-terminal ends of one subunit. Crystal structures of the mouse iNOS oxygenase domain also reveal that L-arg and analogs binds with side chain termini fitted tightly into the narrow part of the active site cavity with the guanidine group lying coplanar to the heme. In mouse iNOs oxygenase domain the guanidinium group from L-arginine forms two H-bond with the side chain carboxyl group of the conserved Glutamate (E 371 in iNOS) localizing the third terminal L-arg nitrogen over the heme iron for putative hydroxylation.

The role of H4b is arguably the most intriguing aspect of structure /function relationships in NOS biochemistry. In the oxygenase domain structure, H4B is found to be sandwiched between two monomers in the dimer interface, but buried within the protein and away from the solvent. H4b stabilizes the dimer by integration into the hydrophobic core of the interface thereby facilitating the substrate interactions by lining the active center channel through H-bonding to heme propionate and stacking its indole against conserved tryptophan (W449 in eNOS and human iNOS W463). In the NOS dimer, H4B is located proximal and perpendicular to the heme with extensive h bonding from heme as well as residues of both monomers. There are two identical H4b binding sites in the NOS dimer structure and the residues from both subunits are responsible for creating and stabilization of the pterin binding sites (93,95,97). This explains why H4b binding promotes dimerization, heme sequestration, and conformational change in the interface region of iNOS (40,55,63,93), but the eNOS dimer is not influenced to the same extent (101) *In vivo*, pterin availability influences monomer/dimer ratios in iNOS and membrane bound eNOS (32, (102). This suggests that H4b has a strong stuctural influence on NOS protein primarily either by promoting dimerization or stabilizing the dimer interaction per se. But recent evidence from various laboratories suggest its role as an electron donor in the first of NO synthesis and as a mediator of heme reactiviy in the second step which is beyond the scope of this review (See a recent review by Alderton, *et al* (103).

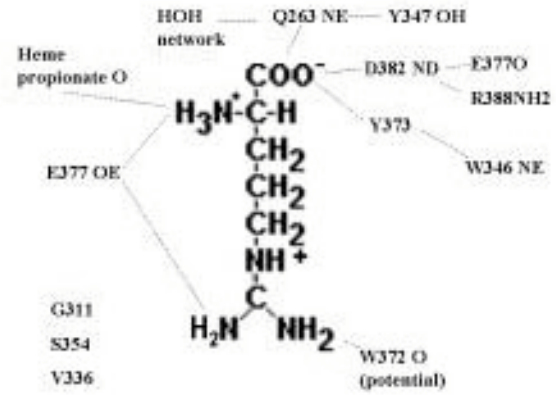

Figure 4. Schematic of arginine binding site showing important substrate contacts.

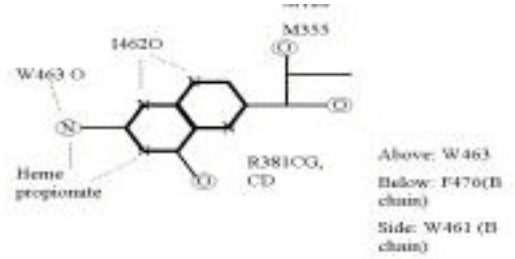

Figure 5. Schematic of terahydrobiopterin binding site showing important cofactor binding contacts. Note that unlike the substrate binding site contacts for each of the two pterin binding sites in a dimer are supplied by both monomers (A and B chains) ; the sites lie along the interface.

The organization of important active site residues is depicted in the following cartoons of the L-arginine and pterin binding sites based on eNOS and iNOS oxygenase domain crystal structures (Figures 4 and 5). We are fortunate that we will be able to interpret our thermodynamic models in the light of a number of mutants, already characterized, that affect substrate binding, catalysis and cofactor binding. For example, E377A and D382A do not bind arginine and W463A and F476A show diminished activation with H_4B.

Recent studies with human iNOS identified four residues including Asp 280, encoded by exons 8 and 9, having critical roles in dimerization and substrate binding. Interestingly, these residues are not in the dimer interface but modulate the dimerization and heme function from a distal site by forming a H-bonding network with the active site residues Glu377 and Asp382; thus influencing the global iNOS oxygenase fold (104). The position corresponding to D382 is worthy of further study since it is the outstanding example of a residue which makes important interactions with substrate but is not conserved; it is a contributor to isoform specificity, but cannot account for it all.

Other residues not directly located in the active site modulate substrate binding through H-bonding networks or involvement in subunit interactions (e.g. Y347, W346, D280, Y267 in human iNOS). The position corresponding to D382 is worthy of further study since it is the outstanding example of a residue which makes important interactions with substrate but is not conserved; it is a contributor to isoform specificity, but cannot account for it all. The periphery of the catalytic site contains additional regions that interact with inhibitors; for example, S356 eNOS, corresponding to N370 iNOS, forms contacts with NNA. The lining of the hydrophobic pocket apparently occupied by substituents such as the methyl group of NMA is formed by F355, V358, and M341 in eNOS, all highly conserved; N340 also forms part of the binding site periphery, and is also conserved, but small flanking sequence differences are present.

Schematics showing features of the arginine (top) and pterin binding sites of NOS (Figures 4and 5); numbers refer to human iNOS. Dotted lines indicate H bonds, which may contribute to local conformational differences. The lining of the substrate channel includes many conserved residues, but there are interesting exceptions. Looking down the channel from the outside, there are columns of side chains. These include R372, N368, and I364 in eNOS; two are conserved, but N368 is aspartic acid in nNOS and iNOS. Another example is the series S248, G249, P336 in eNOS; in this case the cognate of S248 is alanine in iNOS.

The functional implications of the so-called N-terminal hook on NOS dimerization, H_4-biopterin (H_4B) binding, catalytic activity and heme pocket structure have already been described for iNOS by Ghosh et al (56,105). These effects are mediated through the local conformation. No comparable results are available for corresponding residues in nNOS and eNOS; available information suggests that conformational effects on ligand binding are dependent on residues involved in the dimer interface. Moreover, the role of H_4B in producing the active conformation of the NOS structure is still not clear. It promotes dimerization of iNOS from an inactive monomer to an active dimer yet it is not required for nNOS dimerization (56) (D.K. Ghosh, unpublished observation).

Several existing mutants in this region have interesting effects on ligand binding, suggesting that differences here contribute to isoform specificity through conformational effects. Examples include mutation of Cys331/Cys109 (nNOS/iNOS), Lys 88 and Thr99 (human iNOS) to alanine, which cause effects on dimerization, pterin binding, and arginine binding. Cys 331/109 is involved in Zn coordination, which mediates dimer formation and stabilizes the active conformation in most eukaryotic isoforms. Examples of interface residues are shown in the following table, which also includes residues involved in the heme site, the Zn^{+2} site, and the dimer. Some residues present in the dimer interface differ among NOS isoforms as suggested by sequence alignment; these include E96in HiNOS (serine in eNOS and val in nNOS), S124 HiNOS (K in eNOS and Gln in nNOS), and M447 in HiNOS (Y451 HiNOS and Gln in eNOS).

5. REDUCTASE DOMAINS

NOS FMN binding domains are homologous to flavodoxins; alignment of selected NOS and flavodoxin sequences reveals a major insertion in eNOS and nNOS relative to flavodoxins and CPNR corresponding to an ~45 residue subdomain. This insertion represents the most important difference between cNOS and iNOS sequences, and correlates with Ca^{+2}/CaM control (16, 106, 107).

All enzymes containing this domain contain three FMN binding regions (92). The region nearest the C terminal (44) contributes the shielding aromatic. The region nearest the N terminal of the module forms a cluster of hydrogen bonds with the terminal phosphate group of FMN; a third region contributes a second aromatic ring in contact with FMN as well as H-bond partners. The domain consists of a five-stranded parallel β sheet, with the FMN binding site along one edge. The aromatic side F587 and Y625 (in iNOS) are in contact with the isoalloxazine ring system. The subdomain corresponding to the insertion in eNOS lies along the edge of the sheet opposite the FMN binding site and adjacent to CaM.

The FAD and NADPH binding domains of NOS have been modeled using homology with ferredoxin NADPH reductase (FNR). FNR consists of a five stranded parallel β? sheet domain and an eight-stranded β barrel. The FAD isoalloxazine ring lies against the outside of the β barrel and interacts with a conserved aromatic residue inside the barrel. The adenine binding site is located on a loop connecting two barrel strands. A stacking interaction for the isoalloxazine ring is provided by the last residue in the sheet domain, which is otherwise devoted to NADPH binding. This interaction provides a natural mode of communication between the NADPH and FAD binding sites. The adenine binding site, including a stacking aromatic residue, is located on a β>α loop. The nicotinamide moiety was not bound in any solved FNR homolog, but can be modeled as stacked with the FAD isoalloxazine ring system.

Homologous proteins, which contain both FMN and FAD binding domains, have in addition a subdomain within the FAD binding domain which functions to align the FMN and FAD domains for electron transfer. Its structure has been recently solved by the Kim and Masters laboratories in P450 reductase (92). The individual domains in this structure are very similar to the ancestral component domains (flavodoxins and NADPH ferredoxin reductase type enzymes); the new structure established the alignment of the FAD and FMN domains, specifying a close edge to edge orientation of the FMN and FAD isoalloxazine ring systems. This provides us with an excellent model of the homologous NOS reductase domains, although the determinants of calmodulin control are still missing.

6. DOMAIN INTERACTION AND CONTROL

6.1. Dimerization

Structural information about individual domains provides clues about structure/function relationships in NOS holoenzyme. It is now clear that in mammalian isoforms there are several reasons why the dimeric enzyme is the minimal active form. The pterin binding sites are both formed by elements from both monomers, the conformation of the oxygenase domain and catalytic site is stabilized in the active form by dimerization, and the oxygenase domains are reduced by the reductase domains of the complementary monomer.

The original bidomain model (figure 2) postulated for iNOS ((54) has been supported by other experimental methods in absence of a full length reductase crystal structure. For example, yeast two hybrid studies with the oxygenase and reductase domains by Venema *et al* (108) indicates that for iNOS, the oxygenase domain has all the determinates for dimerization and reductase has no role in dimer formation. The same studies also suggest that in nNOS and eNOS there may be significant intermonomer interactions between reductase domains as well as between oxygenase and reductase domain across the dimer. This would imply that all NOS isoforms are not identical in terms of dimerization control, and may explain why H4B binding and subunit association are intimately connected in iNOS dimerization but not in eNOS or nNOS.

Recent studies with urea generated monomers of both the constitutive isoforms indicate that dimerization does not depend on H4B, while the iNOS monomers always dimerize to a large extent in presence of H4-biopterin (45)(D.K. Ghosh, unpublished observation). Recent studies also showed calmodulin promotes dimerization of the oxygenase domain of human eNOS, and that the C-terminal reductase domain is not required for dimer formation (109). Although the implications of these observations are not fully understood, it is now well established that L-arg and H4B promote dimer formation and stabilize existing dimers against proteolytic cleavage or denaturing agents like SDS (55, 56, 110). Recent mutagenesis studies from several different laboratories with holo NOS and oxygenase domain also provide information about NOS dimerization, residues involvement and contributations from both N-terminal and C-terminal regions in subunit interaction. To date there is no evidence that point mutation or small deletions of residues in the reductase domain have any impact on dimerization, although some mutations perturb productive flavin-heme electron flow coupled to NO formation. For example, deletion of the extreme C-terminal 24 residues in iNOS results in loss of NO production due to its inability to bind NADPH, but has no impact on dimerization or H4B and L-arg binding (111); this mutation could also render the FAD site nonfunctional because of loss of the isoalloxazine shielding residue. Point mutation of F1122 by the authors was indeed shown to reduce the activity by approximately half. Similar point mutation of two conserved acidic residues Asp (918) and Glu (919)) and one conserved aromatic residue (Phe892), in nNOS holo enzyme results in a loss of FMN binding and NO synthase activity without altering the substrate recognition or calmodulin binding. Activity can be regained fully after reconstitution with FMN indicating that the mutation affects the FMN module and the loss in activity is due a decrease in the affinity for FMN (112).

On the other hand, mutation in the oxygenase domain is frequently associated with loss and/or defect in dimerization, H4b binding, L-arg binding and catalytic activity. Examples include relatively conserved point mutations like G450A, A453I , D92A N83A , H95A D280A Y267A in iNOS , C99A in eNOS and D314 in nNOS (104,105,113-115) which are associated with loss in activity, dimerization , H4b/l-arg binding and in some cases also heme loss, consistent with the primacy of the oxygenase domain in subunit interaction and catalytic activity. Adjacent to this region, deletion of the variable N-termini in iNOS, eNOS and nNOS did not affect dimerization, H4B /L-arg binding or catalytic activity, but further deletion into the conserved core unit had dramatic effects on these essential properties, resulting in a heme containing inactive monomer (56, 105,116,117).

Very few studies have been done with the isolated reductase domain reconstituted with the oxygenase domain. Earlier studies showed that the isolated reductase domain from eNOS and iNOS can support NO synthesis when mixed with their respective partner oxygenase domain, although the rates are only 1/10 of that found with intact full length (53, 118). More recent studies with nNOS oxygenase and reductase domain indicate that CaM has no role in electron transfer in the reconstituted system and addition CaM may inhibit the activity (119). The relatively low activity of the reconstituted system is expected in light of the greatly increased number of degrees of freedom of the domains, making it much more difficult to achieve domain alignment conducive to productive electron transfer.

6.2. The role of calmodulin in NOS activation

The NOSs are the first enzymes in which calmodulin (CaM) has been shown to control activity by regulating electron flux (6, 7, 120, 121). While elevated calcium levels are required for CaM binding and activation of nNOS and eNOS, basal levels of calcium in the cell allow for the tight binding and activation of iNOS (13). CaM activation of NOS enzymes appear to be more complex than that found in the other 30 target enzymes which often requires only the removal of a pseudo substrate inhibitory domain from their active sites.

In isolated nNOS reductase domains, CaM facilitates electron transfer from NADPH and the flavins to exogenous electron acceptors such as the heme in cytochrome c (47), and has been reported to increase the rate of electron transfer both between FMN and heme and within the reductase domains (46). Although CaM is required for interdomain electron transfer in iNOS it is not a requirement for electron transfer within its reductase domains (122). Amino acids 503-532 of the mouse iNOS consist almost exclusively of the characteristic hydrophobic/basic regions of Ca^{+2}/CaM binding sites. Comparison of this sequence with the corresponding region of nNOS shows only 43% identity of the 21 amino acids residues that are designated as the putative CaM binding sites for nNOS, suggesting that these differences may contribute to the ability of iNOS to bind CaM at low concentrations (123). The NOSs display different affinities toward CaM with the general order being iNOS >> eNOS >> nNOS. Recent works using chimeric NOS having exchanged CaM binding sequence between NOS isoforms as well as synthetic CaM binding sequence peptides suggest that high CaM binding affinity towards iNOS requires additional regions of iNOS (outside of residues 503-532, the so called canonical sequences) (124, 125) although alterations in sensitivity to Ca+2 could be observed by simply swapping canonical binding segments. CaM binding and activation may be an interesting target for the selective inhibition of the different NOS isoforms. It has already been reported that a plant CaM, SCaM-1, acts as an antagonist for nNOS activation and single substitution of Met144 to Valine was found to be responsible for this inhibition (126).

Studies using protein chimeras of CaM and troponin C have helped to define regions of CaM that affect cation binding and target enzyme activation such as neuronal NOS (127-129). CaM and troponin C (TnC) are homologous proteins with 50% direct amino acid identity and remarkably similar crystal structures (130, 131). The N- and C- terminal domains of both proteins are comprised of high affinity, helix-loop-helix, and calcium binding motifs known as EF-hands. Although they are structurally similar, there are several functional differences between CaM and TnC. TnC does not activate most CaM target enzymes and CaM does not substitute for TnC. A number of investigations have been performed where the domains or elements of the EF-hand motif have been exchanged between CaM and TnC. These investigations have led to the suggestion that the CaM latch domain plays a critical role in its activation of nNOS (127); single amino acid mutations in domains 1 and 3 reduce the activation of nNOS (129), and the CaM effects on electron transfer at two points within nNOS can be functionally separated (128). While the functional properties of nNOS have been studied, neither iNOS nor eNOS have been included in these investigations.

FMN binding domain orientation allows FMN to lie close to the isoalloxazine ring system of FAD in the adjacent domain. This places the CaM binding site, at the opposite edge of the β sheet from FMN, in a location remote from reductase cofactors. The major insertion in the FMN binding domain, however, has properties that suggest that it functions as a control element. Inhibitory elements, displaced by CaM from an adjacent site or the CaM binding site itself, are a feature of other CaM controlled enzymes (107). Salerno and coworkers, starting from structural considerations, proposed that the insertion was a control element and provided evidence for an autoinhibitory role (16). Critical to this proposal is the accurate specification of the element's location. Most importantly, the insertion lies in an alpha>beta loop on the opposite edge of the beta sheet from FMN, but *directly adjacent to CaM*. This positioning differentiates the FMN domain insertion from other NOS subdomains that mediate interdomain interactions, and allows it to interact with CaM during activation. Synthetic peptides homologous to the FMN binding domain insertions (particularly to the eNOS insertion) are inhibitors of cNOS activity and CaM binding.

Chimeric NOS enzymes with altered calcium sensitivity have been produced by altering or swapping the AI sequence, inserting an AI, or deleting it entirely; e.g., (132, 133). Most convincingly, elimination of the AI from eNOS produces an iNOS like enzyme with high activity and a low activation requirement for calcium. Site directed mutations in which two residues were altered produced enzymes with significantly lower calcium requirements, while partial deletions sometimes produced enzymes more difficult to activate, probably reflecting a less flexible remnant of the AI.

7. EVOLUTION: DEVELOPMENT OF A MODULAR ENZYME

The evolutionary history of the development of eukaryotic NO synthase isoforms from their prokaryotic ancestors cannot be understood without considering the incorporation of one and two domain components into the large modular enzymes by a series of gene fusion events. The initial evidence for this process was the striking homology noted by Bredt and Snyder (44), and the concept has served as the basis for several very different lines of investigation (22, 51, 134). Briefly, genes coding for two domain ancestors closely related to modern FAD containing ferredoxin/flavodoxin reductases fused with genes coding for FMN containing flavodoxins, leading to the development of modern P450 reductase type enzymes as well as the reductase portions of modular enzymes such as NOS and sulfite and nitrite reductases. This process also included the evolution of a subdomain sized insertion in the FAD beta barrel domain which functions to align the flavin binding domains, positioning the FAD and FMN groups for productive electron transfer (92, 135).

Prokaryotic NOS enzymes contain only the oxygenase domain and are presumably reduced by independent flavoprotein and/or flavoprotein components(29). Most of the sequence information on these enzymes has been obtained through genomic sequencing projects, e.g. (25, 26). The prokaryotic history of the development of the oxygenase domain includes increased coverage of the active site, a process that continued in eukaryotes along with elaboration of the 'wing tips' of the central winged beta sheet. Gene fusion between oxygenase and reductase components led to the prototypical holoNOS. Modularity on this scale is found in prokaryotes, but it is a particular advantage in a cytosolic eukaryotic protein because of the large volume of the cellular compartment, and because it allows concerted localization of the components of a multidomain system.

Successful gene fusions depend critically on the orientation of the component domains in the fused product. This depends in turn on the length and properties (e.g., surface interactions and conformation) of the connecting region, which was derived from non-coding DNA, and on subsequent development of accessory regions (e.g., FAD 'hinge region' subdomain). The domain interaction surfaces would have been well established prior to gene fusion to enable the individual components to interact productively in solution.

Although it has been proposed that these interacting surfaces evolved after the gene fusion events, the correlations which enable potentially interacting domains to be identified using their occurrence in modular proteins (e.g.,(136)) is far more likely to arise from a series of events in which development of interacting surfaces in independently expressed components is followed by segregation of genes within an operon, positioning them for gene fusion. Several lines of evidence support this view. Modularity is far more likely to occur in 'late' systems than in 'early' ones. In the case of NOS components, flavodoxins (and ferredoxins, the alternative electron carriers in many prokaryotes) are major electron distribution points which interact with multiple enzymes involved in the generation of reducing power (PSI), the production of biosynthetic reducing equivalents for carbon fixation and other processes (NADPH ferredoxin/flavodoxin reductases), and other enzymes. Free flavodoxins and ferredoxins can hardly have been developed through a cleavage event from all the putative modular ancestors. Finally, the interaction surfaces in modular proteins are in some sense 'degenerate' compared to those in their independently expressed ancestors. Independently expressed component domains of modular proteins (e.g., NOS oxygenase and reductase domains) function poorly together compared to closely analogous systems in which the components are independently expressed in nature. This is to be expected if the interaction surfaces evolved in independently expressed proteins, since once fusion occurs the covalent association of the domains removes much of the selection pressure maintaining tight association. Regulatory mechanisms may in addition exert selective pressure against maintenance of the tight association of domains, since a major mechanism in control is the regulated transition between productive and unproductive domain alignments.

A related aspect of the evolution of molecular surfaces involves the ability of prokaryotic NOS enzymes to dimerize without benefit of a zinc binding site or N terminal sequences involved in dimer interface formation in eukaryotic enzymes. Mammalian oxygenase domain constructs truncated to positions corresponding to the natural N termini of the prokaryotic enzymes are incapable of dimerization. A plausible explanation is that the evolution of the additional sequence forming the interface structures was favorable because it permitted a more closely associated dimer, particularly desirable in eukaryotic cellular compartments. Once these structures were in place, the selection pressure on the rest of the interface was removed, since tight association was already enforced by the N terminal structures. Degeneration of the rest of the interface left the truncated domains unable to associate into active dimers.

8. DOMAIN ALIGNMENT AND CONTROL: A NATURAL NANOSCALE ELECTRICAL SWITCH

The theory of classical allosteric control in multimeric enzymes delineated the advantages of cooperative multimeric regulated proteins in which interactions between subunits produce a concerted

conformational transition (137). Typically the transition involves a conformational change within a domain which gives rise to inhibition/activation (or binding/release). The interdomain interactions increase the sharpness of the transition through cooperative effects exemplified by the R/T transition in hemoglobin (138).

Other calmodulin modulated systems have been shown to involve displacement of an internal inhibitor (autoinhibitory element) from the active site or from the CaM binding domain itself, allowing a conformational change. This element often has calmodulin-like properties, so that in the absence of Ca^{+2}/CaM the CaM binding site associates with the internal autoinhibitory element, producing an inactive conformation(139). In calcium regulated proteases, the autoinhibitory element is regulated by the substrate (peptide) binding site but not cleaved, and blocks the active site until displaced by calmodulin (140).

In nitric oxide synthases, control is exerted through modulation of electron transfer through the reductase domains to the active site (6, 7, 120, 121). Available information suggests that the primary regulated step is the transfer of electrons from FMN to heme, although there is data to indicate that FAD to FMN electron transfer is also Ca^{+2}/CaM sensitive. Interdomain electron transfer between the prosthetic groups in NOS necessitates transfers over at least 10-15A via tunneling. The primary factors influencing electron transfer rates in this situation are the distance between electron carriers and their redox potentials.

Work by Dutton and colleagues (141, 142) has demonstrated that the amino acid composition of the intervening region has little effect on the observed rate of electron transfer in several model systems, and provides a characteristic distance relationship under which the electron transfer rate falls off by an order of magnitude over each additional 1.7A. While this characteristic distance may be somewhat different in radically different classes of protein, it is unlikely that the overall picture will change. Since calmodulin has no effect on the potentials of the flavin or heme groups in cNOS holoenzymes (143) or in reductase domains expressed with the CaM binding site attached (144), it is probable that the control of electron transfer occurs by controlling the distance between the groups. A less likely alternative involves the possibility of conformational adjustment of potentials; electron transfer could occur in one state with a set of effective potentials, followed by a conformational adjustment producing a new state with new potentials. Equilibrium redox potential measurements measure only the potentials connecting the most stable states, and hence need not necessarily describe all the relevant microscopic potentials. While such adjustments in the environment of an electron carrier certainly occur in response to redox reactions, it is clear that in most cases equilibrium potentials describe the electron transfer rate reasonably well. It is also unlikely that Ca^{+2}/CaM would affect microscopic potentials profoundly but leave the equilibrium potentials unchanged.

The control system in NOS is well represented as an electrical switch, which is activated by calcium with the aid of the molecular biosensor calmodulin. This is an important addition to our growing inventory of bioelectronic devices. These include the largely solid state charge separation mechanisms in the photosynthetic reaction centers (e.g., (145)), respiratory systems in which electron flow is coupled to the pumping of protons or sodium ions across membranes (146) (this is sometime reversed to increase the reducing power of electrons), and the remarkable center ‘o’ of quinol reductases (147). This last device functions as an electron pair splitter, generating one high potential and one low potential electron from each quinol it oxidizes to quinone.

Mitchell (146) proposed center o as a necessary device to his concept of a Q cycle in mitochondria and related systems. Observations on the components of the system, initially spectroscopic, revealed multiple conformational states which led to proposals for ‘switching units’ in which conformational changes, particularly between spectroscopically distinguishable states of the Rieske iron sulfur protein, would be coupled to the redox states of components such as quinone; this requires at least two conformational states in which the iron sulfur group is in contact with its acceptor or donor, but not both (148). Recent crystal structures of quinol cytochrome c reductases have revealed conformational states of the Rieske center consistent with a switch in which the iron sulfur group serves as a one electron shuttle between the center o quinol binding site and cytochrome c1 (149-151); the second electron, from the semiquinone generated in the first electron transfer step, reduces a b cytochrome.

How does activation of the enzyme by calmodulin enable electron transfer in cNOS? The simple answer appears to be that calmodulin binding drives (or permits) domain realignment, positioning the electron carriers, in particular FMN and heme, for productive electron transfer. The autoinhibitory element appears to play a role in enforcing inactive domain alignment, either by acting as a shim separating the domains or as a linker anchoring the FMN binding domain in an unproductive position. CaM binding appears to displace the element from the inhibitory conformation, which may also be destabilized by covalent modifications.

Other regions in cNOS are involved with control and activation. A small insertion in the FAD subdomain which modulates interactions between the FAD and FMN binding domains appears to be correlated with the presence and composition of the major FMN domain insertion. The recent partial crystal structure of the nNOS reductase domains indicates that it is well positioned to interact with it (152). It may form part of the binding site of the AI. An array of negative charges on the C terminal face of the FMN binding domain also correlates with the AI, and is positioned to interact with it directly (16,106).

The C terminal region extending beyond the end of the NADPH binding domain is variable in length and has limited homology between isoforms. Homologous proteins such a NADPH ferredoxin reductase and P450 reductase (153) end with the conserved FAD shielding aromatic

residue at the edge of the NADPH domain or after one additional residue; iNOS, eNOS, and nNOS have 12, 18, and 23 additional residues. Phosphorylation of the serine residue (1179) in eNOS nineteen residues from the FAD stacking aromatic at the start of the c terminal region activates the enzyme, and C terminal truncated constructs are constitutively active in the absence of calmodulin; for example, deletion of 27 residues in the N-terminus of eNOS holoenzyme produces a 5-fold reduction in EC (50) for calcium and a 2-4-fold increase in maximal catalytic activities(154). The potential for interaction between the C terminal region and the autoinhibitory element has been recently discussed (155).

Recent stop flow kinetics results suggest that electron transfer through the reductase domains is activated in the absence of NADPH, and inhibited in its presence unless relieved by calmodulin (156). This led to the suggestion that NADPH introduced a 'conformational lock', which prevented electron transfer. Several possibilities present themselves. In flavin pyridine nucleotide oxidoreductases, the nicotinamide moiety displaces the shielding residue to form a long wavelength charge transfer complex with the flavin isoalloxazine ring system. Formation of the charge transfer complex could change the conformation of the adjacent C terminal tail region to allow it to mediate inhibitory interactions between domains. Alternatively, formation of the charge transfer complex might in itself require an inactive conformation, and the C terminal region might be to stabilize the charge transfer complex. Complicating this situation is the effect of these interactions on FAD electrochemistry. In the charge transfer complex reducing equivalents are shared between FAD and NADPH, and are hence unavailable for transfer to FMN and heme. In addition, the nicotinamide moiety must be replaced by the stacking aromatic before electron transfer can occur, because the stacking aromatic controls the redox potential by destabilizing the bent reduced isoalloxazine state. This produces a low potential flavin site with a stabilized semiquinone, just the requirements for sequential one-electron transfers through the FMN semiquinone/quinol couple to the heme.

So far we have discussed the control of cNOS activity in terms of an active domain alignment promoted by calmodulin and an inactive domain alignment representing the 'off' state. Reality is probably more complex. In the structure of P450 reductase (92), the FMN and FAD isoaloxazine ring systems are edge to edge and nearly in van der waals contact; the substituents on the rings are within 4.4 A. This is clearly well within electron transfer distance. Docking a model of the eNOS or nNOS reductase domains built on this framework with the solved crystal structure of an eNOS domain or a homology model of an nNOS domain cannot produce a distance favorable for FMN/heme electron transfer; the heme iron cannot approach within 25A of the FMN. This suggests several possibilities: the reductase domain may be a poor model for NOS domain orientations, or may even represent an inactive conformation. This latter case would suggest the existence of an active conformation in which the FMN domain was oriented to be accessible to both the FAD and to its electron acceptor.

It seems more likely that electron transfer from NADPH to heme in NOS requires a series of domain realignments in which the FMN cofactor serves as a shuttle between FAD and heme. Ancestral systems using independently expressed ferredoxins and flavodoxins function using shuttle mechanisms in many systems (e.g., (157). Electron transfer requires the close approach of a donor or acceptor to the FMN of flavodoxin or the iron sulfur group of ferredoxin; this is easy to arrange for a single partner in a binary complex, but more difficult to imagine for a donor and acceptor simultaneously in a single complex, since so much of the surface of the ferredoxin Rossmann fold (92)edge is given over to interaction with a single partner. In the case of NOS the release of the 'flavodoxin' (the FMN binding domain) would be only conformational because it is tethered to both the oxygenase (through the dimer interface) and FAD (covalently) domains. The relationship between the NOS shuttle/switch and the center o pair splitter is also obvious.

If this picture is correct, the P450 reductase structure represents the conformation in which FAD to FMN electron transfer is activated. To transfer electrons to heme, the FMN binding domain must pivot to expose an edge of the FMN to the surface of the oxygenase domain dimer through which the heme is most exposed. It is possible that a heme/FMN distance of 15A could then be achieved, at the cost of separating the flavins. After FMN/heme electron transfer, the FMN domain would return to its former state to pick up a second electron from FAD.

Obviously, such a shuttle mechanism could be interrupted either from the FAD/NADPH side or from the heme side (by a chock preventing domain rearrangement). This would facilitate control from multiple sites, and would suggest that activity could be enhanced by increased flexibility in this region or decreased by rigidity. Instead of an 'on' state and an 'off' state, activity requires free movement between two states required for electron transfer. Inhibition of such a system can be accomplished by jamming the enzyme in either of these states or an additional state, which is not part of the catalytic cycle. This could result in interesting effects, including inhibition of multiple steps, and the observation of multiple phases of reduction kinetics resulting from enzymes starting from different conformations.

9. ACKOWLEDGEMENT

DKG is thankful to Dr. Brice Weinberg for sharing the Lab space, facility, helpful suggestions and genuine encouragement during this writing. In addition, DKG also thanks Drs DJ Stuehr and Briane Crane for their contribution to the understanding of NOS structure and function. J.C.S. thanks Susan Smith for helpful suggestions and even more helpful experiments, Bettie Sue Masters and her lab for good ideas, good enzyme, and encouragement,

and Steve Gross for his insights and contributions to joint efforts at understanding control in NOS.

10. REFERENCES

1. Furchgott, R. F., Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid activatible factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide, in *Vasoldilation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium*, Vanhouette, P. M., Ed., Raven Press, New York, pp. 401 (1988)

2. Ignarro, L. J., Buga, G. M., Wood, K. S., Byrns, R. E., and Chadhuri, G., Endothelium derived relaxing factor produced and released from artery and vein is nitric oxide, in *Proc. Natl. Acad. Sci. USA*, Vol. 84, pp. 9265 (1987)

3. Palmer, R. M. J., Ferringe, D. S., and Moncada, S., Nitric oxide release accounts for the biological activity of endothelial relaxing factor, *Nature, 327*, 524 (1987)

4. Gally, J. A., Montague, P. R., Reeke, G. N., Jr., and Edelman, G. M., The NO hypothesis: possible effects of a short-lived, rapidly diffusible signal in the development and function of the nervous system, *Proc Natl Acad Sci U S A, 87*, 3547 (1990)

5. Garthwaite, J., Charles, S. L., and Chess-Williams, R., Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain, *Nature, 336*, 385 (1988)

6. Abu-Soud, H. M., and Stuehr, D. J., Nitric oxide synthases reveal a role for calmodulin in controlling electron transfer, *Proc Natl Acad Sci U S A, 90*, 10769 (1993)

7. Bredt, D. S., and Snyder, S. H., Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme, *Proc Natl Acad Sci U S A, 87*, 682 (1990)

8. Bredt, D. S., and Snyder, S. H., Nitric oxide: a physiologic messenger molecule, *Annu Rev Biochem, 63*, 175 (1994)

9. Arnold, W. P., Mittal, C. K., Katsuki, S., and Murad, F., Nitric oxide activates guanylate cyclase and increases guanosine 3':5'- cyclic monophosphate levels in various tissue preparations, *Proc Natl Acad Sci U S A, 74*, 3203 (1977)

10. Craven, P. A., and Derubertis, F. R., Restoration of responsivenessof purified guanylate cyclase to nitrosoguanidine, nitric oxide, and relate activators by heme and heme proteins: evidence for the involvement of the paramagnetic nitrosyl-heme complex in enzyme activation, *J. BIol. Chem., 253*, 8433 (1978)

11. Gruetter, C. A., Relaxation of bovine coronary artery and activation of coronary artery guanylate cyclase by ntric oxide, nitroproside, and a carcinogenic nitrosamine, *J. Cyclic, 5*, 211 (1979)

12. Mayer, B., Schmidt, K., Humbert, P., and Bohme, E., Biosynthesis of endothelium-derived relaxing factor: a cytosolic enzyme in porcine aortic endothelial cells Ca2+-dependently converts L- arginine into an activator of soluble guanylyl cyclase, *Biochem Biophys Res Commun, 164*, 678 (1989)

13. Knowles, R. G., Merrett, M., Salter, M., and Moncada, S., Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat, *Biochem J, 270*, 833 (1990)

14. McCall, T. B., Palmer, R. M. J., and Moncada, S., Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion, *Biochem. J., 262*, 293 (1989)

15. Curran, R. D., Hepatocytes produce nitric oxides from L-arginine in responsew to inflamatory products of Kupffer cells, *J. Exp. Med., 170*, 1769 (1989)

16. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M., Martasek, P., Roman, L. J., Masters, B. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S., An autoinhibitory control element defines calcium-regulated isoforms of nitric oxide synthase, *J Biol Chem, 272*, 29769 (1997)

17. Saeij, J. P., Stet, R. J., Groeneveld, A., Verburg-van Kemenade, L. B., van Muiswinkel, W. B., and Wiegertjes, G. F., Molecular and functional characterization of a fish inducible-type nitric oxide synthase, *Immunogenetics, 51*, 339 (2000)

18. Kun Kim, D., Kyung Hong, E., Ho Lee, K., Il Kim, J., and Keun Song, W., Molecular cloning and expression of nitric oxide synthase gene in chick embryonic muscle cells, *Cell Biochem Funct, 17*, 261 (1999)

19. Korneev, S. A., Piper, M. R., Picot, J., Phillips, R., Korneeva, E. I., and O'Shea, M., Molecular characterization of NOS in a mollusc: expression in a giant modulatory neuron, *J Neurobiol, 35*, 65 (1998)

20. Moroz, L. L., Norby, S. W., Cruz, L., Sweedler, J. V., Gillette, R., and Clarkson, R. B., Non-enzymatic production of nitric oxide (NO) from NO synthase inhibitors, *Biochem Biophys Res Commun, 253*, 571 (1998)

21. Moroz, L. L., Gillette, R., and Sweedler, J. V., Single-cell analyses of nitrergic neurons in simple nervous systems, *J Exp Biol, 202*, 333 (1999)

22. Moroz, L. L., On the origin and early evolution of neuronal NO signaling: A comparative analysis., in *Nitric oxide and free radicals in peripheral neurotransmission.*, S, K., Ed., Springer-Verlag, New York, pp. 1 (2000)

23. Davies, S., Nitric oxide signalling in insects, *Insect Biochem Mol Biol, 30*, 1123 (2000)

24. Golderer, G., Werner, E. R., Leitner, S., Grobner, P., and Werner-Felmayer, G., Nitric oxide synthase is induced in sporulation of Physarum polycephalum, *Genes Dev, 15*, 1299 (2001)

25. Fujita, Y., Ogasawara, N., Sadaie, Y., Fujita, M., Yoshida, K., Yoshikawa, H., Miwa, Y., Yamamoto, H., Sekiguchi, J., Kumano, M., Yamane, K., Murata, M., and Ohki, R., [The genome of Bacillus subtilis and the features of its genes], *Tanpakushitsu Kakusan Koso, 44*, 1449 (1999)

26. White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., Haft, D. H., Gwinn, M. L., Nelson, W. C., Richardson, D. L., Moffat, K. S., Qin, H., Jiang, L., Pamphile, W., Crosby, M., Shen, M., Vamathevan, J. J., Lam, P., McDonald, L., Utterback, T., Zalewski, C., Makarova, K. S., Aravind, L., Daly, M. J., Fraser, C. M., and *et al.*, Genome sequence of the radioresistant bacterium Deinococcus radiodurans R1, *Science, 286*, 1571 (1999)

27. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S.,

Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S. K., Codani, J. J., Connerton, I. F., Danchin, A., and *et al.*, The complete genome sequence of the gram-positive bacterium Bacillus subtilis, *Nature, 390*, 249 (1997)

28. Adak, S., Bilwes, A. M., Panda, K., Hosfield, D., Aulak, K. S., McDonald, J. F., Tainer, J. A., Getzoff, E. D., Crane, B. R., and Stuehr, D. J., Cloning, expression, and characterization of a nitric oxide synthase protein from Deinococcus radiodurans, *Proc Natl Acad Sci U S A, 99*, 107 (2002)

29. Adak, S., Aulak, K. S., and Stuehr, D. J., Direct evidence for nitric oxide production by a nitric-oxide synthase- like protein from Bacillus subtilis, *J Biol Chem, 277*, 16167 (2002)

30. Hibbs, J. B., Taintor, R. R., and Vavrin, Z., Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite., *Science, 235*, 473 (1987)

31. Marletta, M. A., Nitric oxide synthase structure and mechanism, *J Biol Chem, 268*, 12231 (1993)

32. Stuehr, D. J., N-hydroxy-L-arginine is an intermediate in the biosynthesis of NO from arginine, *J. BIol. Chem., 266*, 6259 (1991)

33. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S., Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide, *Proc Natl Acad Sci U S A, 89*, 11141 (1992)

34. Stuehr, D. J., and Ikeda-Saito, M., Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome P-450-like hemeproteins that contain a flavin semiquinone radical, *J Biol Chem, 267*, 20547 (1992)

35. White, K. A., and Marletta, M. A., Nitric oxide synthase is a cytochrome P-450 type hemoprotein, *Biochemistry, 31*, 6627 (1992)

36. Tayeh, M. A., and MArletta, M. A., Macrophage oxidation of L-arginine to NO, nitrate and nitrite, *J. Biol. Chem., 264*, 19654 (1989)

37. Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G., and Bohme, E., Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase, *FEBS Lett, 288*, 187 (1991)

38. Kwon, N. S., Nathan, C. F., and Stuehr, D. J., Reduced biopterin as a cofactor in the generation of NO by murine macrophages, *J Biol Chem, 264*, 20496 (1989)

39. Giovanelli, J., Campos, K. L., and Kaufman, S., Tetrahydrobiopterin, a cofactor for rat cerebellar nitric oxide synthase, does not function as a reactant in the oxygenation of arginine, *Proc Natl Acad Sci U S A, 88*, 7091 (1991)

40. Tzeng, E., Billiar, T. R., Robbins, P. D., Loftus, M., and Stuehr, D. J., Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer, *Proc Natl Acad Sci U S A, 92*, 11771 (1995)

41. Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A., Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen, *Biochemistry, 38*, 15689 (1999)

42. Crane, B. R., Arvai, A. S., Ghosh, S., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A., Structures of the N(omega)-hydroxy-L-arginine complex of inducible nitric oxide synthase oxygenase dimer with active and inactive pterins, *Biochemistry, 39*, 4608 (2000)

43. Hevel, J. M., White, K. A., and Marletta, M. A., Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein, *J Biol Chem, 266*, 22789 (1991)

44. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H., Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase, *Nature, 351*, 714 (1991)

45. Stuehr, D. J., Kwon, N. S., and Nathan, C. F., FAD and GSH participate in macrophage synthesis of nitric oxide, *Biochem Biophys Res Commun, 168*, 558 (1990)

46. Abu-Soud, H. M., Yoho, L. L., and Stuehr, D. J., Calmodulin controls neuronal nitric-oxide synthase by a dual mechanism. Activation of intra- and interdomain electron transfer, *J Biol Chem, 269*, 32047 (1994)

47. Sheta, E. A., McMillan, K., and Masters, B. S., Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase, *J Biol Chem, 269*, 15147 (1994)

48. Masters, B. S., Nitric oxide synthases: why so complex?, *Annu Rev Nutr, 14*, 131 (1994)

49. Masters, B. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P., Neuronal nitric oxide synthase, a modular enzyme formed by convergent evolution: structure studies of a cysteine thiolate-liganded heme protein that hydroxylates L-arginine to produce NO. as a cellular signal [published erratum appears in FASEB J 1996 Jul;10(9):1107], *Faseb J, 10*, 552 (1996)

50. McMillan, K., and Masters, B. S., Prokaryotic expression of the heme- and flavin-binding domains of rat neuronal nitric oxide synthase as distinct polypeptides: identification of the heme-binding proximal thiolate ligand as cysteine-415, *Biochemistry, 34*, 3686 (1995)

51. Masters, B. S., McMillan, K., Nishimura, J., Martasek, P., Roman, L. J., Sheta, E., Gross, S. S., and Salerno, J., Understanding the structural aspects of neuronal nitric oxide synthase (NOS) using microdissection by molecular cloning techniques: molecular dissection of neuronal NOS, *Adv Exp Med Biol, 387*, 163 (1996)

52. Salerno, J. C., Martasek, P., Roman, L. J., and Masters, B. S., Electron paramagnetic resonance spectroscopy of the heme domain of inducible nitric oxide synthase: binding of ligands at the arginine site induces changes in the heme ligation geometry, *Biochemistry, 35*, 7626 (1996)

53. Ghosh, D. K., Abu-Soud, H. M., and Stuehr, D. J., Reconstitution of the second step in NO synthesis using the isolated oxygenase and reductase domains of macrophage NO synthase, *Biochemistry, 34*, 11316 (1995)

54. Ghosh, D. K., and Stuehr, D. J., Macrophage NO synthase: characterization of isolated oxygenase and reductase domains reveals a head-to-head subunit interaction, *Biochemistry, 34*, 801 (1995)

55. Ghosh, D. K., Abu-Soud, H. M., and Stuehr, D. J., Domains of macrophage N(O) synthase have divergent roles in forming and stabilizing the active dimeric enzyme, *Biochemistry, 35*, 1444 (1996)

56. Ghosh, D. K., Wu, C., Pitters, E., Moloney, M., Werner, E. R., Mayer, B., and Stuehr, D. J.,

Characterization of the inducible nitric oxide synthase oxygenase domain identifies a 49 amino acid segment required for subunit dimerization and tetrahydrobiopterin interaction, *Biochemistry, 36*, 10609 (1997)
57. Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., and Bredt, D. S., Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy, *Cell, 82*, 743 (1995)
58. Cho, K. O., Hunt, C. A., and Kennedy, M. B., The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein, *Neuron, 9*, 929 (1992)
59. Jaffrey, S. R., and Snyder, S. H., PIN: an associated protein inhibitor of neuronal nitric oxide synthase, *Science, 274*, 774 (1996)
60. Rodriguez-Crespo, I., Straub, W., Gavilanes, F., and Ortiz de Montellano, P. R., Binding of dynein light chain (PIN) to neuronal nitric oxide synthase in the absence of inhibition, *Arch Biochem Biophys, 359*, 297 (1998)
61. Hemmens, B., Woschitz, S., Pitters, E., Klosch, B., Volker, C., Schmidt, K., and Mayer, B., The protein inhibitor of neuronal nitric oxide synthase (PIN): characterization of its action on pure nitric oxide synthases, *FEBS Lett, 430*, 397 (1998)
62. Chen, P. F., Tsai, A. L., and Wu, K. K., Cysteine 184 of endothelial nitric oxide synthase is involved in heme coordination and catalytic activity, *J Biol Chem, 269*, 25062 (1994)
63. Abu-Soud, H. M., Loftus, M., and Stuehr, D. J., Subunit dissociation and unfolding of macrophage NO synthase: relationship between enzyme structure, prosthetic group binding, and catalytic function, *Biochemistry, 34*, 11167 (1995)
64. Schmidt, H. H., Pollock, J. S., Nakane, M., Gorsky, L. D., Forstermann, U., and Murad, F., Purification of a soluble isoform of guanylyl cyclase-activating-factor synthase, *Proc Natl Acad Sci U S A, 88*, 365 (1991)
65. Siddhanta, U., Presta, A., Fan, B., Wolan, D., Rousseau, D. L., and Stuehr, D. J., Domain swapping in inducible nitric-oxide synthase. Electron transfer occurs between flavin and heme groups located on adjacent subunits in the dimer, *J Biol Chem, 273*, 18950 (1998)
66. McMillan, K., and Masters, B. S., Optical difference spectrophotometry as a probe of rat brain nitric oxide synthase heme-substrate interaction, *Biochemistry, 32*, 9875 (1993)
67. Frey, C., Narayanan, K., McMillan, K., Spack, L., Gross, S. S., Masters, B. S., and Griffith, O. W., L-thiocitrulline. A stereospecific, heme-binding inhibitor of nitric- oxide synthases, *J Biol Chem, 269*, 26083 (1994)
68. Smith, S. M., Sham, C., Roman, L., Martasek, P., and Salerno, J. C., Titration of low K(d) binding sites: binding of arginine analogs to nitric oxide synthases, *Nitric Oxide, 5*, 442 (2001)
69. Tsai, A. L., Berka, V., Chen, P. F., and Palmer, G., Characterization of endothelial nitric-oxide synthase and its reaction with ligand by electron paramagnetic resonance spectroscopy, *J Biol Chem, 271*, 32563 (1996)
70. Presta, A., A.M.Weber-Main, Stankovich, M. T., and Stuehr, D. J., Comparative effects of substrates and pterin cofactor on the heme midpoint potential in inducible and neuronal nitric oxide synthases, *J. Am. Chem. Soc., 120*, 9460 (1998)
71. Salerno, J. C., Frey, C., McMillan, K., Williams, R. F., Masters, B. S., and Griffith, O. W., Characterization by electron paramagnetic resonance of the interactions of L-arginine and L-thiocitrulline with the heme cofactor region of nitric oxide synthase, *J Biol Chem, 270*, 27423 (1995)
72. Salerno, J. C., McMillan, K., and Masters, B. S., Binding of intermediate, product, and substrate analogs to neuronal nitric oxide synthase: ferriheme is sensitive to ligand-specific effects in the L-arginine binding site, *Biochemistry, 35*, 11839 (1996)
73. Salerno, J. C., Martasek, P., Williams, R. F., and Masters, B. S., Substrate and substrate analog binding to endothelial nitric oxide synthase: electron paramagnetic resonance as an isoform-specific probe of the binding mode of substrate analogs, *Biochemistry, 36*, 11821 (1997)
74. Migita, C. T., Salerno, J. C., Masters, B. S., Martasek, P., McMillan, K., and Ikeda-Saito, M., Substrate binding-induced changes in the EPR spectra of the ferrous nitric oxide complexes of neuronal nitric oxide synthase, *Biochemistry, 36*, 10987 (1997)
75. Jung, C., Stuehr, D. J., and Ghosh, D. K., FT-Infrared spectroscopic studies of the iron ligand CO stretch mode of iNOS oxygenase domain: effect of arginine and tetrahydrobiopterin, *Biochemistry, 39*, 10163 (2000)
76. Ingledew, W. J., Smith, S. M., Salerno, J. C., and Rich, P. R., Neuronal nitric oxide synthase ligand and protein vibrations at the substrate binding site. A study by FTIR, *Biochemistry, 41*, 8377 (2002)
77. Abu-Soud, H. M., Gachhui, R., Raushel, F. M., and Stuehr, D. J., The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydrobiopterin on its stability, *J Biol Chem, 272*, 17349 (1997)
78. Bec, N., Gorren, A. C., Voelker, C., Mayer, B., and Lange, R., Reaction of neuronal nitric-oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin, *J Biol Chem, 273*, 13502 (1998)
79. Ledbetter, A. P., McMillan, K., Roman, L. J., Masters, B. S., Dawson, J. H., and Sono, M., Low-temperature stabilization and spectroscopic characterization of the dioxygen complex of the ferrous neuronal nitric oxide synthase oxygenase domain, *Biochemistry, 38*, 8014 (1999)
80. Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko, GA, and Sligar SG. The catalytic pathway of cytochrome p450cam at atomic resolution., Science 287,1615 (2000)
81. Tierney, D. L., Huang, H., Martasek, P., Masters, B. S., Silverman, R. B., and Hoffman, B. M., ENDOR spectroscopic evidence for the position and structure of NG- hydroxy-L-arginine bound to holo-neuronal nitric oxide synthase, *Biochemistry, 38*, 3704 (1999)
82. Wang, J., Rousseau, D. L., Abu-Soud, H. M., and Stuehr, D. J., Heme coordination of NO in NO synthase, *Proc Natl Acad Sci U S A, 91*, 10512 (1994)
83. Wang, J., Stuehr, D. J., and Rousseau, D. L., Tetrahydrobiopterin-deficient nitric oxide synthase has a modified heme environment and forms a cytochrome P-420 analogue, *Biochemistry, 34*, 7080 (1995)
84. Couture, M., Stuehr, D. J., and Rousseau, D. L., The ferrous dioxygen complex of the oxygenase domain

of neuronal nitric- oxide synthase, *J Biol Chem, 275*, 3201 (2000)
85. Fan, B., Wang, J., Stuehr, D. J., and Rousseau, D. L., NO synthase isozymes have distinct substrate binding sites, *Biochemistry, 36*, 12660 (1997)
86. Scheele, J. S., Bruner, E., Kharitonov, V. G., Martasek, P., Roman, L. J., Masters, B. S., Sharma, V. S., and Magde, D., Kinetics of NO ligation with nitric-oxide synthase by flash photolysis and stopped-flow spectrophotometry, *J Biol Chem, 274*, 13105 (1999)
87. Scheele, J. S., Kharitonov, V. G., Martasek, P., Roman, L. J., Sharma, V. S., Masters, B. S., and Magde, D., Kinetics of CO ligation with nitric-oxide synthase by flash photolysis and stopped-flow spectrophotometry, *J Biol Chem, 272*, 12523 (1997)
88. Tetreau, C., Tourbez, M., Gorren, A., Mayer, B., and Lavalette, D., Dynamics of carbon monoxide binding with neuronal nitric oxide synthase, *Biochemistry, 38*, 7210 (1999)
89. Griffith, O. W., and Gross, S. S., Inhibitors of nitric oxide synthase, in *Methods of Nitric Oxide Research*, Stammler, J., Ed., John Wiley. New York (1998)
90. Gross, S. S., Macrophage and endothelial cell nitric oxide synthase: cell type selective inhibition by NG aminoguanidine, NG-nitroarginine, and NG-methyl arginine, *Biochem Biophys Res Commun, 170*, 96 (1990)
91. Babu, B. R., Frey, C., and Griffith, O. W., L-arginine binding to nitric-oxide synthase. The role of H-bonds to the nonreactive guanidinium nitrogens, *J Biol Chem, 274*, 25218 (1999)
92. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S., and Kim, J. J., Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes, *Proc Natl Acad Sci U S A, 94*, 8411 (1997)
93. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A., Structure of nitric oxide synthase oxygenase dimer with pterin and substrate, *Science, 279*, 2121 (1998)
94. Crane, B. R., Arvai, A. S., Gachhui, R., Wu, C., Ghosh, D. K., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A., The structure of nitric oxide synthase oxygenase domain and inhibitor complexes *Science, 278*, 425 (1997)
95. Li, H., Raman, C. S., Glaser, C. B., Blasko, E., Young, T. A., Parkinson, J. F., Whitlow, M., and Poulos, T. L., Crystal structures of zinc-free and -bound heme domain of human inducible nitric-oxide synthase. Implications for dimer stability and comparison with endothelial nitric-oxide synthase, *J Biol Chem, 274*, 21276 (1999)
96. Poulos, T. L., Li, H., and Raman, C. S., Heme-mediated oxygen activation in biology: cytochrome c oxidase and nitric oxide synthase, *Curr Opin Chem Biol, 3*, 131 (1999)
97. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L., Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center, *Cell, 95*, 939 (1998)
98. Fischmann, T. O., Hruza, A., Niu, X. D., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C., Structural characterization of nitric oxide synthase isoforms reveals striking active-site conservation, *Nat Struct Biol, 6*, 233 (1999)
99. Crane, B. R., Rosenfeld, R. J., Arvai, A. S., Ghosh, D. K., Ghosh, S., Tainer, J. A., Stuehr, D. J., and Getzoff, E. D., N-terminal domain swapping and metal ion binding in nitric oxide synthase dimerization, *Embo J, 18*, 6271 (1999)
100. Li, H., Raman, C. S., Martasek, P., Masters, B. S., and Poulos, T. L., Crystallographic studies on endothelial nitric oxide synthase complexed with nitric oxide and mechanism-based inhibitors, *Biochemistry, 40*, 5399 (2001)
101. Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R., Endothelial nitric-oxide synthase. Expression in Escherichia coli, spectroscopic characterization, and role of tetrahydrobiopterin in dimer formation, *J Biol Chem, 271*, 11462 (1996)
102. Toth, M., Kukor, Z., and Sahin-Toth, M., Differential response of basal and tetrahydrobiopterin-stimulated activities of placental type III nitric oxide synthase to sodium dodecyl sulphate: relation to dimeric structure, *Mol Hum Reprod, 4*, 1165 (1998)
103. Alderton, W. K., Cooper, C. E., and Knowles, R. G., Nitric oxide synthases: structure, function and inhibition, *Biochem J, 357*, 593 (2001)
104. Ghosh, D. K., M.B. Rashid, M. B., Crane, B. R., Taskar, V., Mast, M., Misukonis, M. A. J., J.B. Weinberg, and Eissa, N. T., Characterization of Key Residues in the Region Encoded by Exons 8-9 of Human Inducible Nitric Oxide Synthase: A Critical Role for Asp 280 in Substrate Binding and Subunit Interaction., *Proc.Natl.Acad.Sci, USA, 98*, 10392 (2001)
105. Ghosh, D. K., B. R. Crane, S. Ghosh, D. Wolan, R. Gachhui, C. Crooks, A. Presta, J. A. Tainer, E. D. Getzoff, and D. J. Stuehr ; Inducible nitric oxide synthase: role of the N-terminal beta-hairpin hook and pterin-binding segment in dimerization and tetrahydrobiopterin interaction. EMBO J. 18:626(1999)

106. Harris, D., Smith, S. M. E., Brown, C., and Salerno, J. C., Nitric oxide synthases: structure, function, and control, in *Oxygen Homestasis and Its Dynamics*, Ishimura, Y., Ed., Springer-Verlag, Tokyo, pp. 289 (1997)
107. Jarrett, H. W., and Madhavan, R., Calmodulin binding proteins also have a calmodulin like binding site within their structures, *J. Biol. Chem., 266*, 362 (1991)
108. Venema, R. C., Ju, H., Zou, R., Ryan, J. W., and Venema, V. J., Subunit interactions of endothelial nitric-oxide synthase. Comparisons to the neuronal and inducible nitric-oxide synthase isoforms, *J Biol Chem, 272*, 1276 (1997)
109. Hellermann, G. R., and Solomonson, L. P., Calmodulin promotes dimerization of the oxygenase domain of human endothelial nitric-oxide synthase, *J Biol Chem, 272*, 12030 (1997)
110. Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., and Mayer, B., Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS- resistant dimer, *Embo J, 14*, 3687 (1995)
111. Xie, Q. W., Cho, H., Kashiwabara, Y., Baum, M., Weidner, J. R., Elliston, K., Mumford, R., and Nathan, C., Carboxyl terminus of inducible nitric oxide synthase.

Contribution to NADPH binding and enzymatic activity, *J Biol Chem, 269*, 28500 (1994)
112. Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J., Role of reductase domain cluster 1 acidic residues in neuronal nitric- oxide synthase. Characterization of the FMN-FREE enzyme, *J Biol Chem, 274*, 22313 (1999)
113. Cho, H. J., Martin, E., Xie, Q. W., Sassa, S., and Nathan, C., Inducible nitric oxide synthase: identification of amino acid residues essential for dimerization and binding of tetrahydrobiopterin, *Proc Natl Acad Sci U S A, 92*, 11514 (1995)
114. Chen, P. F., Tsai, A. L., and Wu, K. K., Cysteine 99 of endothelial nitric oxide synthase (NOS-III) is critical for tetrahydrobiopterin-dependent NOS-III stability and activity, *Biochem Biophys Res Commun, 215*, 1119 (1995)
115. Sagami, I., and Shimizu, T., The crucial roles of Asp-314 and Thr-315 in the catalytic activation of molecular oxygen by neuronal nitric-oxide synthase. A site-directed mutagenesis study, *J Biol Chem, 273*, 2105 (1998)

116. Rodriguez-Crespo, I., Moenne-Loccoz, P., Loehr, T. M., and Ortiz de Montellano, P. R., Endothelial nitric oxide synthase: modulations of the distal heme site produced by progressive N-terminal deletions, *Biochemistry, 36*, 8530 (1997)
117. Boyhan, A., Smith, D., Charles, I. G., Saqi, M., and Lowe, P. N., Delineation of the arginine- and tetrahydrobiopterin-binding sites of neuronal nitric oxide synthase, *Biochem J, 323*, 131 (1997)
118. Chen, P. F., Tsai, A. L., Berka, V., and Wu, K. K., Endothelial nitric-oxide synthase. Evidence for bidomain structure and successful reconstitution of catalytic activity from two separate domains generated by a baculovirus expression system, *J Biol Chem, 271*, 14631 (1996)
119. Rozhkova, E. A., Fujimoto, N., Sagami, I., Daff, S. N., and Shimizu, T., Interactions between the isolated oxygenase and reductase domains of neuronalnitric-oxide synthase: assessing the role of calmodulin., *J Biol Chem, 277*, 16888 (2002)
120. Mayer, B., John, M., and Bohme, E., Purification of a Ca2+/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin, *FEBS Lett, 277*, 215 (1990)
121. Busse, R., and Mulsch, A., Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin, *FEBS Lett, 265*, 133 (1990)
122. Newton, D. C., Montgomery, H. J., and Guillemette, J. G., The reductase domain of the human inducible nitric oxide synthase is fully active in the absence of bound calmodulin, *Arch Biochem Biophys, 359*, 249 (1998)
123. Cho, H. J., Xie, Q. W., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., and Nathan, C., Calmodulin is a subunit of nitric oxide synthase from macrophages, *J Exp Med, 176*, 599 (1992)
124. Ruan, J., Xie, Q., Hutchinson, N., Cho, H., Wolfe, G. C., and Nathan, C., Inducible nitric oxide synthase requires both the canonical calmodulin- binding domain and additional sequences in order to bind calmodulin and produce nitric oxide in the absence of free Ca2+, *J Biol Chem, 271*, 22679 (1996)
125. Venema, R. C., Sayegh, H. S., Kent, J. D., and Harrison, D. G., Identification, charaterization, and comparison of the calmodulin- binding domains of the endothelial and inducible nitric oxide sythases, *J Biol Chem, 271*, 6435 (1996)
126. Kondo, R., Tikunova, S. B., Cho, M. J., and Johnson, J. D., A point mutation in a plant calmodulin is responsible for its inhibition of nitric-oxide synthase, *J Biol Chem, 274*, 36213 (1999)
127. Su, Z., Blazing, M. A., Fan, D., and George, S. E., The calmodulin-nitric oxide synthase interaction. Critical role of the calmodulin latch domain in enzyme activation, *J Biol Chem, 270*, 29117 (1995)
128. Gachhui, R., Abu-Soud, H. M., Ghosha, D. K., Presta, A., Blazing, M. A., Mayer, B., George, S. E., and Stuehr, D. J., Neuronal nitric-oxide synthase interaction with calmodulin-troponin C chimeras, *J Biol Chem, 273*, 5451 (1998)
129. George, S. E., Su, Z., Fan, D., and Means, A. R., Calmodulin-cardiac troponin C chimeras: effect of domain exchange in calcium binding and enzyme activation, *J. Biol.Chem., 268*, 25213 (1993)
130. Chattopadhyaya, R., W.E.Meador, A.R.Means, and A.F.Quiocho, Calmodulin refined at 1.7Å resolution, *J.Mol.Biol., 228*, 1177 (1992)
131. Strynadka, N. C. J., and James, M. N. G., Crystal structures of the helix -loop-helixcalcium binding proteins., *Annu.Rev.Biochem., 58*, 951 (1989)
132. Nishida, C. R., and Ortiz de Montellano, P. R., Autoinhibition of endothelial nitric-oxide synthase. Identification of an electron transfer control element, *J Biol Chem, 274*, 14692 (1999)
133. Nishida, C. R., P.R.O. de Montellano Nishida CR, d. M. P., among, C. o. e. t. i. n.-o. s.-S. o. a. e., isoforms, n.-o. s., and 2001, J. B. C.-J. J. B. C.-J., Control of electron transfer in nitric-oxide synthases - Swapping of autoinhibitory elements among nitric-oxide synthase isoforms, *J Biol Chem, 276*, 20116 (2001)
134. McMillan, K., Salerno, J. C., and Masters, B. S., Nitric oxide synthases: analogies to cytochrome P450 monooxygenases and characterization of recombinant rat neuronal nitric oxide synthase hemoprotein, *Methods Enzymol, 268*, 460 (1996)
135. Masters, B. S. S., Miller, R. T., Roman, L. J., Nishmura, J. S., Panda, S., Harris, D.E., H., P., Shea, T. M., Zhang, J., Kim, J.-J., and Salerno, J. C., Structural Aspects of the Flavoprotein Domains of Isoforms of Nitric Oxide Synthase in: Flavins and Flavoproteins, in *Proc. 13th Int. Symp. Flavins and Flavoproteins*, Ghisla S, K. P., Macheroux P, Sund H,, Ed., Agency for Scientific Publ., Berlin, Konstanz, Germany (1999)
136. Marcotte, E. M., Pellegrini, M., Ng, H. L., Rice, D. W., Yeates, T. O., and Eisenberg, D., Detecting protein function and protein-protein interactions from genome sequences, *Science, 285*, 751 (1999)
137. Monod, J., From enzymatic adaptation to allosteric transitions, *Science, 154*, 475 (1966)
138. Monod, J., Wyman, J., and Changeux, J. P., On the Nature of Allosteric Transitions: A Plausible Model, *J. Mol. Biol., 12*, 88 (1965)
139. Jarrett, H. W., and Madhavan, R., Calmodulin-binding proteins also have a calmodulin-like binding site within their structure. The flip-flop model, *J Biol Chem, 266*, 362 (1991)

140. Smith, M. K., Colbran, R. J., Brickey, D. A., and Soderling, T. R., Functional determinants in the autoinhibitory domain of calcium/calmodulin-dependent protein kinase II. Role of His282 and multiple basic residues, *J Biol Chem, 267*, 1761 (1992)
141. Page, C. C., Moser, C. C., Chen, X., and Dutton, P. L., Natural engineering principles of electron tunnelling in biological oxidation-reduction, *Nature, 402*, 47 (1999)
142. Moser, C. C., Page, C. C., Farid, R., and Dutton, P. L., Biological electron transfer, *J Bioenerg Biomembr, 27*, 263 (1995)
143. Salerno, J., and Smith, S. M. E., Coupled Equilibria in Nitric Oxide synthases, in *Fourth International Conference on the Biochemistry and Molecular Biology of Nitric Oxide*, Vol. 4, NO Journal (abstract), Los Angeles, CA, pp. 224 (2000)
144. Daff, S., Noble, M. A., Craig, D. H., Rivers, S. L., Chapman, S. K., Munro, A. W., Fujiwara, S., Rozhkova, E., Sagami, I., and Shimizu, T., Control of electron transfer in neuronal NO synthase, *Biochem Soc Trans, 29*, 147 (2001)
145. Dutton, P. L., Leigh, J. S., Jr., and Reed, D. W., Primary events in the photosynthetic reaction centre from Rhodopseudomonas spheroides strain R26: triplet and oxidized states of bacteriochlorophyll and the identification of the primary electron acceptor, *Biochim Biophys Acta, 292*, 654 (1973)
146. Mitchell, P., Vectorial chemiosmotic processes, *Annu Rev Biochem, 46*, 996 (1977)
147. Mitchell, P., The protonmotive Q cycle: a general formulation, *FEBS Lett, 59*, 137 (1975)
148. Salerno, J. C., Cytochrome electron spin resonance line shapes, ligand fields, and components stoichiometry in ubiquinol-cytochrome c oxidoreductase, *J Biol Chem, 259*, 2331 (1984)
149. Yu, C. A., Zhang, L., Deng, K. P., Tian, H., Xia, D., Kim, H., Deisenhofer, J., and Yu, L., Structure and reaction mechanisms of multifunctional mitochondrial cytochrome bc1 complex, *Biofactors, 9*, 103 (1999)
150. Berry, E. A., Guergova-Kuras, M., Huang, L. S., and Crofts, A. R., Structure and function of cytochrome bc complexes, *Annu Rev Biochem, 69*, 1005 (2000)
151. Darrouzet, E., Moser, C. C., Dutton, P. L., and Daldal, F., Large scale domain movement in cytochrome bc(1): a new device for electron transfer in proteins, *Trends Biochem Sci, 26*, 445 (2001)
152. Zhang, J., Martasek, P., Paschke, R., Shea, T., Masters, B. S. S., and Kim, J. J. P., Crystal structure of the FAD/NADPH-binding domain of rat neuronal nitric-oxide synthase -Comparisons with NADPH-cytochrome P450 oxidoreductase, *J. Biol. Chem., 276*, 37506 (2001)
153. Porter, T. D., and Kasper, C. B., NADPH-cytochrome P450 reductase: FMN and FAD domains evolved from different flavoproteins, *Biochemistry, 25*, 1682 (1986)
154. Lane, P., and Gross, S. S., Disabling a C-terminal autoinhibitory control element in endothelial nitric-oxide synthase by phosphorylation provides a molecular explanation for activation of vascular NO synthesis by diverse physiological stimuli, *J Biol Chem, 277*, 19087 (2002)
155. Roman, L. J., Martasek, P., and Masters, B. S. S., Intrinsic and extrinsic modulation of nitric oxide synthase activity, *Chemical Reviews, 102*, 1179 (2002)
156. Daff, S., Craig, D., and Chapman, S., Calmodulin activates neuronal NO synthase reductase complex by releasing a tight NADPH-dependent conformational lock, *Nitric Oxide, 6*, 366 (2002)
157. Lambeth, J. D., Seybert, D. W., Lancaster, J. R., Jr., Salerno, J. C., and Kamin, H., Steroidogenic electron transport in adrenal cortex mitochondria, *Mol Cell Biochem, 45*, 13 (1982)

Key Words: Nitric Oxide, NO synthase, H4-biopterin, X-ray structure, Domain alignment, Flavoprotein reductase CaM, Structure-function , EPR, Arginine analogs , NO synthase Inhibitors, Review

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