Published in final edited form as:

J Biol Inorg Chem. 2012 June ; 17(5): 675–685. doi:10.1007/s00775-012-0887-y.

Role of an Isoform-Specific Serine Residue in FMN-Heme Electron Transfer in Inducible Nitric Oxide Synthase

Wenbing Li^{†,1}, Weihong Fan^{†,1}, Li Chen[†], Bradley O. Elmore[†], Mike Piazza[‡], J. Guy Guillemette[‡], and Changjian Feng^{†,*}

[†]College of Pharmacy, University of New Mexico, Albuquerque, NM 87131, USA

[‡]Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

Abstract

In the crystal structure of a calmodulin (CaM) bound FMN domain of human inducible nitric oxide synthase (iNOS), the CaM-binding region together with CaM forms a hinge, and pivots on a R536(NOS)/E47(CaM) pair [Xia et al. (2009) *J. Biol. Chem.* 284, 30708–30717]. Notably, isoform-specific human iNOS S562 and C563 residues form H-bonds with R536 residue through their backbone oxygens. In this study, we investigated role of the S562 and C563 residues in the FMN–heme interdomain electron transfer (IET), rates of which can be used to probe the interdomain FMN/heme alignment. Human iNOS S562K and C563R mutants of oxygenase/FMN (oxyFMN) construct were constructed by introducing charged residues at these sites as found in human nNOS and eNOS isoforms, respectively. The IET rate constant of S562K mutant is notably decreased by one-third, and its flavin fluorescence intensity per μM is also diminished by $\sim 24\%$. These results indicate that a positive charge at position-562 destabilizes the H-bond mediated NOS/CaM alignment, resulting in slower FMN–heme IET in the mutant. On the other hand, the IET rate constant of the C563R mutant is similar to wild-type. Moreover, human iNOS oxyFMN R536E mutant was constructed to disrupt the bridging CaM/NOS interaction, and its FMN–heme IET rate is indeed decreased by 96 %. These results demonstrated new role of the isoform-specific serine residue of the key CaM/FMN(NOS) bridging site in regulating the FMN–heme IET (possibly by tuning alignment of the FMN and heme domains).

Keywords

Electron transfer; nitric oxide synthase; calmodulin; laser flash photolysis; heme; flavin

Introduction

Nitric oxide synthases (NOSs) are the enzymes responsible for nitric oxide (NO) biosynthesis in mammals. NO functions in key physiological processes including neurotransmission and blood pressure regulation [1, 2]. The NOS enzyme is tightly regulated. Deviant NO production by NOS clearly contributes to an increasing number of human pathologies, including cancer, hypertension and ischemic injury caused by stroke [2–4]. Selective NOS modulators are required for therapeutic intervention because of the ubiquitous nature of NO in human physiology, and the fact that three NOS isoforms are each

*Corresponding Author: Changjian Feng, cfeng@unm.edu, phone: 505-925-4326, fax: 505-925-4549..

¹Both authors contributed equally to this work.

Supporting Information Available. Table of primers for constructions of the human iNOS oxyFMN S562K, C563R and R536E mutants; gel electrophoresis of the purified mutants; UV-vis spectra of the proteins; summary of the processes occurring upon CO photolysis; analysis of the FMN content by HPLC; flavin fluorescence spectra of wt iNOS oxyFMN with added EDTA.

capable of producing NO *in vivo*. It is challenging to design selective NOS inhibitors due to high active site conservation among the NOS isoforms [5–8]. Clinical agents that directly and selectively modulate NOS isoform activity remain elusive.

Mammalian NOS is a homodimeric flavo-hemoprotein. Each subunit contains a C-terminal electron-supplying reductase domain with binding sites for NADPH (the electron source), FAD and FMN, and an N-terminal catalytic heme-containing oxygenase domain. The substrate, L-arginine (L-Arg), and a cofactor, (6R)-5,6,7,8-tetrahydrobiopterin (H₄B), both bind near the heme center in the oxygenase domain [9]. The oxygenase and reductase domains are connected by a calmodulin(CaM)-binding region, occupation of which is required for electron transfer from the reductase to the oxygenase domain. In Ca²⁺-independent iNOS isoform, the linker is bound to CaM tightly (but reversibly [10]). In contrast, in nNOS and eNOS the CaM binding is [Ca²⁺] dependent [11]. CaM binding to eNOS/nNOS acts as a “molecular switch” to enable electron flow from flavin cofactors in the reductase domain to the heme [12–14], and thus activate NO production.

There are crystal structures available for truncated NOS domains, including the oxygenase domains of each of the NOS isoforms [9, 15, 16], rat nNOS reductase constructs [17, 18], CaM bound human iNOS FMN domain [19], and CaM bound to peptides corresponding to the CaM-binding regions in nNOS, iNOS and human eNOS [20]. These crystal structures provide important information on the control mechanism of NO synthesis by NOS. However, no structures of the complete NOS holoenzyme are available; this is likely due to dynamic structural rearrangements and domain motions during NOS catalysis (see below).

The NOS interdomain electron transfer (IET) processes couple the reactions in the domains, and thus represent the key steps in NOS function [11, 21, 22]. Tight coupling of the enzyme is important for normal cardiovascular function and prevention of disease, because uncoupled or partially coupled NOS results in synthesis of reactive oxygen species, such as superoxide and peroxynitrite [23–25]. In particular, the CaM-controlled intersubunit FMN–heme IET is essential for coupling the electron transfer in the reductase domain with the NO synthesis in the catalytic heme domain [14], and it is thus of current interest to study the CaM-dependant FMN–heme IET in the NOS isoforms [21, 26–28]. The effect of CaM binding on redox potentials of NOS cofactors is a matter of debate. It has been shown that CaM binding has little effect on the thermodynamics of redox processes in NOS [29–31]. On the other hand, CaM binding was reported to substantially shift redox potentials of flavin cofactors in an NOS reductase construct [32]. Nonetheless, it is generally accepted that the regulation by CaM is accomplished dynamically through controlling redox-linked conformational changes required for effective IET. A laser flash photolysis approach, recently developed by us [33], has been used for direct determination of rates of the FMN–heme IET between catalytically significant redox couples of the two centers in oxygenase/FMN (oxyFMN) constructs [33–38] and full-length NOS enzymes [35, 39, 40].

How surfaces of CaM interact with the NOS domains remains enigmatic, and is mechanistically important in CaM-regulated NOS function. An “FMN-domain tethered shuttle” model (Figure 1) was recently proposed [34, 41], and is strongly supported by our IET kinetics [33–35, 37–39, 42] and spectroscopic [43, 44] studies and those of others [45–52]. In this model, eNOS/nNOS catalysis requires shuttling of the FMN domain between its structurally different electron-accepting (input) and electron-donating (output) states. In the two states, the FMN domain is engaged in distinctly different interdomain interactions, and the module swings back and forth to contact the flavodoxin-NADPH reductase (FNR) module and the NOS heme domain. The CaM binding is important in the eNOS/nNOS activation as it: (i) allows the conversion of the two states to occur, and (ii) facilitates the proper alignment of the FMN and heme domains in the output state [21, 35, 39]. Moreover,

emerging evidence indicates that the requirement of CaM for iNOS is associated with proper alignment of the FMN and heme domains in the output state [35, 38, 53]. Our kinetics studies indicate that the iNOS holoenzyme remains mainly in its input state, and that the basic control mechanisms acting in the NOS isoforms are similar [35].

While inter-protein interaction sites between CaM and the CaM-binding linkers of eNOS [20], iNOS and nNOS have been resolved, there is only one structure for CaM in complex with the FMN domain of human iNOS (Figure 2) [19]. In the structure, the only inter-protein interactions between the iNOS FMN domain and CaM involve conserved R536 of iNOS, which makes a salt bridge with E47 of CaM and forms H-bonds with backbone oxygens of S562 and C563 in the FMN domain (Figure 2A). The R536/E47 ionic bond was predicted to regulate the IET from FAD to FMN and from FMN to heme by adjusting the relative orientation and distance among the three redox cofactors [19]. Indeed, kinetic studies of nNOS mutants at the conserved arginine demonstrated that the Arg(NOS)-Glu(CaM) bridging interaction is a key factor that enables CaM to activate nNOS [54, 55]. Importantly, the H-bond forming residues S562 and C563 in human iNOS are isoform-specific among the NOS isoforms (Figure 2B): they are Lys and His, and Arg and Lys for human nNOS and eNOS, respectively. Here, at least one positively charged residue exists at these two positions in eNOS and nNOS, whereas the residues are both neutral in iNOS. We thus propose that the charged residues in eNOS/nNOS may likely interfere with the bridging Arg-Glu ionic bond (and disturb the H-bond network); the human iNOS S562 and C563 residues may be involved in alignment of CaM/FMN(NOS) domains, and ultimately, the NOS FMN/heme domains.

In the present study we have investigated role of the isoform-specific H-bond forming residues S562 and C563 of human iNOS in the FMN–heme IET process. We utilized a bi-domain NOS oxyFMN construct, in which only the oxygenase and FMN domains, along with the CaM binding region, are present [56]. The homologous dimeric oxyFMN construct is a minimal electron transfer complex designed to favor the interactions between the FMN and heme domains [56]. Biochemical studies showed that the oxyFMN construct is a validated model of the NOS output state [33, 34, 43, 56]. We designed and constructed human iNOS oxyFMN mutants of S562K (by introducing the positively charged residue in human nNOS; Figure 2B), and C563R (to study if the charge at the equivalent C563 site in human eNOS induces similar effect). The R536E mutant was also constructed to disrupt the bridging CaM/NOS interaction. The FMN–heme IET rates and fluorescence spectroscopic properties of the mutants were determined to probe the mutational effect on the interdomain FMN/heme alignment. This is based on the fact that: (i) rate of the FMN–heme IET measured by laser flash photolysis can be used as a probe for the formation of the interdomain FMN/heme complex [21]; (ii) NOS flavin fluorescence is a sensitive probe of environment of the FMN cofactor. The results provide new insight into role of the isoform-specific residue S562 of the bridging Arg(NOS)-Glu(CaM) site in modulating the iNOS FMN–heme IET.

Materials and Methods

Construction of S562K, C563R and R536E Mutants Plasmids of Human iNOS oxyFMN

The human iNOS oxyFMN mutants were prepared by site-directed mutagenesis on a pCWori⁺ vector containing cDNA of wild-type (wt) iNOS oxyFMN construct, which consists of residues 71–723 of the full-length human iNOS enzyme [35]. The primers for the mutations are listed in Table S1 of Supporting Information. Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA). The mutated plasmids were confirmed by DNA sequencing at the University of New Mexico DNA Research Services.

Protein Expression and Purification

The iNOS oxyFMN plasmid was co-transfected with CaM expression vector (p209) into *E. coli* BL21(DE3) cells by electroporation (MicroPulser, Bio-Rad). The iNOS protein must be co-expressed with CaM *in vitro* because of its tendency to aggregate when residues of the highly hydrophobic CaM-binding region are exposed to an aqueous environment. The transformed cells were grown at 37 °C in terrific broth in the presence of 100 mg/L of ampicillin and 34 mg/L chloramphenicol. The protein expression was induced by adding the induction cocktail (0.5 mM isopropyl β -D-thiogalactopyranoside, 0.4 mM 8-aminolevulinic acid, and a pinch of riboflavin) when the cell culture reached an OD₆₀₀ of 1. After incubation at 25 °C for 40 h, the cells were harvested at 4 °C. 50 – 60 g wet cell pellets can be obtained from 4 L culture media.

The cells were re-suspended in the lysis buffer (pH 7.5) containing 40 mM Tris-HCl, 10 % glycerol, 250 mM NaCl, three complete protease tablets (Roche), 10 μ M H₄B, and 50 mg/ml lysozyme. The cells were lysed using a digital sonifier (Branson, Model 250). The lysate was centrifuged in a Type 70Ti rotor using an ultracentrifuge (L7, Beckman) at 30,000 rpm for 40 min at 4 °C. The supernatant was then loaded into a Co²⁺-chelating column (Talon metal affinity resin, Clontech) pre-equilibrated with five bed-volume equilibration buffer (40 mM Tris-HCl, 10 % glycerol, 250 mM NaCl, pH 7.5). The column was washed with ten bed-volume wash buffer (40 mM Tris-HCl, 10 % glycerol, 250 mM NaCl, 15 mM imidazole, pH 7.5) and then eluted with a 15 – 150 mM imidazole gradient in the elution buffer (40 mM Tris-HCl, 10 % glycerol, 250 mM NaCl, pH 7.5). The eluted protein was pooled, concentrated to about 500 μ L and dialyzed into the storage buffer (50 mM Tris-HCl, 10 % glycerol, 150 mM NaCl, 1 mM DTT, 4 μ M H₄B, pH 7.5). The proteins were concentrated to ~ 250 μ M and stored at –80 °C. The protein molar concentration was determined based on heme content via difference spectra of the NOS-ferrous-CO adduct, where $\epsilon = 74 \text{ mM}^{-1}\text{cm}^{-1}$ for ΔA_{444} [57].

Laser Flash Photolysis

CO photolysis experiments were conducted using an Edinburgh LP920 laser flash photolysis spectrometer, in combination with a Q-switched Continuum Surelite I-10 Nd:YAG laser and a Continuum Surelite OPO. A 446 nm laser pulse (out of the OPO module) was focused onto the sample cell to trigger the IET reactions. A 50 W halogen lamp was used as the light source for measuring the kinetics at ms – s time scales. A LVF-HL filter (Ocean Optics, FL) with band pass peaked at selected wavelength (580 or 465 nm) was placed before the partially reduced protein sample to protect it from photo-bleaching and further photo-reduction by the white monitor beam [28].

Briefly, a solution of 350 μ L containing 20 μ M 5-deazariboflavin (dRF) and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol) was degassed in a laser photolysis cuvette by a mixture of CO and Ar (with a volume ratio of ~ 1:3) for 90 min. L-Arg was present to keep oxidized heme in the catalytically relevant high spin state. Aliquots of concentrated NOS protein were subsequently injected through a septum to achieve the desired concentration, and the protein solution was kept in ice and further purged by passing the CO/Ar mixture over the solution surface for 60 min (to remove minor oxygen contamination before being subjected to illumination). The protein solution in cuvette was then illuminated for an appropriate period to obtain a partially reduced form of NOS [Fe(II)-CO][FMNH*]. The sample was subsequently flashed with a 446 nm laser excitation to trigger the FMN-heme IET, which can be followed by the loss of absorbance of FMNH* at 580 nm, and/or the loss of absorbance of Fe(II) at 465 nm [35]; see Figure 3 for processes occurring in iNOS upon laser excitation. All the experiments were repeated at least twice. Transient absorbance

changes were averaged and analyzed using OriginPro 8.5 (OriginLab Corporation, MA). The laser flash photolysis experiments were conducted at room temperature.

Fluorescence Spectra of NOS

The NOS flavin fluorescence spectra were measured by a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at room temperature. 1 – 7 μM of the iNOS protein in degassed buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 10 % glycerol, pH 7.6) was filtered by a 0.2 μm membrane filter. A quartz cuvette with 1 cm path length was used for the experiments. All samples were excited at 446 nm, and the fluorescence spectra were recorded from 480 to 700 nm. The flavin fluorescence intensities at 525 nm were plotted *versus* protein concentrations. Linear regression analysis of the data gives slope of the line for each sample (*i.e.*, NOS flavin fluorescence per μM).

Analysis of the FMN Content

The NOS flavin content was analyzed using an analytical HPLC system (Agilent 1200), equipped with a C₁₈ reversed-phase column (250 $\times$ 4 mm) fitted with a 4 $\times$ 4 mm C₁₈ guard column. Briefly, the iNOS protein (5 – 6 μM in water) was heated for 5 min at 95 °C, chilled for 5 min on ice, and then centrifuged for 10 min. The supernatant was kept at 0 °C in the dark until HPLC analysis. FMN was detected by absorbance at 445 nm, and its concentration was obtained by using a calibration line of free FMN (0.1 – 100 μM).

Modeling of the CaM-FMN(iNOS) Hinge Region

The models of the hinge regions of the human iNOS mutant proteins were constructed by starting with the X-ray crystal structure of the human iNOS FMN domain/CaM complex (PDB ID: 3HR4). Ser562, Cys563 or Arg536 were replaced (keeping the rest of the structure unchanged) by Lys, Arg, and Glu respectively, using the Rotamers tool in UCSF Chimera (version 1.5.3, <http://www.cgl.ucsf.edu/chimera>) [58]. The Richardson Rotamer library was used and the rotamer was selected based on probability.

Results

Protein Expression and General Properties

The human iNOS oxyFMN S562K, C563R and R536E mutants were overexpressed in *E. coli* with yields similar to those obtained for the wt protein. The SDS PAGE (Figure S1 in Supporting Information) confirms the size of the recombinant proteins. The iNOS proteins have typical spectral features: (i) the UV-vis absorption spectrum of the oxidized sample in the presence of L-Arg has the Soret maximum at 396 nm, typical of a high spin 5-coordinated heme protein, and flavin peaks at 450 and 480 nm and a characteristic high spin ferriheme charge transfer band at 650 nm (Figure S2); (ii) the integrity of the protein was further confirmed by the lack of conversion to a P420 type species upon dithionite reduction in the presence of CO (red trace in Figure S2).

Electron Transfer between the Heme and FMN Domains in Partially Reduced iNOS oxyFMN Proteins

As expected [35], CO dissociation by laser photolysis of the [Fe(II)-CO][FMNH[•]] form results in a spectral “transition” (*i.e.* a change in direction of absorption changes over the time) in the 580 nm traces of the S562K and C563R mutants. Figure 4 shows traces of the S562K mutant: a rapid decay (with a rate constant of $205.1 \pm 1.2 \text{ s}^{-1}$) below the pre-flash baseline (Figure 4A), followed by a much slower recovery to the baseline (with a rate constant of $1.70 \pm 0.02 \text{ s}^{-1}$) at a longer time scale (Figure 4B). The rapid absorbance decay

at 580 nm (Figure 4A) is due to consumption of FMNH[•] in the following IET process [33, 35]:

In eq 1, FMN_{hq} stands for the FMN hydroquinone species generated by the IET reaction. The rate constant of the rapid decay at 580 nm is independent of signal amplitude (data not shown), *i.e.* concentration of the reduced protein, confirming an intraprotein process. It is also important to note that the observed IET rate constant for the wt human iNOS oxyFMN (Table 1) is in good agreement with the reported value that was determined on another laser flash photolysis apparatus for iNOS oxyFMN preparations obtained in a different laboratory [35].

We could hardly observe the spectral transition in the 580 nm traces for the R536E mutant. Importantly, our previous studies clearly showed that the FMN–heme IET in iNOS can be also monitored by absorbance decrease at 465 nm, which is due to net oxidation of heme in eq 1 [35] (Figure 3). Indeed, using the current laser flash photolysis apparatus, we obtained similar IET rate constants for the wt iNOS oxyFMN protein by measuring transient traces at 580 and 465 nm (Table 1). This is also the case for the S562K and C563R mutants; a typical 465 nm trace of the S562K mutant is shown in Figure 5. Note that the 465 nm trace has larger amplitude compared to the 580 nm trace in Figure 4, which is due to much larger absorption coefficients of the heme species than the flavin species in NOS [35]. This offers an alternative to determine the IET rates of NOS samples whose IET can be barely observed at 580 nm.

The IET rate of R536E mutant was thus determined by monitoring absorption change at 465 nm. Similarly, upon CO photolysis, the absorption at 465 nm of the partially reduced R536E decays below the baseline due to oxidation of Fe(II) in eq 1 [35] with a rate constant of $13.6 \pm 0.2 \text{ s}^{-1}$ (Figure 6A), followed by a slower recovery toward baseline (Figure 6B). We did not attempt to estimate the rate constant for the slow recovery phase because of the significant overlap between the IET and CO rebinding processes at this time scale (Figure 6B). The comparable IET and CO rebinding rates most likely contributes to the observed small amplitude of the kinetics trace because the two processes compete for free Fe(II) species, and their absorption changes go in opposite directions [35, 39].

The IET rate constants of the wt and mutants iNOS oxyFMN proteins are listed in Table 1. Note that there is about a one-third decrease in the IET rate constant for the S562K mutant compared to the wt protein, while the C563R mutant has similar IET rate constant. On the other hand, the IET rate of the R536E mutant is decreased significantly by 96 %. The slower IET rates in the S562K and R536E mutants are not caused by loss of FMN, as all mutants and wt contain similar FMN amount (0.8 FMN per protein chain; see Figure S3).

Fluorescence Spectra of the NOS Flavin Cofactor

Flavins bound to a protein can exhibit either enhanced or quenched fluorescence to a degree that is both specific and characteristic of the particular protein. The characteristic fluorescence of NOS flavin at 525 nm is a sensitive probe for the local environment of the FMN cofactor (including solvent exposure) [48, 59]. Additionally, the association between the NOS heme and FMN domains triggered by CaM binding can place the FMN molecule close enough to the paramagnetic heme for the FMN fluorescence being quenched [51]. Indeed, addition of EDTA, a calcium chelator, into the wt human iNOS oxyFMN sample resulted in a correlated increase in the NOS FMN fluorescence (Figure S4). A similar result was obtained when the three mutant iNOS oxyFMN proteins were treated with EDTA (data

not shown). The fluorescence results and our previous IET kinetics data [37] indicate that EDTA deactivation of CaM causes the FMN domain to move away from heme. Heme quenching and solvent exposure may thus both affect NOS flavin fluorescence.

We measured the fluorescence spectra of the human iNOS oxyFMN proteins under identical conditions (*i.e.*, protein concentration, buffer, and fluorometer settings) (Figure 7A). A change in the fluorescence intensities of the mutants can be noted from the observed spectra, indicating that the mutations have an effect on local FMN environment. Additionally, the fluorescence intensities (per μM) of the oxidized proteins were determined (Figure 7B, Table 1). It is evident that all the mutants exhibit lower fluorescence per μM than the wt protein, and that R536E possesses the lowest flavin fluorescence per μM .

Discussion

Compelling evidence indicates that the requirement of CaM for iNOS is associated with proper alignment of the FMN and heme domains in the output state [53]. The mechanism of CaM binding and activation to the iNOS enzyme is poorly understood [60], and it is thus of significant importance to investigate the detailed molecular mechanism of CaM-dependent promotion of the FMN/heme interactions in iNOS, in order to understand how CaM specifically regulates the NOS isoforms functions by key CaM/NOS interactions.

Based on the crystal structures of a CaM-bound human iNOS FMN domain, the R536(NOS)/E47(CaM) bridging interaction (Figure 2A) was predicted to regulate the IET from FMN to heme by adjusting the relative orientation and distance between the two cofactors [19]. The importance of the bridging interaction in CaM activation of the nNOS isoform is supported by recent kinetic studies of nNOS mutants at the conserved arginine [54, 55]. In line with the previous results, the present study showed that the Arg to Glu mutation at the conserved human iNOS R536 residue remarkably decreases the FMN–heme IET rate (by $\sim 96\%$), indicating that the FMN/heme alignment is disrupted by introducing an opposite charge at this site via repelling interaction between E536(NOS) and E47(CaM); see also Figure 8 below.

In this study, we have also further investigated roles of isoform-specific human iNOS S562 and C563 residues in the FMN–heme IET, whose backbone oxygens form H-bonds with the conserved R536 residue (Figure 2). The FMN–heme IET rate constant of the S562K mutant is notably decreased by one-third (compared to the wt), while the C563R mutant has similar IET rate constants as the wt (Table 1). The flavin fluorescence intensity (per μM) of the S562K mutant is also decreased by $\sim 24\%$ (Figure 7B). These results support a model that introducing a positive charge at S562 disfavors the H-bond mediated NOS/CaM alignment, resulting in disturbance of the interdomain FMN/heme alignment and slower FMN–heme IET. On the other hand, the IET kinetics properties of the C563R mutant are similar to the wt. The negligible effect of Cys to Arg mutation is rather surprising. Our modeling studies (see below) suggested that the C563R mutation causes a possible structural perturbation elsewhere, which could account for the fact that the C563R mutation can be accommodated in the complex without a major change in the IET of the protein. The residue at this position in wild type human eNOS (K543) is also positively charged. This indicates that position-563 is accommodating and has more tolerance of variation in types of amino acids.

The fluorescence results (Figure 7 and Table 1) indicate that the FMN environment in the mutants differ from the wt. The diminished fluorescence (per μM) in the mutants is possibly attributed to the altered local FMN environment in which FMN becomes more solvent accessible. We are aware of the discrepancy between the similar IET rates and the decreased fluorescence intensity of the C563R mutant (compared to the wt). Therefore, a direct

correlation of changes between the IET rates and the flavin fluorescent properties of each mutant protein may not exist. However, this is not necessarily surprising since a modified flavin environment does not need to equate to a change in the alignment of the FMN and heme domains. The mutant associated decrease in flavin fluorescence could be due to a combination of solvent accessibility and heme quenching. Heme proximity alone can't be assumed sufficient for electron transfer. The proper alignment of the heme and flavin centers for IET does not necessarily result in the highest possible degree of quenching. In the absence of NOS holoenzyme crystal structures, it is difficult to envisage how CaM interacts with the NOS domains, and if the interactions are different in the NOS proteins. It is thus important to utilize other techniques to provide insight into the interdomain FMN/heme alignment. We have recently used pulsed EPR techniques to directly measure the FMN...heme distance [26] and their relative orientation [61] in iNOS. Such study is important because protein electron transfer is primarily controlled by the distance and orientation between the redox centers [62]. Thus, a comparative study of the interdomain FMN...heme distance and orientation in the NOS samples is needed to determine if the FMN/heme alignment differs significantly. Such studies are in progress. Additionally, the present work was conducted on the bi-domain oxyFMN construct, a model of the NOS output state for NO production [56]. It is necessary to examine the full-length enzyme so that effects of the amino acid replacements on the NOS enzyme activity and the IET kinetics can be assessed. These experiments need considerable efforts and are outside of the scope of the present study.

We further evaluated the effects of the individual mutants on the IET rates by generating model structures based on the reported crystal structure of a CaM bound human iNOS construct (that includes the CaM binding region and FMN domain) [19]. The resulting model structures are represented in Figure 8. For the S562K model, the highest probability rotamers have their positive epsilon amino group lie between 3 and 6 Å from the side chain amino group of R536. This potentially introduces a positive charge in vicinity that would create an unfavorable electrostatic repulsion interaction between the epsilon amino group of K562 and the side chain guanidine group of R536. This could disrupt the hydrogen bonding network between the backbone of residue 562 and the side chain of R536 that stabilizes the interaction between R536 of iNOS and E47 of CaM. Such disturbance could account for the one-third decrease in the IET rate constant for the S562K mutant. On the other hand, the C563R mutation appears to cause a possible structural perturbation elsewhere in the FMN domain according to our model. This would unlikely disturb the hydrogen bonding network in the hinge region (Figure 2A), which would account for a rate constant that is similar to the wt protein. Not surprisingly, our model indicates that the R536E mutation would cause the salt bridging and hydrogen binding interactions (that bridge the FMN domain to CaM) to be broken, which would account for the drastic 96 % decrease in the IET rate constant.

Biological electron transfer systems depend on proper interactions between proteins/ domains that shuttle electron(s) [63–65]. Imperfect FMN/heme alignment in the R536E mutant is more likely the cause of the diminished IET rate. Note that R536 is at the end of CaM-binding region. On the other hand, the notable but smaller decrease in IET rate of the S562K mutant clearly indicates that specific interactions of S562 *outside* the CaM-binding region are compatible with efficient FMN–heme IET by stabilizing the IET-competent conformation of the FMN/heme complex. The interactions between CaM surfaces and NOS domains outside the CaM-binding region are secondary to that of the CaM-binding linker in tuning the FMN–heme IET, as indicated by a much smaller decrease in IET rate of S562K mutant (compared to the R536E mutant). This is consistent with previous studies showing that CaM binding to NOS is the primary factor in modulating the FMN–heme IET [38].

It is important to note that the S562K mutant mimics the nNOS environment near the bridging site (Figure 2B). At least one charged residue exists at the two H-bond forming residues positions in eNOS and nNOS, whereas the residues are both neutral in iNOS. Based on the solved CaM-bound iNOS FMN domain structure, the charged residues in eNOS/nNOS may interfere with the bridging Arg-Glu ionic bond by repelling the conserved Arg (Figure 8). Here, the positively charged eNOS R542/K543 and nNOS K783 residues would disrupt the hydrogen bonds of the conserved arginine that exist in iNOS. This would weaken the association between CaM and the eNOS/nNOS isoform, giving the IET rates in the order of eNOS < nNOS < iNOS. Similar order has been observed for the IET rates of the full length NOS isoforms (eNOS < nNOS $\approx$ iNOS) [35, 39, 40]. Due to the critical role of R536-mediated interactions in controlling the relative orientation between FMN domain and CaM [19], it will be intriguing to test a new model that the difference in the H-bond forming residues contributes to the different IET rates and catalytic activity in the NOS isoforms. The IET kinetics and spectroscopic studies of the nNOS and eNOS mutants at the equivalent isoform-specific sites should provide insight into role of the H-bond forming residues in the NOS isoform regulation.

In summary, we have demonstrated a new regulatory role in the FMN-heme IET for an isoform-specific serine residue positioned in a key CaM/FMN(NOS) bridging site. The IET kinetics results, along with X-ray crystal structural data of a CaM-bound human iNOS construct [19], indicate that human iNOS S562 is involved in the FMN/heme alignment by forming H-bond with the pivotal bridging NOS/CaM site, and that the H-bond mediated effect is site-specific.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health (GM081811 and HL091280 to C.F.), AHA Grant-in-Aid (09GRNT2220310 to C.F.) and NSERC Discovery Grant (183521 to J.G.G.). This project was supported by grants from the National Center for Research Resources (5P20RR016480-12) and the National Institute of General Medical Sciences (8 P20 GM103451-12) from the National Institutes of Health. CF acknowledges the support of UNM HSC RAC grant.

Abbreviations

NOS	nitric oxide synthase
iNOS	inducible NOS
nNOS	neuronal NOS
CaM	calmodulin
FNR	flavodoxin-NADPH reductase
FMN	flavin mononucleotide
FMNH[•]	FMN semiquinone
FMN_{hq}	FMN hydroquinone
oxyFMN	bi-domain NOS construct in which only the heme-containing oxygenase and FMN domains along with the CaM-binding linker are present
IET	intraprotein interdomain electron transfer

k_{et}	rate constant of the FMN–heme IET
wt	wild-type
H ₄ B	6R-5,6,7,8-tetrahydrobiopterin
dRF	5-deazariboflavin

References

- Schmidt H, Walter U. *Cell*. 1994; 78:919–925. [PubMed: 7923361]
- Moncada S, Higgs EA. *Br J Pharmacol*. 2006; 147:S193–S201. [PubMed: 16402104]
- Zhou L, Li F, Xu H-B, Luo C-X, Wu H-Y, Zhu M-M, Lu W, Ji X, Zhou Q-G, Zhu D-Y. *Nat Med*. 2010; 16:1439–1443. [PubMed: 21102461]
- Steinert JR, Chernova T, Forsythe ID. *The Neuroscientist*. 2010; 16:435–452. [PubMed: 20817920]
- Vallance P, Leiper J. *Nature Reviews Drug Discovery*. 2002; 1:939–950.
- Delker SL, Ji H, Li H, Jamal J, Fang J, Xue F, Silverman RB, Poulos TL. *J. Am. Chem. Soc.* 2010; 132:5437–5442. [PubMed: 20337441]
- Xue F, Li H, Delker SL, Fang J, Martásek P, Roman LJ, Poulos TL, Silverman RB. *J. Am. Chem. Soc.* 2010; 132:14229–14238. [PubMed: 20843082]
- Feng CJ, Fan W, Ghosh DK, Tollin G. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics*. 2011; 1814:405–408.
- Crane BR, Arvai AS, Ghosh DK, Wu CQ, Getzoff ED, Stuehr DJ, Tainer JA. *Science*. 1998; 279:2121–2126. [PubMed: 9516116]
- Stevenstruss R, Marletta MA. *Biochemistry*. 1995; 34:15638–15645. [PubMed: 7495792]
- Roman LJ, Martasek P, Masters BSS. *Chem. Rev.* 2002; 102:1179–1189. [PubMed: 11942792]
- Abu-Soud HM, Yoho LL, Stuehr DJ. *J. Biol. Chem.* 1994; 269:32047–32050. [PubMed: 7528206]
- Abu-Soud HM, Stuehr DJ. *Proc Natl Acad Sci U S A*. 1993; 90:10769–10772. [PubMed: 7504282]
- Panda K, Ghosh S, Stuehr DJ. *J. Biol. Chem.* 2001; 276:23349–23356. [PubMed: 11325964]
- Raman CS, Li HY, Martasek P, Kral V, Masters BSS, Poulos TL. *Cell*. 1998; 95:939–950. [PubMed: 9875848]
- Li HY, Shimizu H, Flinspach M, Jamal J, Yang WP, Xian M, Cai TW, Wen EZ, Jia QA, Wang PG, Poulos TL. *Biochemistry*. 2002; 41:13868–13875. [PubMed: 12437343]
- Zhang J, Martasek P, Paschke R, Shea T, Masters BSS, Kim JJP. *J. Biol. Chem.* 2001; 276:37506–37513. [PubMed: 11473123]
- Garcin ED, Bruns CM, Lloyd SJ, Hosfield DJ, Tiso M, Gachhui R, Stuehr DJ, Tainer JA, Getzoff ED. *J. Biol. Chem.* 2004; 279:37918–37927. [PubMed: 15208315]
- Xia C, Misra I, Iyanagi T, Kim J-JP. *J. Biol. Chem.* 2009; 284:30708–30717. [PubMed: 19737939]
- Aoyagi M, Arvai AS, Tainer JA, Getzoff ED. *EMBO J*. 2003; 22:766–775. [PubMed: 12574113]
- Feng CJ, Tollin G. *Dalton Trans*. 2009:6692–6700. [PubMed: 19690675]
- Alderton WK, Cooper CE, Knowles RG. *Biochem. J*. 2001; 357:593–615. [PubMed: 11463332]
- Xia Y, Roman LJ, Masters BSS, Zweier JL. *Circulation*. 1998; 98:387–388.
- Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BSS, Karoui H, Tordo P, Pritchard KA. *Proc Natl Acad Sci U S A*. 1998; 95:9220–9225. [PubMed: 9689061]
- Chen CA, Druhan LJ, Varadharaj S, Chen YR, Zweier JL. *J. Biol. Chem.* 2008; 283:27038–27047. [PubMed: 18622039]
- Astashkin AV, Elmore BO, Fan W, Guillemette JG, Feng CJ. *J. Am. Chem. Soc.* 2010; 132:12059–12067. [PubMed: 20695464]
- Stuehr DJ, Tejero J, Haque MM. *FEBS J*. 2009; 276:3959–3974. [PubMed: 19583767]
- Feng CJ. *Coord. Chem. Rev.* 2012; 256:393–411. [PubMed: 22523434]
- Noble MA, Munro AW, Rivers SL, Robledo L, Daff SN, Yellowlees LJ, Shimizu T, Sagami I, Guillemette JG, Chapman SK. *Biochemistry*. 1999; 38:16413–16418. [PubMed: 10600101]

30. Gao YT, Smith SME, Weinberg JB, Montgomery HJ, Newman E, Guillemette JG, Ghosh DK, Roman LJ, Martasek P, Salerno JC. *J. Biol. Chem.* 2004; 279:18759–18766. [PubMed: 14715665]
31. Daff S, Noble MA, Craig DH, Rivers SL, Chapman SK, Munro AW, Fujiwara S, Rozhkova E, Sagami I, Shimizu T. *Biochem. Soc. Trans.* 2001; 29:147–152. [PubMed: 11356143]
32. Dunford AJ, Rigby SEJ, Hay S, Munro AW, Scrutton NS. *Biochemistry.* 2007; 46:5018–5029. [PubMed: 17411075]
33. Feng CJ, Thomas C, Holliday MA, Tollin G, Salerno JC, Ghosh DK, Enemark JH. *J. Am. Chem. Soc.* 2006; 128:3808–3811. [PubMed: 16536556]
34. Feng CJ, Tollin G, Holliday MA, Thomas C, Salerno JC, Enemark JH, Ghosh DK. *Biochemistry.* 2006; 45:6354–6362. [PubMed: 16700546]
35. Feng CJ, Dupont A, Nahm N, Spratt D, Hazzard JT, Weinberg J, Guillemette J, Tollin G, Ghosh DK. *J. Biol. Inorg. Chem.* 2009; 14:133–142. [PubMed: 18830722]
36. Li W, Chen L, Fan W, Feng CJ. *FEBS Lett.* 2012; 586:159–162. [PubMed: 22198200]
37. Li W, Fan W, Elmore BO, Feng CJ. *FEBS Lett.* 2011; 585:2622–2626. [PubMed: 21803041]
38. Feng CJ, Fan W, Dupont A, Guy Guillemette J, Ghosh DK, Tollin G. *FEBS Lett.* 2010; 584:4335–4338. [PubMed: 20868689]
39. Feng CJ, Tollin G, Hazzard JT, Nahm NJ, Guillemette JG, Salerno JC, Ghosh DK. *J. Am. Chem. Soc.* 2007; 129:5621–5629. [PubMed: 17425311]
40. Feng CJ, Taiakina V, Ghosh DK, Guillemette JG, Tollin G. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics.* 2011; 1814:1997–2002.
41. Ghosh DK, Salerno JC. *Front Biosci.* 2003; 8:D193–D209. [PubMed: 12456347]
42. Feng CJ, Roman LJ, Hazzard JT, Ghosh DK, Tollin G, Masters BSS. *FEBS Lett.* 2008; 582:2768–2772. [PubMed: 18625229]
43. Sempombe J, Elmore BO, Sun X, Dupont A, Ghosh DK, Guillemette JG, Kirk ML, Feng CJ. *J. Am. Chem. Soc.* 2009; 131:6940–6941. [PubMed: 19405537]
44. Sempombe J, Galinato MGI, Elmore BO, Fan W, Guillemette JG, Lehnert N, Kirk ML, Feng CJ. *Inorg. Chem.* 2011; 50:6859–6861. [PubMed: 21718007]
45. Sanae R, Kurokawa F, Oda M, Ishijima S, Sagami I. *Biochemistry.* 2011; 50:1714–1722. [PubMed: 21244098]
46. Ilagan RP, Tejero JS, Aulak KS, Ray SS, Hemann C, Wang Z-Q, Gangoda M, Zweier JL, Stuehr DJ. *Biochemistry.* 2009; 48:3864–3876. [PubMed: 19290671]
47. Li H, Das A, Sibhatu H, Jamal J, Sligar SG, Poulos TL. *J. Biol. Chem.* 2008; 283:34762–34772. [PubMed: 18852262]
48. Ilagan RP, Tiso M, Konas DW, Hemann C, Durra D, Hille R, Stuehr DJ. *J. Biol. Chem.* 2008; 283:19603–19615. [PubMed: 18487202]
49. Welland A, Garnaud PE, Kitamura M, Miles CS, Daff S. *Biochemistry.* 2008; 47:9771–9780. [PubMed: 18717591]
50. Haque MM, Panda K, Tejero J, Aulak KS, Fadlalla MA, Mustovich AT, Stuehr DJ. *Proc Natl Acad Sci U S A.* 2007; 104:9254–9259. [PubMed: 17517617]
51. Salerno JC, Ghosh DK, Ray K, Adrados M, Nahm N, Li H, Poulos TI, Lakowicz J. *Nitric Oxide.* 2010; 22:S12.
52. Guan Z-W, Haque MM, Wei C-C, Garcin ED, Getzoff ED, Stuehr DJ. *J. Biol. Chem.* 2010; 285:3064–3075. [PubMed: 19948738]
53. Newman E, Spratt DE, Mosher J, Cheyne B, Montgomery HJ, Wilson DL, Weinberg JB, Smith SME, Salerno JC, Ghosh DK, Guillemette JG. *J. Biol. Chem.* 2004; 279:33547–33557. [PubMed: 15138276]
54. Tejero J, Haque MM, Durra D, Stuehr DJ. *J. Biol. Chem.* 2010; 285:25941–25949. [PubMed: 20529840]
55. Masters BS, Roman LJ, Martasek P, Feng CJ. *Nitric Oxide.* 2010; 22:S11.
56. Ghosh DK, Holliday MA, Thomas C, Weinberg JB, Smith SME, Salerno JC. *J. Biol. Chem.* 2006; 281:14173–14183. [PubMed: 16461329]
57. McMillan K, Masters BSS. *Biochemistry.* 1993; 32:9875–9880. [PubMed: 7691172]

58. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE. J. Comput. Chem. 2004; 25:1605–1612. [PubMed: 15264254]
59. Jachymova M, Martasek P, Panda S, Roman LJ, Panda M, Shea TM, Ishimura Y, Kim JJP, Masters BSS. Proc Natl Acad Sci U S A. 2005; 102:15833–15838. [PubMed: 16249336]
60. Spratt DE, Taiakina V, Palmer M, Guillemette JG. Biochemistry. 2007; 46:8288–8300. [PubMed: 17580957]
61. Astashkin AV, Fan W, Elmore BO, Guillemette JG, Feng CJ. The Journal of Physical Chemistry A. 2011; 115:10345–10352. [PubMed: 21834532]
62. Page CC, Moser CC, Chen XX, Dutton PL. Nature. 1999; 402:47–52. [PubMed: 10573417]
63. Tollin G. Electron Transfer in Chemistry. 2001; IV:202–231.
64. Leys D, Scrutton NS. Curr Opin Struct Biol. 2004; 14:642–647. [PubMed: 15582386]
65. Hoffman BM, Celis LM, Cull DA, Patel AD, Seifert JL, Wheeler KE, Wang JY, Yao J, Kurnikov IV, Nocek JM. Proc Natl Acad Sci U S A. 2005; 102:3564–3569. [PubMed: 15738411]

Figure 1.

Tethered shuttle model. Flavodoxin-type FMN-binding domain (yellow) shuttles between the flavodoxin-NADPH reductase (FNR)-type two-domain “dehydrogenase unit” and the heme-containing oxygenase domain. Top: input state; bottom: output state. An input state corresponds to the existing intact reductase structure [18] (*i.e.*, docked FAD/FMN state). In an output state the FMN binding domain associates with the oxygenase domain (*i.e.*, docked FMN/heme state). Free FMN domain conformations also exist in between these two docked states. The novel aspect of NOS is the involvement of CaM. CaM binding to eNOS/nNOS unlocks the input state, thereby enabling the FMN domain to shuttle between the FAD and heme domains. CaM is also required for proper alignment of the FMN and heme domains in iNOS. Adapted from ref. [39].

A

B

iNOS	S	R	V	R	T	I	L	F	A	T	E	T	G	K	S	E	A	L	A	W	D	L	G	A	L	F	S	C	A	F	N	566	
nNOS	K	R	V	K	A	T	I	L	Y	A	T	E	T	G	K	S	Q	A	Y	A	K	T	L	C	E	I	F	K	H	A	F	D	787
eNOS	K	R	V	K	A	T	I	L	Y	G	S	E	T	G	R	A	Q	S	Y	A	Q	Q	L	G	R	L	F	R	K	A	F	D	546

Figure 2.

(A) Structure of a human iNOS FMN domain/CaM complex (PDB ID: 3HR4). The FMN domain is gold, the CaM-binding linker is yellow, the bound CaM is green, and Ca^{2+} ions are grey spheres. The CaM-binding region together with CaM forms a hinge, and pivots on a R536(NOS)/E47(CaM) pair. In full-length iNOS, the dimer of the heme domain (not existing in the crystal structure) is connected to the other end of the CaM-binding linker. (B) Human NOS isoform sequence alignment of region near the conserved R536 (highlighted in red). Note that the H-bonding residues at 562 and 563 in human iNOS differ among the three NOS isoforms, as highlighted in yellow.

Figure 3.

Summary of processes occurring upon CO photolysis in the partially reduced form $[\text{Fe(II)-CO}][\text{FMNH}^\bullet]$ of iNOS protein. A pulse of 446 nm laser is used to dissociate CO from the Fe(II)–CO complex with the formation of a transient free Fe(II) species (reaction 1). The laser-induced CO dissociation results in a drop of the midpoint potential of the heme, and rapidly converts a good electron acceptor (the Fe(II)–CO complex) into an electron donor (the free Fe(II) species), favoring electron transfer from Fe(II) to FMNH $^\bullet$ (reaction 3). This IET process can be followed at 580 nm (that is due to FMNH $^\bullet$ reduction), and 465 nm, which is due to heme oxidation. A CO/Ar ($\sim 1:3$ v/v) mixture is used to slow down the CO rebinding (reaction 2), making it a poor competitor for the IET (reaction 3). The 465 nm trace has larger amplitude (compared to the 580 nm trace), which is due to much larger absorption coefficients of the heme species than the flavin species in NOS [35]. This makes it possible to determine the IET kinetics of NOS samples whose IET can be barely observed at 580 nm. Adapted from [28].

Figure 4.

Transient traces at 580 nm at (A) 0 – 0.04 s and (B) 0 – 2.0 s obtained for the [Fe(II)-CO] [FMNH•] form of human iNOS oxyFMN S562K mutant flashed by a 446 nm laser excitation. Anaerobic solution contained ~ 10 μ M protein, ~ 20 μ M dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol). Solid line corresponds to a single-exponential fit to the data.

Figure 5.

Transient traces at 465 nm at 0 – 0.04 s obtained for the [Fe(II)-CO][FMNH*] form of human iNOS oxyFMN S562K mutant flashed by a 446 nm laser excitation. Anaerobic solution contained ~ 10 μ M protein, ~ 20 μ M dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol). Solid line corresponds to a single-exponential fit to the data. Note that amplitude of the 465 nm trace is stronger than that of the 580 nm trace (Figure 4).

Figure 6.

Transient traces at 465 nm at (A) 0 – 0.4 s and (B) 0 – 4.0 s obtained for the [Fe(II)-CO] [FMNH[•]] form of human iNOS oxyFMN R536E mutant flashed by a 446 nm laser excitation. Note the significant difference in time scale of panel A (compared to Figure 4). Anaerobic solution contained 10 μ M protein, $\sim$ 20 μ M dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM Bis-Tris propane, 400 mM NaCl, 2 mM *L*-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol).

Figure 7.

(A) Fluorescence spectra of wt, S562K, C563R and R536E human iNOS oxyFMN proteins. The protein concentrations were 5 μM each, and the measurements were performed under the same conditions. (B) Plot of flavin fluorescence intensity (at 525 nm) versus the protein concentration; the fluorometer PMT voltage was lowered (relative to that for panel A) to cover the whole range of protein concentrations (1 – 7 μM). The slope value for each protein (obtained by linear regression analysis of the data) is listed in Table 1, and depicted in the inset of panel B. Fluorescence intensities of as-isolated proteins were determined at the indicated concentrations by excitation at 446 nm and monitoring emission at 525 nm. Buffer: 40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, and 10 % glycerol, pH 7.6. Data are representative of two experiments.

Figure 8.

Comparison of the tentative interaction network in the hinge region of the CaM-bound human iNOS mutants. The wild-type figure generated from PDB file 3HR4 [19] shows key residues in its hinge region. Salt bridges and hydrogen bonds between Arg536(NOS) and its adjacent residues in CaM (Asn42 and Glu47, labeled in green) and the human iNOS FMN domain (Ser562, Cys563, Ala564, Phe565 and Pro695) are shown as yellow lines. The S562K, C563R and R536E models were generated (see Methods section), and the most likely interactions between the residues are represented as solid lines, if existed.

Table 1

The FMN–heme IET rate constants (k_{et}) and NOS flavin fluorescence (per μM) of the human iNOS oxyFMN proteins

iNOS oxyFMN proteins	k_{et} (s^{-1}) ^a		Flavin fluorescence/ μM ^b
	580 nm trace	465 nm trace	
wt	340.3 $\pm$ 5.9	312.5 $\pm$ 3.4	78.9 $\pm$ 0.7
S562K	206.7 $\pm$ 1.4	177.6 $\pm$ 6.4	60.0 $\pm$ 0.5
C563R	350.9 $\pm$ 3.3	330.0 $\pm$ 2.5	52.4 $\pm$ 1.1
R536E	Not observed	13.6 $\pm$ 0.2	22.9 $\pm$ 1.0

^aBuffer: 40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H₄B, 1 mM Ca²⁺ and 10 % glycerol, pH 7.6. Final iNOS protein concentration: $\sim$ 10 μM . The FMN–heme IET kinetics in wt, S562K and C563R human iNOS oxyFMN proteins could be monitored at both 580 and 465 nm, while it was only observable for the R536E mutant at 465 nm.

^bBuffer: 40 mM Bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 10 % glycerol, pH 7.6.