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Structural and mechanistic aspects of flavoproteins: electron transfer through the nitric oxide synthase flavoprotein domain

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Abstract

Nitric oxide synthases belong to a family of dual-flavin enzymes that transfer electrons from NAD(P)H to a variety of heme protein acceptors. During catalysis, their FMN subdomain plays a central role by acting as both an electron acceptor (receiving electrons from FAD) and an electron donor, and is thought to undergo large conformational movements and engage in two distinct protein–protein interactions in the process. This minireview summarizes what we know about the many factors regulating niric oxide synthase flavoprotein domain function, primarily from the viewpoint of how they impact electron input/output and conformational behaviors of the FMN subdomain.

Keywords

conformational equilibrium; electron flux; electron transfer; flavoprotein; global kinetic model; heme protein; heme reduction; nitric oxide; protein–protein interaction; semiquinone

Introduction

Flavoproteins are a versatile group of biological catalysts that may represent 1–3% of all genes in prokaryotic and eukaryotic genomes [1,2]. Nitric oxide synthases (NOS; EC 1.14.13.39) are members of a dual-flavin reductase family, which transfer electrons from NADPH to a variety of heme protein acceptors [3–5]. The electron transfer occurs in a linear manner from NADPH to FAD to FMN. During catalysis, the FMN subdomain plays a central role by acting as both an electron acceptor (receiving an electron from $FADH_2$) and an electron donor (transferring an electron typically from $FMNH^-$), and is thought to undergo large conformational movements in the process. How this process occurs and is regulated in dual-flavin enzymes like NOS is a topic of current interest.

Characteristics of NOS

NOS enzymes catalyze the NADPH- and O_2-dependent conversion of L-arginine (Arg) to citrulline and nitric oxide (NO) via the intermediate N-hydroxyarginine (Scheme 1) [6–9]. There are three mammalian NOS enzymes: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). nNOS and eNOS are reversibly activated by the Ca^{2+}-binding protein calmodulin (CaM) to enable their participation in biological signaling cascades. By contrast, iNOS binds CaM regardless of the Ca^{2+} concentration and can remain continuously active [7,10].

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NOS enzymes are homodimers (Fig. 1). Their subunits are modular and are comprised of an N-terminal 'oxygenase domain' (NOSoxy) that binds iron protoporphyrin IX (heme), (*6R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) and Arg, and a C-terminal flavoprotein or reductase domain that binds NADPH, FAD and FMN. The two domains are separated by a CaM-binding motif. During catalysis, NADPH-derived electrons transfer into the FAD and FMN in each NOS subunit and then on to the ferric heme in the partner subunits of the homodimer (Fig. 1). Heme reduction, which is rate limiting for NO synthesis [11–13], enables O_2 binding and substrate oxidation to occur within the NOSoxy domain [14–16]. The individual NOS domains and subdomains can be expressed separately, which has facilitated biochemical and structural studies. The protein structural elements that bind heme, Arg, H_4B, CaM, NADPH, FAD and FMN have been identified based on crystallography, mutagenesis and homology studies [17–22].

NOS enzymes have novel features

NOS are heme-thiolate enzymes and catalyze oxygen activation by a mechanism similar to that of the cytochrome P450 (CYP) enzymes (Fig. 2). The oxygen activation involves a two-step heme reduction with protons donated to help break the O–O bond and generate reactive heme-oxy enzyme species. However, in NOS, the second electron is provided to the heme-dioxy species by a bound H_4B cofactor rather than by the flavoprotein domain [16]. The H_4B radical is then reduced within the enzyme by the flavoprotein domain in order to continue catalysis [23]. NOSoxy domains also have a unique protein fold compared with CYPs, a shorter heme-binding loop and a distinct proximal heme environment with different hydrogen bonding to the cysteine heme ligand [17–19,24]. The attached flavoprotein and heme domains of NOS are also an unusual feature shared by only a handful of prokaryotic CYP proteins [4,8,25].

In comparison, the NOS flavoprotein domain is related to a family of dual-flavin enzymes that contain FAD and FMN, and transfer NADPH-derived electrons to separate hemeprotein partners or to attached heme domains [5,14,20,22,26]. Other members from eukaryotes include cytochrome P450 reductase (CYPR) and methionine synthase reductase. Typically (except bacterial CYP_{BM3}), these flavoproteins are isolated in their 1-electron reduced forms containing oxidized FAD and a stable FMN semiquinone radical ($FMNH^{\bullet}$). After reduction by NADPH occurs, they utilize a 3-2-1 electron-transfer cycle in which their FMN group redox cycles between its electron-accepting semiquinone form ($FMNH^{-}$) and its fully reduced, electron-donating hydroquinone form ($FMNH_2$ or $FMNH^{-}$). However, the NOS flavoprotein displays a number of unique features within this enzyme family. These include NOS electron-transfer reactions being suppressed in the native state by up to three unique protein regulatory inserts: an autoinhibitory insert in the FMN domain [27–30], a C-terminal tail (CT) [31–33] and possibly a small insertion or β-finger in the connecting domain [34,35] (Fig. 3A,B). CaM binding to NOS relieves the suppression at three points in the electron-transfer sequence [36–40] (Fig. 3C). NOS electron- transfer activity can also be impacted by phosphorylation [41–46] and by extrinsic proteins like caveolin-1 [47,48], dynamin-2 [49] and heat-shock protein 90 [50]. Finally, NOS enzyme activity is controlled by self-generated NO, which binds to the NOS heme as an intrinsic feature of catalysis [12,13,51] (Fig. 4). This forces the NOS heme reduction rate (k_r in Fig. 4) to remain relatively slow in order to minimize an inherent NO dioxygenase activity in NOS that destroys the NO it makes (futile cycle, Fig. 4).

In summary, NOS enzymes display at least four features that distinguish them from other dual-flavin and heme-thiolate enzymes: (a) the FMN subdomain interacts with its partner donor and acceptor domains all within an enzyme dimer; (b) electron transfer is suppressed in the basal state and the suppression is relieved by CaM binding; (c) bound H_4B provides the second electron for oxygen activation in place of the flavoprotein, and then redox cycles within NOS; and (d) heme–NO binding is an intrinsic feature of catalysis that constrains the rate of heme

reduction by the flavoprotein domain. How these features shape NOS flavoprotein domain function is discussed below.

Key function of the FMN subdomain

Figure 5 depicts a three-state, two-equilibrium model that can describe FMN subdomain function in a NOS dimer. The FMN subdomain receives electrons from the NADPH/FAD subdomain (FNR) in subunit 1 (green), and then shuttles electrons to the NOSoxy domain in subunit 2 (black). This process is thought to require relatively large (70 Å) movement of the FMN subdomain [22], and to involve two reversible and temporally distinct protein binding interactions:

Equilibrium A describes the FNR–FMN subdomain interaction that is required to generate $FMNH^-$ or $FMNH_2$:

$$FADH_2\ (\text{or}\ FADH^\bullet) + FMNH^\bullet \leftrightarrow FADH^\bullet\ (\text{or}\ FAD) + FMNH_2\ (\text{or}\ FMNH^-)$$

Equilibrium B describes the FMN–NOSoxy interaction that enables heme reduction:

$$FMNH^- + Fe^{3+}\ \text{heme} \leftrightarrow FMNH^\bullet + Fe^{2+}\ \text{heme}$$

Large movements of the FMN subdomain are constrained by two hinge elements (green, H1 & H2) that connect it to the electron-donating (FNR) and electron-accepting (NOSoxy) components within the NOS dimer. The CaM-binding site (gray box) in the H2 hinge enables CaM to influence the movements. The same face on the FMN subdomain (red) is expected to interact with each partner subdomain to receive and give electrons. Thus, at either end of a larger movement, the FMN subdomain likely engages in distinct short-range conformational sampling motions with each of its partner subdomains [52,53]. Basic tenets of this model have previously been used to describe FMN subdomain function in other dual-flavin enzymes that shuttle electrons to hemeprotein partners [54,55] and even across subunits as in the dimeric CYPR–BM3 [56,57].

Studying conformational equilibrium A

Equilibrium A is critical because it helps define electron entry and exit from the FMN subdomain. Obtaining the K_{eq}A and associated k_{on} and k_{off} kinetic parameters for the FNR–FMN subdomain complex is a worthwhile and important goal. To date, conformational studies on the NOS flavoprotein domain have involved ensemble measures with the bound FMN poised in its oxidized, semiquinone and hydroquinone states. These studies measured fluorescence intensity of the oxidized flavins, the interaction of bound $FMNH^\bullet$ with a soluble paramagnetic agent by EPR spectroscopy, and rates and extent of reaction of bound $FMNH_2$ ($FMNH^-$) with cytochrome *c* in single turnover or pre-steady-state conditions by stopped-flow spectroscopy [58–62]. In general, these methods can report on any dual-flavin enzyme that is poised in the 0-, 1- and 4-electron reduced states which, practically speaking, are the reduced states most attainable for experimentation. Some strengths and limitations of the measures have been discussed recently [63]. The flavin fluorescence and EPR methods provide semiquantitative information regarding equilibrium A that is useful for comparative studies, whereas the stopped-flow/cytochrome *c* method can provide quantitative estimates of K_{eq}A and in some cases measures of k_{off} for the FMN subdomain (Fig. 5), as recently reported for eNOS and nNOS (described below) [58]. Experimentally, it is challenging to study equilibrium A because dual-flavin enzymes are difficult to poise in all the intermediate states that are likely to be populated during catalysis. For example, this includes the 2- and 3-electron reduced state,

with accompanying variations in NADP(H) binding site occupancy. Recently, Salerno and colleagues discussed a kinetic modeling approach that might help to address these issues [64].

Electron flux and equilibrium A

In general, electron flux through a protein depends on the rates of electron input and output, with either process being rate limiting. In the case of the NOS flavoprotein (or for dual-flavin enzymes in general), the question becomes, how is the electron flux affected by the rate of $FMNH_2$ formation and by the rate of $FMNH_2$ (or $FMNH^-$) reaction with the electron acceptor? Electron flux through NOS enzymes can be measured by the steady-state activities of cytochrome *c* reduction, NO synthesis and/or accompanying rates of NADPH or O_2 consumption. Among these, cytochrome *c* reductase activity is the most straightforward way to measure electron flux through the flavoprotein domain. This is because cytochrome *c* is reduced very slowly by the FNR subdomain [62], and instead is reduced by the FMN subdomain only when it contains $FMNH_2$ or $FMNH^-$, in a quasi-irreversible single-electron transfer reaction that is rapid, not rate limiting, and that can occur only when the FMN subdomain is in an open or deshielded conformation away from its partner subdomains [58, 59,63]. By contrast, electron flux measures that rely on a 'downstream' event like NOS heme reduction (or subsequent NO synthesis activity) are more complicated to interpret, because heme reduction is relatively slow, CaM dependent and subject to thermodynamic constraints [65], and NO synthesis activity is a culmination of many steps that are prone to influences beyond conformational equilibrium A [51].

The features that make cytochrome *c* reductase activity an excellent measure of electron flux also make it a useful predictor (but never proof) of changes in equilibrium A in dual-flavin enzymes. Figure 6 contains curves showing how electron flux through the FMN subdomain of a dual-flavin enzyme, as measured by cytochrome *c* reductase activity, might change with the value of $K_{eq}A$, according to a simple kinetic model (Fig. 6A). One can compare the model with the equilibrium A that is depicted in Fig. 5, with $k_1 = k_{off}$ and $k_2 = k_{on}$. The calculated k_{obs} values shown in Fig. 6B assume that there are fast rates of electron input (k_3) and output (k_4 = 1000 s^{-1}) relative to the rates of conformational change for the FMN subdomain ($k_1 + k_2$ = 10 s^{-1}), and also that any change in the FMN redox state ($FMNH_2$ versus $FMNH^\bullet$) does not change the k_1 or k_2 values. Each curve in Fig. 6 was calculated using a different electron input rate (k_3, the rate of $FMNH_2$ formation). Calculations of the concentrations of each species with time were carried out using GEPASI v. 3.30 [66]. The model predicts that there is always a K_{eq} position for maximum electron flux through the enzyme. On either side of this optimum, the electron flux drops off because either the formation rate (k_2) or dissociation rate (k_1) of the FNR–FMN subdomain complex becomes slower. At relatively fast rates of $FMNH_2$ formation, electron flux through the flavoprotein is primarily a function of the rates of conformational change (k_1, k_2) that determine $K_{eq}A$. However, when the rate of conformational change begins to approach the rate of $FMNH_2$ formation (either from speeding up k_1 and k_2 or by slowing $FMNH_2$ formation), then the rate of electron input (k_3) becomes an important factor for governing the electron flux, and consequently electron flux would be more sensitive to changes in the rate of $FMNH_2$ formation. Thus, one could envision three ways that electron flux through the FMN subdomain might be controlled in a dual-flavin enzyme: changing the ratio or speed of k_1 and k_2, changing the rate of $FMNH_2$ formation or by a combination of these effects. In addition, further tuning could be achieved if the changes in the FMN redox state that occur during catalysis ($FMNH_2$ versus $FMNH^\bullet$) do cause the k_1 or k_2 values to change.

Factors that may modulate equilibrium A and/or the $FMNH_2$ formation rate

Although the model in Fig. 6 is conceptually useful, the situation is more complicated in dual-flavin enzymes because of a number of factors, including the k_1 and k_2 of $K_{eq}A$ possibly being influenced by changes in the FAD and FMN reduction state or by changes in NADP(H)-binding site occupancy during catalysis. Another factor to consider is the thermodynamic driving force to generate $FMNH_2$. The midpoint potential of the $FMNH_2$/FMNH couple in NOS enzymes (and in most other dual-flavin enzymes) is similar to the $FADH_2$/FADH couple and is somewhat more negative than the FADH/FAD couple [67]. These data indicate that a relatively poor driving force exists for $FMNH_2$ buildup, which then occurs to different incomplete extents as the flavoprotein cycles through its 3- and 2-electron reduced states during catalysis. This, in turn, can impact the rate of electron exit and flux through the flavoprotein. Two studies have investigated how changes in the flavin midpoint potentials may alter $FMNH_2$ formation and the resultant electron flux through the FMN subdomain of NOS [68,69].

Factors that may alter equilibrium A conformational rates k_1 and k_2 and/or alter the rate of electron input into the NOS flavoprotein are listed in Table 1. These include proteins, small molecules, NOS regulatory inserts and point mutations. For many of the factors, our only indication currently that they might alter $K_{eq}A$ is from their changing the steady-state cytochrome *c* reductase activity. Thus, more work needs to be done to obtain measures of $K_{eq}A$ and the associated k_1 and k_2 values for dual-flavin enzymes, particularly when they are poised in all catalytically relevant intermediate redox states (1-, 2-, or 3-electron reduced), perhaps ultimately using single molecule spectroscopic approaches.

Relationship between CT, bound NADPH and equilibrium A in NOS

Among the factors listed in Table 1, only the roles of CaM, the CT and bound NADPH have been studied in detail. An interesting and possibly novel connection appears to link regulation of $K_{eq}A$ by the CT and bound NADPH. Basically, the CT of nNOS and eNOS contain a conserved Arg residue whose side chain makes a salt bridge interaction with the 2′-phosphate of bound NADP(H) [22]. Mutagenesis studies suggest that this interaction helps transduce the effect of bound NADP(H) on $K_{eq}A$ (it causes $K_{eq}A$ to decrease), presumably by strengthening the CT to act as a clasp for the FMN subdomain [60]. NADP(H) binding may have a similar influence on $K_{eq}A$ in the related enzyme CYPR [55,70], although it has no CT regulatory element. This suggests that multiple modes of regulation are in play, even for the relatively fundamental circumstance of NADP(H) binding. Some other modes have been explored in the FNR, CYPR and NOS enzymes [61,71–76].

Is there a correlation between NOS reductase activity and $K_{eq}A$?

That a relationship exists between the $K_{eq}A$ and the cytochrome *c* reductase activity of the CaM-free reductase domain of neuronal NOS (nNOSr) was first considered based on measures taken with the 4-electron reduced nNOS flavoprotein in three different states (NADPH-free/CaM-free, NADPH-bound/CaM-free and CaM-bound) [59]. Subsequent measures made with CT point mutants of nNOS (R1400E, R1400S or F1395S) [60,61], and nNOS mutants possessing graded CT truncations [33], allowed the relationship to be examined over a wider range of $K_{eq}A$ than was previously possible. Figure 7 shows that a good correlation appears to exist ($R = 0.96$) between the cytochrome *c* reductase activities of the various CaM-free nNOS flavoproteins and their degree of FMN deshielding, which is directly related to the $K_{eq}A$ for each flavoprotein (greater FMN deshielding = higher $K_{eq}A$). Curiously, several of the CaM-free mutant enzymes depicted in Fig. 7 appear to be in a super-deshielded state compared with the CaM-bound wild-type nNOSr. This may be at odds with more recent data [63,77], indicating that the FMN deshielding level in CaMbound nNOSr is near its maximal value, because it is similar in magnitude to the isolated FMN subdomain, which should exhibit the

maximal possible FMN deshielding. This discrepancy may reflect the inherent difficulty in precisely measuring FMN shielding and the value for K_{eq}A in nNOS, because of its small dynamic range (FMN shielding values only range between 50 and near 100%) [63,77]. At this point, the data suggest that K_{eq}A and the associated k_{on} and k_{off} conformational rates are primary factors in regulating the cytochrome *c* reductase activity of NOS enzymes, particularly in the CaM-free state.

Do the conformational motions describing equilibrium A limit electron flux through NOS enzymes?

Daff and colleagues [59] first proposed that the conformational opening of the FNR–FMN subdomain complex (k_{off} in Fig. 5 and k_1 in Fig. 6) might limit electron flux through the NOS flavoprotein, and they presented the first data to support such a mechanism. There have been several subsequent investigations, culminating in a recent report by Ilagan *et al.* [58] that provides the first ensemble rate measures (Table 2) for the conformational steps in the nNOS and eNOS flavoproteins (dissociation and association of the 4-electron reduced FNR–FMN subunit complex, k_{on} and k_{off} in Fig. 5). Remarkably, the results suggest that k_{off} is the sole kinetic parameter that limits steady-state electron flux to cytochrome *c* for both the CaM-free eNOS and nNOS flavoproteins (Table 2). So the answer to the question posed above is yes, in the CaM-free nNOS and eNOS, the rate of $FMNH_2$ formation appears to be relatively fast and not rate limiting, and instead a specific conformational step (k_{off}, dissociation of the reduced FMN subdomain) is rate limiting for cytochrome *c* reductase activity. How these conformational movements are regulated in NOS, and whether similar conformational motions may limit electron flux through other dual-flavin enzymes, are exciting questions that could be approached through similar experimental means.

Does the rate of electron input (rate of $FMNH_2$ formation) limit electron flux through NOS enzymes?

As noted above, for CaM-free eNOS and nNOS, the answer to this question appears to be no [58]. But in the CaM-bound enzymes, or in other dual-flavin enzymes, it remains an open question. Electron input into NOS has been studied by monitoring flavin reduction kinetics [33,60–62,78]. Hydride transfer from NADPH to FAD is relatively fast and does not limit the rate of $FMNH_2$ formation or electron flux through NOS, except in mutants that retard this hydride transfer [62]. FMN reduction is often difficult to discern because of its similar spectral properties to the bound FAD. In addition, the observed rate of FMN reduction in a dual-flavin enzyme may depend to a variable extent on the K_{eq}A parameter k_{on}, which is the formation rate of the FNR–FMN subdomain complex (Fig. 5). The kinetics of interflavin electron transfer (FAD and FMN) in dual-flavin enzymes has been studied using a T-jump method [79] and by observing rates of flavin semiquinone formation ($FADH^{\bullet}$ and/or $FMNH^{\bullet}$) during pre-equilibrium reduction reactions with NADPH [61,80–85]. In such studies, k_{obs} ranged from 20 to 100 s^{-1} at 10°C for nNOS, but appeared to be slower in eNOS. Several factors appear to influence the rate of flavin reduction in NOS enzymes (Table 1). CaM increased the rates of NOS flavin reduction in most studies. The mechanism appears to involve specific domains of CaM [86,87]. A faster interflavin electron transfer may conceivably help explain how CaM increases electron flux through NOS enzymes (cytochrome *c* reductase activity; the effect of changing k_3 in Fig. 6). Indeed, some correlation exists between the rates of flavin reduction and the cytochrome *c* reductase activity of nNOS bound to a series of CaM analogs [88,89]. However, it is difficult to interpret these data because a means to exclusively alter the rate of $FMNH_2$ formation without causing coincident changes in conformational equilibrium A and in the k_{on} and k_{off} parameters is still unavailable. Indeed, CaM shifts equilibrium A in NOS enzymes to the more open conformation, and therefore likely increases the k_{off} parameter of

equilibrium A [58] and may possibly increase the k_{on} parameter as well. Unfortunately, the shift in K_{eq}A caused by CaM prevented an accurate measure of the k_{off} parameter in CaM-bound eNOS and nNOS [58], and thus prevented assessment of the relative importance of conformational change rates versus rates of $FMNH_2$ formation in limiting electron flux through the CaM-bound NOS enzymes. In general, as the k_{on} and k_{off} of equilibrium A increase, it becomes more probable that the rate of electron input (specifically, $FMNH_2$ formation) or some other step like $NADP^+$ release, as in F1395S nNOS [61] and the analogous CYPR mutants [73], will limit electron flux through NOS or other dual-flavin enzymes. Further work should continue to clarify this issue.

Creating an intrinsic set point for equilibrium A

Common structural features in dual-flavin enzymes may determine their set points for K_{eq}A. Among these are complementary charge pairing interactions that are present to various extents in the FNR–FMN subdomain interface, including the interface in NOS (Fig. 8). Point mutations that neutralize charge pairing or introduce charge-repelling interactions may increase the K_{eq}A set point to various degrees, at least as judged by the increase in cytochrome *c* reductase activity that they cause [90]. Remarkably, CaM-free eNOS and nNOS have significantly different set points for K_{eq}A [58] (Table 2), but CaM binding shifted the K_{eq}A of eNOS to a value closer to that of nNOS (Table 2). Their different basal set points for equilibrium A explain why eNOS has much slower electron flux through its FMN subdomain (as measured by cytochrome *c* reductase activity) [58]. The structural basis for their different set points is unclear at this point, but may certainly involve apparent differences in their CT and autoinhibitory insert elements, or elsewhere in the enzyme.

Changing the set point for K_{eq}A may influence electron flux through the NOS flavoprotein in interesting ways (Fig. 6). For example, the basal set points of eNOS and nNOS, although different from one another, appear to both lie to the left of their optimum, and support a suboptimal electron flux. CaM binding shifts their K_{eq}A set points to a value that supports increased electron flux. According to this model, introducing a mutation that shifts the intrinsic set point, say, by weakening the FNR–FMN subunit interaction, would be expected to boost electron flux through either of the CaM-free NOS enzymes. However, this is only true to a point, because the mutation could conceivably cause the K_{eq}A to shift so far that upon CaM binding, the mutant K_{eq}A would lie beyond the optimum, and therefore would actually support a slower electron flux in the CaM-bound versus CaM-free state. Real-life examples may already exist, in particular the FNR–FMN subdomain interface mutant R1229E nNOS [77] and the nNOS CT truncation mutant tr1397 [32,33]. In these cases, the rate of $FMNH_2$ formation may be limited by a conformational change, namely, the k_{on} for FNR–FMN subdomain complex formation may be so slow that it becomes rate limiting for $FMNH_2$ formation during the steady state (also see k_2 in Fig. 6A). A means to measure the reduction state of the bound FMN ($FMNH_2$ versus $FMNH^{\bullet}$) during steady-state catalysis in dual-flavin enzymes would be generally useful, as was done in other flavoproteins modified to contain reporter flavin analogs [91]. In any case, the set point for K_{eq}A is a fundamental parameter whose varied settings [58] could both up- and downregulate electron flux through the dual-flavin enzymes.

Conformational equilibrium B

We know comparatively little about the FMN–NOSoxy interaction and the associated equilibrium described by K_{eq}B (Fig. 5). A crystal structure of this domain–domain interaction is not available. Nevertheless, a conserved electropositive surface on the NOSoxy domain is proposed to provide a potential docking site for the FMN subdomain [18], and this idea is supported by limited mutagenesis studies [92]. Combining the known structures of nNOS flavoprotein, the NOSoxy dimer and CaM when it is bound to the eNOS binding peptide,

Garcin *et al.* [22] constructed a model for full-length nNOS that indicates that an allowable large motion of the FMN module could bring the FMN cofactor within an acceptable electron-transfer distance from the heme in the partner NOSoxy domain. Although this model suggests feasibility, whether it is an accurate depiction of the FMN–NOSoxy interaction is still unclear. However, recent crystal structures of CYPR mutants now support the feasibility of the long-range movement that is required for the FMN subdomain to support heme reduction in NOS enzymes [85].

Measuring the FMN–NOSoxy interaction and $K_{eq}B$

NO synthesis activity is too complex to be a reliable indicator of the FMN–NOSoxy interaction. Measuring heme reduction is better but is still indirect and may have inherent limitations. Measuring the rate and extent of back electron transfer from the ferrous NOS heme to FMNsq following flash photolysis of CO can indicate precisely the rate of electron transfer, but cannot reveal the extent of the FMN–NOSoxy interaction [93–95]. Recently, Ilagan *et al.* [63] investigated $K_{eq}B$ by studying single-turnover electron-transfer reactions between a fully-reduced FMN–NOSoxy construct of nNOS and excess cytochrome *c*. Their evidence shows that $K_{eq}B$ is poised at values far below unity in nNOS, such that the dissociated conformation predominates and the $K_{eq}B$ value is little changed in the presence or absence of bound CaM. Thus, broad differences appear to exist in the set points of $K_{eq}A$ and $K_{eq}B$ in NOS enzymes, and in how the two set points are regulated. The FMN–NOSoxy complex formation described by $K_{eq}B$ appears to be infrequent and/or transient in practically all circumstances, such that the FMN subdomain may interact far less with NOSoxy than it does with the FNR subdomain in a NOS homodimer. These concepts are consistent with the poor ability of isolated nNOS flavoprotein and nNOSoxy domains to interact with one another and catalyze heme reduction or NO synthesis when they are mixed together [96], and is consistent with NOS enzymes having slow rates of heme reduction compared with other flavo-heme proteins [51]. Moreover, this likely distinguishes NOS from related flavoproteins that do not have attached heme acceptor domains and thus make higher affinity interactions between their FMN subdomains and their detached electron acceptor partners (e.g. the interaction of CYPR with heme oxygenase 1) [97,98]. Additional measurements of $K_{eq}B$ and the associated conformational rates in NOS enzymes will certainly improve our understanding of this essential FMN subdomain interaction.

Relationship of $K_{eq}A$ to equilibrium B and to NOS heme reduction

At the limit, $K_{eq}A$ can impact $K_{eq}B$, heme reduction and NO synthesis because the reduced FMN subdomain must become dissociated from the FNR sub-domain in order to interact with NOSoxy and to reduce the heme (Fig. 5). However, the lowest possible rates for the FMN subdomain dissociation step (k_{off}) in the CaM-bound eNOS and nNOS are ~1 and 20 s^{-1}, respectively [58] (Table 2), and these rates are still 4–10 times faster than the observed rates of heme reduction in the CaM-bound eNOS or nNOS at the same temperature and conditions (0.1 and 5 s^{-1}, respectively) [99,100]. This indicates that the electron transfer from the reduced FMN subdomain to the NOS heme is considerably less efficient than is its electron transfer to cytochrome *c*, which has turnover numbers of 1 and 20 s^{-1} for CaM-bound eNOS and nNOS, respectively, under the same conditions [58]. Indeed, greatly increasing the $K_{eq}A$ in nNOS via CT truncations enables only a small NO synthesis by the CaM-free enzyme [33]. This, and a variety of other evidence [33,51,68,90,99,101–103] suggest that shifting $K_{eq}A$ toward the FMN-deshielded state is not enough on its own to support heme reduction and NO synthesis in nNOS. Instead, additional and distinct effects on the FMN–NOSoxy interaction must be required, and the effects of CaM binding cannot be totally ascribed to the flavoprotein domain as suggested by others. Interestingly, these additional CaM effects need not cause a significant change in $K_{eq}B$ [63], but could rather have more subtle effects on structural elements that

restrict motions of the FMN subdomain or present physical barriers that prevent the FMN subdomain from docking in a subset of conformations that allow electron transfer to the NOSoxy heme.

Factors that may regulate equilibrium B

Table 1 lists factors that may influence K_{eq}B in NOS enzymes, mostly as indicated by their effects on NO synthesis activity or on the heme reduction rate. A few are discussed below.

Calmodulin

CaM has been assumed to promote the FMN–NOSoxy interaction, as judged by its ability to trigger NOS heme reduction and NO synthesis. Early hypotheses that the autoinhibitory insert and CT elements were critical in the process are not supported by deletion studies showing that NOS mutants missing either one or both of these control elements for the most part require CaM for NO synthesis, and then achieve an NO synthesis activity that is ≥ 50% of wild-type [28,30,31,102,104,105]. Studies with CaM variants [60,86–89,106–110] indicate that several structural features of CaM may be important. However, the recent results of Ilagan *et al.* [63] suggest that CaM binding may not alter K_{eq}B to a great extent, implying it may primarily function through additional mechanisms.

Connecting hinge domains

The composition of the two hinges that connect the FMN subdomain in NOS enzymes (H1 and H2 in Fig. 5) defines the allowable movements of the FMN subdomain and thus controls the FMN–NOSoxy interaction (equilibrium B). This in turn may greatly impact the extent and rate of heme reduction in NOS enzymes. Precedent includes flavocytochrome b_2, where altering its hinge length caused a 10-fold change in the heme reduction rate [111–114]. The FMN–FNR subdomain hinge (H1 in Fig. 5) is one of the least conserved motifs and is shorter in eNOS than in nNOS. Swapping the H1 hinge of nNOS into eNOS increased its heme reduction rate and increased its NO synthesis activity fourfold [99]. This confirms that the NOS H1 is a structural element that helps define the FMN–NOSoxy interaction, but whether it impacts K_{eq}B is still unclear. Analogous studies have been carried out on the H1 hinge of CYPR [55,85].

Challenge of H_4B reduction

During NO synthesis, the NOS FMN subdomain must provide an electron to reduce the ferric heme and the H_4B radical at two distinct points during the catalytic cycle (Fig. 2). A recent study found that reduction of the H_4B radical in nNOS requires CaM binding and occurs at a rate similar to ferric heme reduction [23]. These results, along with distance constraints suggesting that direct electron transfer from the FMN subdomain to the H_4B radical would be too slow, led the authors to propose a through-heme model for H_4B radical reduction by the FMN subdomain in NOS (Fig. 9). This mechanism essentially has the heme porphyrin ring acting as a wire to deliver an electron from the FMN subdomain to the H_4B radical. It eliminates the problem of electron transfer over a long distance, and also eliminates the need to invoke a separate docking site for the FMN subdomain on NOSoxy or the need for the flavoprotein to sense when an electron is required by the heme versus the H_4B radical at discreet steps in the reaction cycle (Fig. 2). Because reduction of the H_4B radical presents a novel function for the FMN subdomain, it will be important to further test the validity, kinetics and thermodynamics of the through-heme pathway in NOS enzymes.

Conclusions

Although the NOS flavoprotein domain has fundamental structural, thermodynamic and mechanistic features in common with the dual-flavin family of reductases, there are unique aspects related to NO synthesis that constrain and shape its function. Both common and unique features govern electron flux through the NOS flavoprotein domain. Many of these appear to act by influencing a conformational equilibrium (K_{eq}A) that defines the interaction between the FMN subdomain and the FNR subdomain, although some may also influence the rate of electron import into the FMN subdomain and the resulting formation of $FMNH_2$. The extent to which K_{eq}A or the rate of $FMNH_2$ formation influences electron flux through the NOS flavoprotein can vary depending on the circumstances. However, the K_{eq}A, and specifically the dissociation rate of the reduced FMN subdomain, appears to be the primary factor that determines electron flux through the CaM-free nNOS and eNOS flavoproteins. A second conformational equilibrium (K_{eq}B) defines the interaction of the reduced FMN subdomain with the NOSoxy domain that is required for heme reduction and NO synthesis. This equilibrium appears to have a different set point and regulation compared to K_{eq}A, but has not been as thoroughly studied. An intrinsic heme–NO binding event occurs in NOS enzymes during catalysis and is likely to restrict the electron transfer function (heme reduction) of the NOS FMN subdomain relative to its function in related dual-flavin enzyme systems.

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Abbreviations

CaM	calmodulin
CT	C-terminal tail
CYP	cytochrome P450
CYPR	cytochrome P450 reductase
eNOS	endothelial nitric oxide synthase
$FADH^{\bullet}$	one-electron reduced (semiquinone) FAD
$FADH_2$	two-electron reduced (hydroquinone) FAD
$FMNH^{\bullet}$	one-electron reduced (semiquinone) FMN
$FMNH_2/FMNH^{-}$	two-electron reduced (hydroquinone) FMN
FNR	ferredoxin $NADP^{+}$ reductase-like subdomain
H_4B	(6*R*)-5,6,7,8-tetrahydro-L-biopterin
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
nNOSr	reductase domain of neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase
NOSoxy	oxygenase domain of NOS

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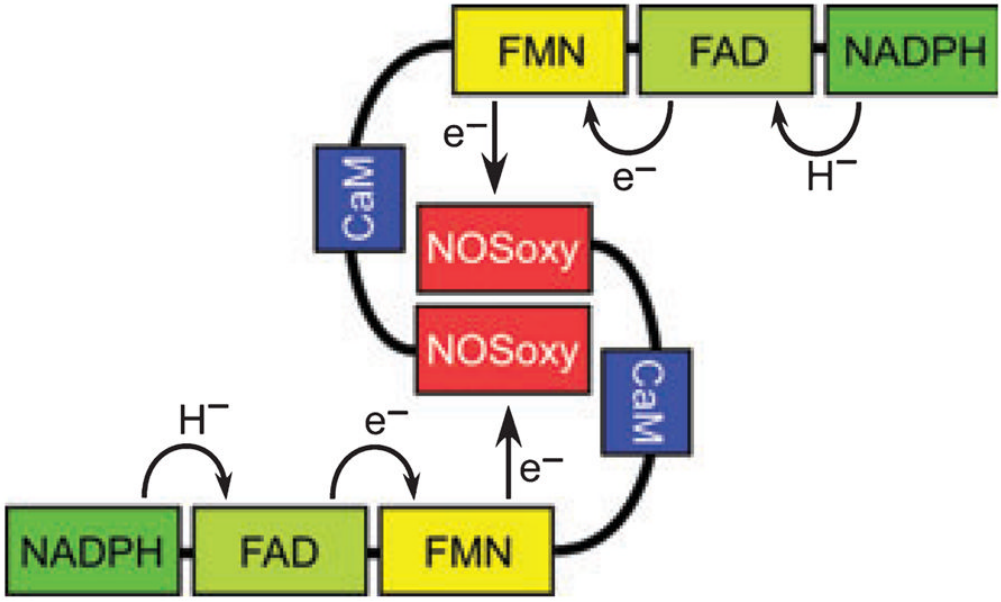


Fig. 1.
Domain arrangement and electron flow in the NOS dimer.

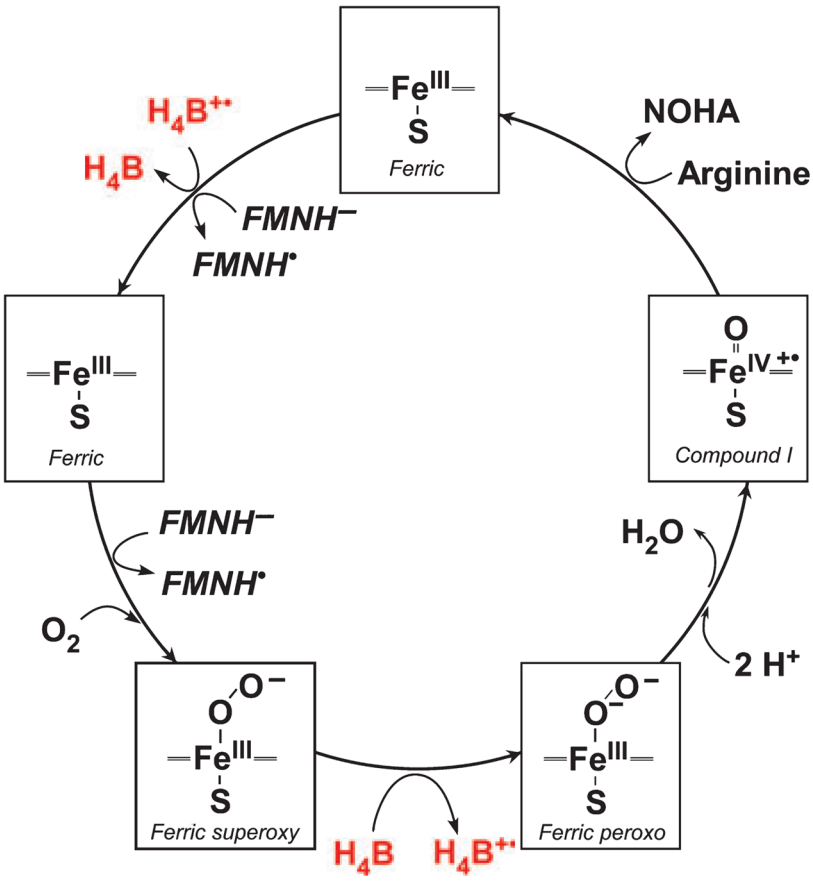


Fig. 2.

Simplified model of arginine hydroxylation in NOS enzymes. Ferric heme receives an electron from $FMNH_2/MNH^-$ enabling oxygen binding and formation of a ferrous dioxygen species. A second electron must be delivered from H_4B to eventually form a high valent iron-oxo species that hydroxylates Arg. The $H_4B^{+\bullet}$ radical has to be reduced before the next catalytic cycle can proceed.

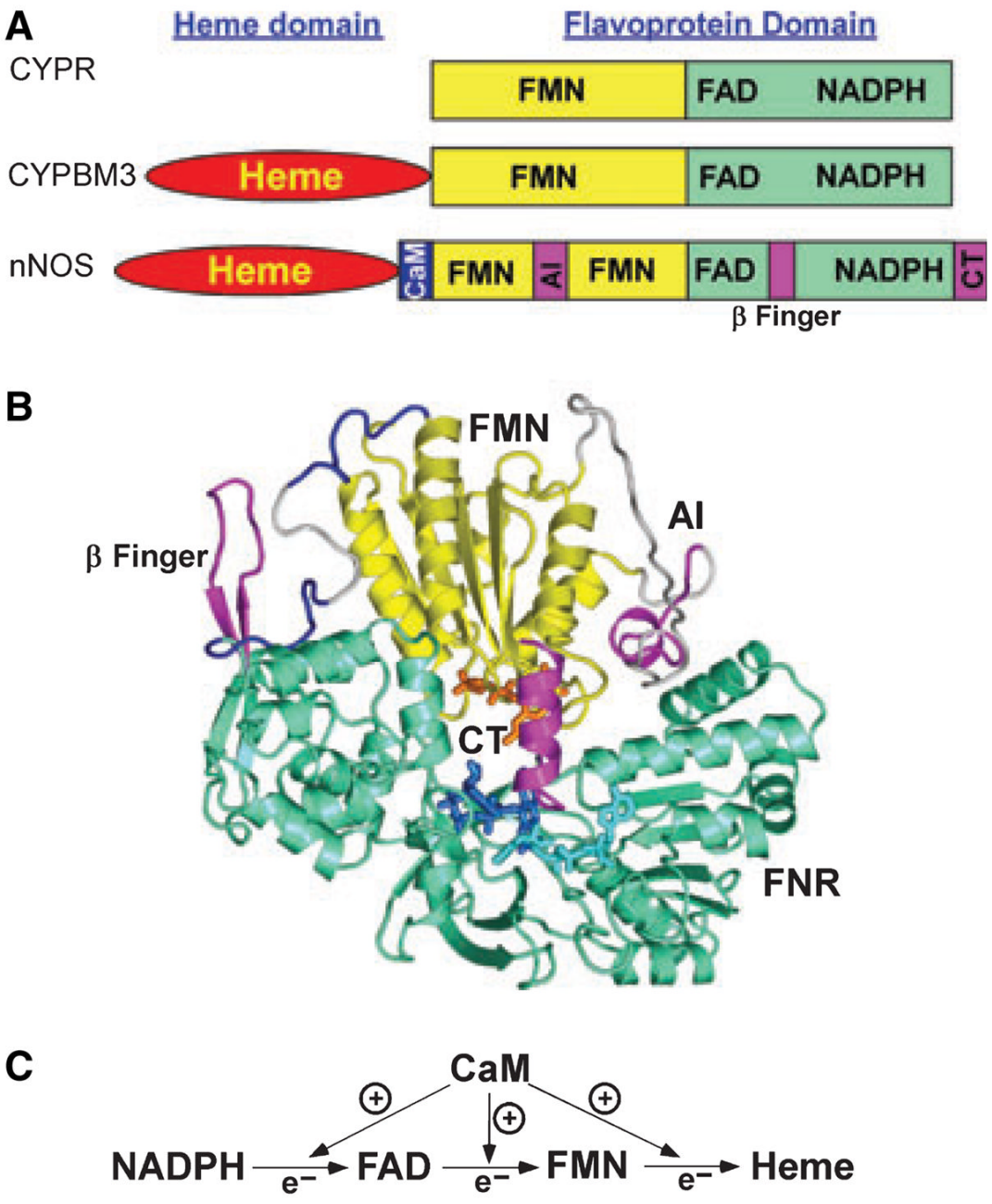


Fig. 3.

(A) Domain organization in NOS and related enzymes. NOS includes regulatory elements that are absent in other closely related proteins. (B) Structure of nNOS flavoprotein domain. The FNR and FMN subdomain are shown in green and yellow, respectively. Regulatory elements (β-finger; AI, autoinhibitory insert; CT, C-terminal tail) are shown in pink. The coenzymes FMN (orange), FAD (dark blue) and $NADP^+$ (cyan) are shown as sticks. Modeled fragments, not visible in the crystal structure, are shown in light gray. The visible parts of the hinge element between FMN and FNR subdomains are shown in dark blue. (C) CaM exerts an enhancing effect in three electron-transfer steps.

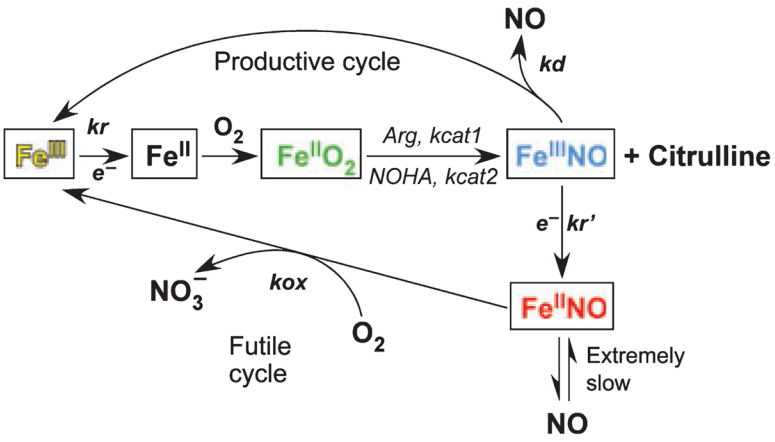


Fig. 4.

Global kinetic model for NOS catalysis. Ferric enzyme reduction (k_r) is rate limiting for the biosynthetic reactions (central linear portion). *kcat1* and *kcat2* are the conversion rates of the $Fe^{II}O_2$ species to products in the Arg and NOHA reactions, respectively. The ferric heme–NO product complex ($Fe^{III}NO$) can either release NO (k_d) or become reduced (k_r) to a ferrous heme–NO complex ($Fe^{II}NO$), which reacts with O_2 (k_{ox}) to regenerate the ferric enzyme. Adapted from Stuehr *et al.* [51].

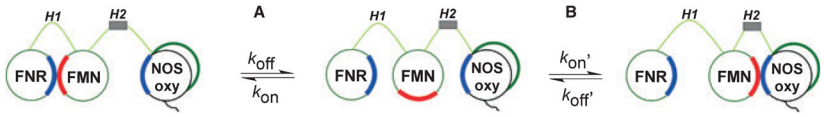


Fig. 5.

Model of NOS FMN subdomain function in electron transfer and heme reduction. Electron transfer in NOS can be regarded as a three-state model. Equilibrium A indicates the change between a conformation in which FNR and FMN subdomains are interacting (left) and a conformation where the FMN subdomain is deshielded and available for interaction with electron acceptors such as cytochrome *c* (center). Equilibrium B indicates the transition from the FMN deshielded conformation to a FMN–NOSoxy domain interacting state. See text for details.

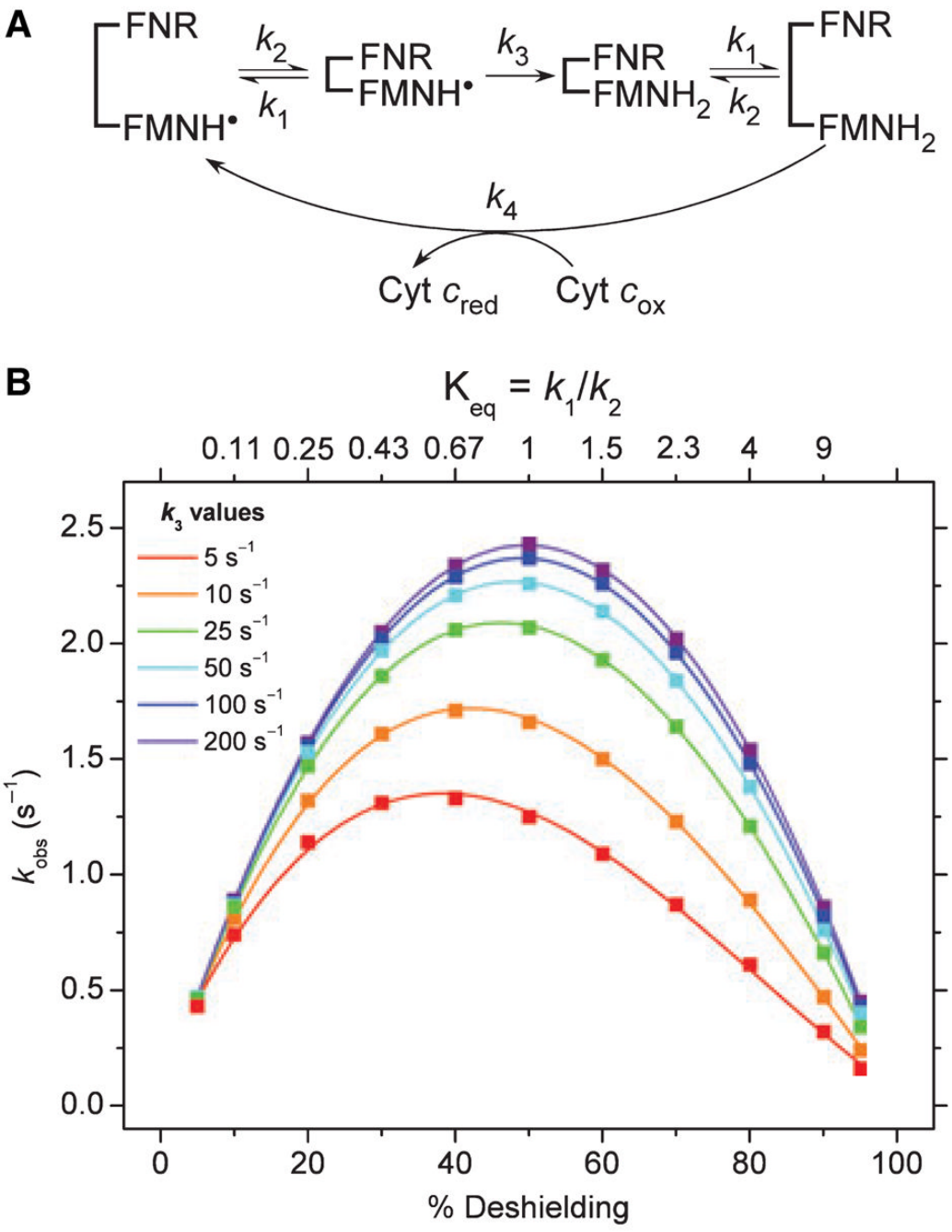


Fig. 6.

Model and simulations of cytochrome *c* reduction by NOS enzymes. (A) Scheme of cytochrome *c* reduction. The model uses four kinetic rates: dissociation (k_1) and association (k_2) of the FMN and FNR subdomains; $FMNH^{\bullet}$ reduction rate (k_3) and cytochrome *c* reduction rate (k_4). For simplicity, k_1 and k_2 are assumed to be independent of the flavin reduction state, *k*4 is assumed to be much faster than the conformational equilibrium so the backwards rates are negligible, oxidized cytochrome *c* concentration is constant and in 100-fold excess. (B) Apparent rates of steady-state cytochrome *c* reduction for different $FMNH^{\bullet}$ reduction (k_3) values. k_{obs} values were determined by fitting the apparent change in the concentration of

reduced cytochrome *c* versus time to a straight line. The percentage of deshielding is $(k_1/(k_1 + k_2)) \times 100$. See text for details.

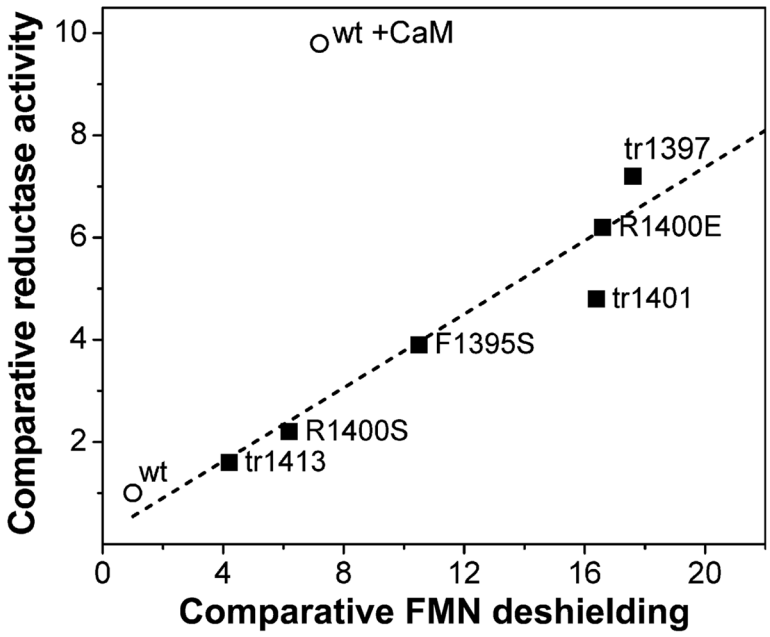


Fig. 7.

Correlation between nNOS cytochrome *c* reductase activity and FMN deshielding. The figure plots relative cytochrome *c* reductase activities of various CaM-free nNOS flavoproteins and CaM-bound wild-type versus their degree of FMN deshielding. All values are relative to NADPH-bound wild-type enzyme, which was given activity and shielding values of unity. Line is a least squares best fit. Adapted from Tiso *et al.* [33].

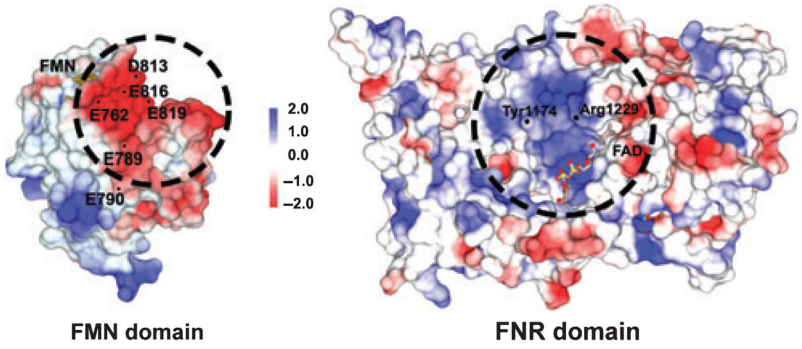


Fig. 8.

Complementary charges in the FMN–FNR subdomain interface. The electrostatic potential surfaces of the FMN (left) and FNR (right) subdomains show complementary negative charges in the FMN surface that interact with a positively charged surface patch in the FNR module. Adapted from Panda *et al*. [90].

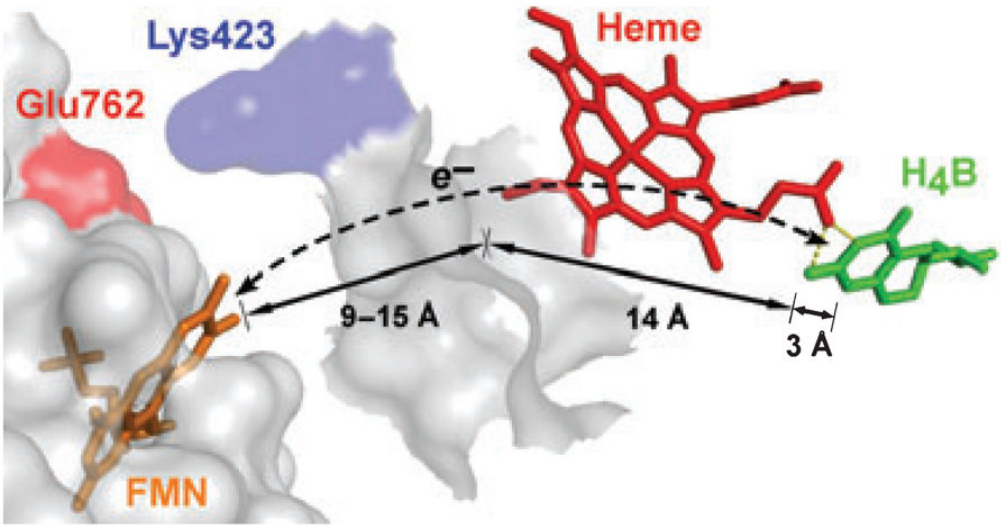
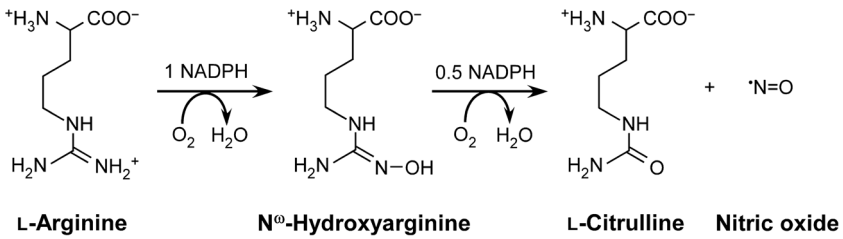


Fig. 9.

Through-heme model for H_4B radical reduction in NOS. H_4B is 17 Å away from the putative FMN-docking surface. Placing the FMN domain in conformations where Lys423 and Glu762 are in close contact enables feasible distances (9–15 Å) for FMN to heme electron transfer but too long (26–32 Å) for direct FMN to H_4B electron transfer. It is proposed that electron transfer proceeds through heme (dashed line) involving two short-distance (< 15 and 3 Å) electron transfer steps. Adapted from Wei *et al.* [23].



Scheme 1.
Reaction catalyzed by NOS.

Table 1

Factors that may alter conformational equilibrium A, B and/or the rate of electron input in nitric oxide synthase (NOS) enzymes.[a] AI, autoinhibitory insert; B2R, bradykinin receptor B_2; CaM, calmodulin; CT, C-terminal tail; HSP-90, heat-shock protein 90; iNOS, inducible nitric oxide synthase; ND, not determined; NA, not applicable; ?, different modifications (mutation, deletion) gave different results.

	K_{eq}A				K_{eq}B		
	Cyt *c* reduction		Flavin reduction rate		Heme reduction	NO synthesis	
Factor	**−CaM**	**+CaM**	**−CaM**	**+CaM**	**+CaM**	**+CaM**	**Ref**
CaM	NA	↑[b]	NA	↑	↑	↑	[37–39,59]
NADPH	↓[b]	=[b]	↑	↑	=	↑	[58,59,61,90,99]
CT[c]	↓	↓[d]	↓	↓e,i =n	↑n =e,i	↓i ↑e,n[e]	[31–33,102,105]
AI(e/n)[c]	↓	↓e↑n	ND	ND	ND	↓e ↑n	[28,102,105,115]
β-finger (e/n/iNOS)	?	?	ND	ND	ND	?	[34,35]
R1229E nNOS	↑[b]	=[b]	↑	↑	ND	↓	[77]
F1395S nNOS	↑[b]	=[b]	↑	=	↓	↓	[61,72,116]
R1400E nNOS	↑[b]	↑[b]	↑	↓	↑	↓	[60]
S1412D nNOS	↑	↑	↑	=	↑	↓	[88]
S1179D eNOS	↑	↑	ND	ND	ND	↑	[117]
Caveolin-1	↓	↓	ND	ND	ND	↓	[47,48]
HSP-90	=[f]	↑[f]	ND	ND	ND	↑	[50,118–122]
Dynamin-2	ND	↑	ND	ND	ND	↑	[49,123]
B2R	ND	=	ND	ND	ND	↓	[124,125]

[a] Unless otherwise stated, cytochrome *c* reduction and NO synthesis changes correspond to steady-state measurements, flavin reduction and heme reduction rates are derived from stopped-flow experiments. e, i or n refer to studies on eNOS, iNOS or nNOS, respectively. For an extensive list of proteins that interact with NOS the reader is referred to other reviews [10,126,127]. Regarding NOS phosphorylation, only the phosphorylation mimics S1179D eNOS and S1412D nNOS are shown; for more detailed information, see Hayashi *et al*. [44] and Mount *et al*. [128].

[b] Pre-steady-state cytochrome *c* reduction measurements.

[c] The effect of the element is inferred from deletion mutants, therefore the effects reported in the table are the opposite of the observed effects.

[d] All but one report indicate decreased cytochrome *c* reduction + CaM in ΔCT nNOS [33].

[e] All but one report indicates increased NO synthesis in ΔCT eNOS [105].

[f] Only eNOS data [121], not determined for iNOS or nNOS.

Table 2

Parameters describing conformational equilibrium A for the 4-electron reduced nNOS and eNOS flavoproteins. [a] CaM, calmodulin; ND, not determined.

Protein	Condition	K_{eq}A	k_{on} (s^{-1})	k_{off} (s^{-1})
eNOS	−CaM	0.1	4	0.5
	+CaM	8–9	ND	≥ 0.9–1.2[b]
nNOS	−CaM	1	8	8
	+CaM	8–9	ND	≥ 14–21[b]

[a] Data are taken from Ilagan *et al*. [58]. Equilibrium A is depicted in Fig. 5. All measures were performed at 10 °C.

[b] Estimated from the initial rates of cytochrome *c* reduction activity.