Phosphorylation Controls Endothelial Nitric Oxide Synthase by Regulating its Conformational Dynamics*

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Running Title: Ser1179 controls catalysis in eNOS

Abstract

The activity of endothelial NO synthase (eNOS) is triggered by calmodulin (CaM) binding and is often further regulated by phosphorylation at several positions in the enzyme. Phosphorylation at Ser1179 occurs in response to diverse physiologic stimuli and increases the NO synthesis and cytochrome c reductase activities of eNOS, thereby enhancing its participation in biological signal cascades. Despite its importance, the mechanism by which Ser1179 phosphorylation increases eNOS activity is not understood. To address this we used stopped-flow spectroscopy and computer modeling approaches to determine how the phosphomimetic mutation (S1179D) may impact electron flux through eNOS and the conformational behaviors of its reductase domain, both in the absence and presence of bound CaM. We found that S1179D substitution in CaM-free eNOS had multiple effects: It increased the rate of flavin reduction, altered the conformational equilibrium of the reductase domain, and increased the rate of its conformational transitions. We found these changes were equivalent in degree to those caused by CaM binding to wild-type eNOS, and the S1179D substitution together with CaM binding caused even greater changes in these parameters. The modeling indicated that the changes caused by the S1179D substitution, despite being restricted to the reductase domain, are sufficient to explain the stimulation of both the cytochrome c reductase and NO synthase activities of eNOS. This helps clarify how Ser1179 phosphorylation regulates eNOS and provides a foundation to compare its regulation by other phosphorylation events.

Keywords: eNOS, Fast reaction kinetics, heme reduction, Electron transfer, flavoprotein, kinetic model, simulation

Nitric oxide (NO) synthase enzymes (EC 1.14.13.39) are homodimers with subunits...
comprised of an N-terminal oxygenase domain (NOSoxy) that is linked to a C-terminal reductase domain (NOSred) by an intervening calmodulin (CaM) binding sequence (1-4). Many of the structural and catalytic features of NOSred are shared among a family of eukaryotic dual-flavin enzymes whose members include cytochrome P450 reductase (CPR), methionine synthase reductase, and novel reductase-1 (5-7). These enzymes are all comprised of an FMN-binding domain that is attached to a FAD/NADPH-binding domain [FNR (ferredoxin NADP-reductase)] by a flexible hinge of various lengths (8-10). Their electron transfer (ET) reactions all involve a hydride transfer from NADPH to the FAD cofactor bound within the FNR domain, which then passes electrons sequentially to the FMN domain. Once the FMN domain receives two electrons, its FMN hydroquinone (FMNhq) can transfer an electron to a heme group that is located either within the enzyme itself (i.e., NOS) in a partner hemeprotein acceptor (i.e., CPR), or in a non-native hemeprotein acceptor such as cytochrome c (6,11-14). Importantly, ET through diflavin enzymes relies on the transient interactions and motions of the FMN domain, which must move between an FNR-bound, conformationally-closed “input” state, and an unbound, conformationally-open “output” state (15-17). We and others have proposed a four-state kinetic model (Fig. 1) that links the electron flux through any dual-flavin enzyme to the conformational equilibria and stochastic motions of its FMN domain (18,19). Simulations of this model have proven useful in understanding how conformational aspects regulate ET and catalysis by dual-flavin enzymes (15,16,18,20).

Endothelial nitric oxide (NO) synthase (eNOS) generates NO for cell signaling and functions broadly in health and disease (21-23). Its activity is regulated through several mechanisms, including post-translational modifications that in some cases may control its ET reactions. In particular, a conserved Ser residue (Ser1179 in bovine eNOS) that is located in the C-terminal tail of eNOS can undergo phosphorylation in response to physiologic signals like shear stress (24-26). Phosphorylation of Ser1179 enables eNOS to bind CaM at resting cell Ca2+ concentrations and bestows eNOS with greater NO synthesis and cytochrome c reductase activities, and these same changes occurred when Ser1179 was substituted with Asp to mimic the site-specific phosphorylation (24-27). Although these findings are consistent with Ser1179 phosphorylation (or Asp substitution) increasing ET flux in eNOS, the mechanism of action for enhanced catalysis has not been investigated.

To address this issue, we determined if conformational parameters known to control eNOS electron flux become altered by the Ser1179Asp modification, and if so, how the changes compare to those caused by CaM binding to eNOS, and whether they can explain the increases seen in S1179D eNOS catalytic activities. Our experimental results, together with computer simulations of the kinetic model (Fig. 1), reveals how the Ser1179 modification can act through a common mechanism to stimulate both the reductase and NO synthesis activities of eNOS.

RESULTS
Steady-state cytochrome c reductase and NO synthesis activities: The rate of NADPH-dependent cytochrome c reduction is a useful means to study electron flux through the NOS reductase domain. The cytochrome c reductase activity of eNOS is repressed in its native state, but the repression is relieved by CaM binding (20,26). We found the reductase activities of S1179D eNOSr and S1179D eNOS to be greater than wild-type in both the CaM-free and CaM-bound states.

The NO synthesis and corresponding NADPH oxidation activities of the eNOS proteins are reported in Table 1. Consistent with previous reports (26,27), the NO synthesis activity of S1179D eNOS remained dependent on CaM but was two times greater than wild-type when measured at 25 or 10 °C, and was accompanied by proportional increases in enzyme NADPH consumption. Our S1179D eNOS proteins
displayed the expected increases in their catalytic activities and thus were fit for further study.

**Kinetics of Flavin and Heme Reduction:** We next examined the kinetics of flavin and heme reduction in S1179D eNOS, which have not been previously reported. The reactions mixed fully-oxidized eNOSr or full-length eNOS proteins with excess NADPH at 10 °C under anaerobic conditions in a stopped-flow spectrophotometer, and flavin reduction was followed as an absorbance decrease at 457 nm, while heme reduction was followed as build up of the ferrous heme-CO complex at 444 nm (28), respectively. For flavin reduction, all traces fit well to a triple exponential equation as utilized previously (15,29,30), and the fitted rates are listed in Table 2. The initial fast phase $k_1$ is considered to primarily reflect hydride transfer from NADPH to FAD, followed by slower $k_2$ and $k_3$ phases that reflect inter-flavin electron transfer and NADP$^+$ dissociation steps, and further reduction by a second NADPH (30,31). CaM binding increased the rate of flavin reduction in eNOSr, as reported previously (20,28). In comparison, the S1179D eNOSr displayed relatively fast rates of flavin reduction in its CaM-free state that matched the rates we observed for CaM-bound eNOSr, and we observed only a small additional increase in the flavin reduction rate upon CaM binding (particularly in $k_1$) (Table 2, Fig. 3). We also found that the S1179D mutation doubled the rate of heme reduction in CaM-bound eNOS, from $0.005 \pm 0.001$ to $0.011 \pm 0.001$ s$^{-1}$, $n = 7$ (data not shown), consistent with it doubling the NO synthesis activity of eNOS. Our results directly demonstrate that the increased catalytic activities of S1179D eNOS are associated with an increase in ET both into and out of the eNOS reductase domain.

**Determining eNOSr conformational behaviors:** We next compared how CaM binding and the S1179D substitution may impact the conformational behaviors of eNOSr, using an established approach (16,20) that involves monitoring of the reduction of cytochrome $c$ in reactions that mix the fully-reduced form of each eNOSr enzyme with excess cytochrome $c$. Representative stopped-flow absorbance traces are shown in Fig. 4. All enzymes quickly achieved steady-state catalysis, as indicated by their reaction traces becoming nearly linear either during or soon after they had reduced the first molar equivalent of cytochrome $c$ (the molar equivalents are relative to the moles of eNOSr enzyme present in the reaction, and the first molar equivalent is indicated by the dashed box in each panel of Fig. 4). This was confirmed by our finding that the reductase activities calculated from tangent lines drawn to the linear portions of each trace in Fig. 4 (listed in Table 3) gave values that were within 10% of the steady-state reductase activities that we measured at 10 °C using conventional spectroscopic assays that were run over several minutes (Fig. 2). Interestingly, the elapsed time required to reduce the first equivalent of cytochrome $c$ ranged 20-fold among the enzymes, from 0.07 to 1.8 s, with the slowest being eNOSr (Table 3). Together, these results suggest that the S1179D substitution or CaM binding do not alter the general catalytic behavior of eNOSr, but do allow the enzyme to achieve a steady state more quickly and with higher activity.

**Conformational equilibrium setpoint:** The cytochrome $c$ reductase activity of dual-flavin reductase enzymes is sensitive to the equilibrium (Keq) between its un-reactive closed and reactive open conformational states (15-17,20,32,33). We utilized the stopped-flow traces of cytochrome $c$ reduction in Fig. 4 to estimate the conformational Keq setpoints of the fully-reduced wild-type and S1179D eNOSr enzymes in the presence or absence of bound CaM (Keq values are listed in Table 3) (15,16,20). In the reactions of Fig. 4, the burst kinetics in each panel are due to the rapid reaction of cytochrome $c$ with the subpopulation of eNOSr molecules that are in an open conformation, whose reduced FMN domain reacts with the excess cytochrome $c$ at a rate of about 225 s$^{-1}$ (12). The subsequent slower absorbance gain involves the reaction of the remaining eNOSr subpopulation that must open from a closed conformational state in order to react with the cytochrome $c$. The ratio of these two subpopulations for any given eNOSr protein can be determined from their individual absorbance contributions toward reduction of the first equivalent of cytochrome $c$, and thus are used to determine the eNOSr conformational Keq (15,16,20).
For the CaM-free eNOSr (Fig. 4A), ~13% of the absorbance change occurred in the mixing dead time (burst phase) during reduction of the first equivalent of cytochrome c, subsequently leaving ~87% of the absorbance change to be observable within the first equivalent. This result gives an estimated open/closed conformational Keq of ~0.15, which matches our previous Keq estimates determined for CaM-free eNOSr (16,20), and confirms that the fully-reduced, CaM-free eNOSr predominantly populates a closed conformation that is unreactive for fast ET to cytochrome c. In comparison, the absorbance trace for CaM-bound eNOSr (Fig. 4B) shows that 60% of the first equivalent of cytochrome c was reduced within the mixing dead time, giving an estimated conformational Keq ~1.5. Thus, CaM binding shifts the conformational Keq such that eNOSr predominantly populates an open, reactive conformation, consistent with our previous report (20). The kinetic trace for S1179D eNOSr (Fig. 4C) indicates that most of the first equivalent of cytochrome c was reduced within the mixing dead time, giving an estimated conformational Keq of ~2.4, meaning that CaM binding caused a further shift in the equilibrium toward the open reactive conformation, beyond what is caused by the S1179D substitution itself. This finding corroborates a recent study that used fluorescence spectroscopy to investigate the effect of CaM binding on the conformational distribution of S1179D eNOS (34).

**Rates of conformational switching and interflavin ET:** To study these aspects we used an established approach (16,19,20) that involves performing computer simulations of the four-state kinetic model in Fig. 1 to fit the observed kinetic traces of cytochrome c reduction from Fig. 4. This allows one to derive values for the conformational switching rates (i.e., the rates of conformational closing $k_1$, $k_3$ and conformational opening $k_2$, $k_4$ in Fig 1) and the rate of interflavin ET ($k_5$ in Fig.1) that best fit the experimental traces. As explained previously (16), the analysis first generates combinations of allowable conformational switching and interflavin ET rate pairs that each support the experimentally-observed rate of electron flux to cytochrome c (i.e., the steady-state reductase activity that we measured at 10 °C). The allowable rate pairs are graphed for the CaM-free and CaM-bound forms of wild-type and S1179D eNOSr enzymes in Fig. 5. In general, faster conformational switching rates can be paired with slower rates of interflavin ET, and vice versa, in order to support the observed steady-state rate of electron flux through each eNOSr protein to cytochrome c. The blue horizontal and vertical tangents in these graphs give the lower boundary rate values for either kinetic parameter, and the lower boundary rate values are noted in Table 4. Further iterative simulations are then done to identify the best-fit rate pair, which is defined as the rate pair that generates the best fit of each experimental trace in Fig. 4, including the closest match of the time it takes for each enzyme to reduce its first equivalent of cytochrome c. The best-fit rate pairs are located on the traces in Fig. 5 as blue boxes, and the best-fit rates are all reported in Table 4, while the simulated best-fit traces for each enzyme are shown in Fig. 6. Our simulation results indicate that the increased cytochrome c reductase activities of CaM-bound eNOSr, and of S1179D eNOSr with and without bound CaM, can be explained by their having faster conformational dynamics (opening and closing rates) and faster rates of interflavin ET, in addition to their having shifts in the conformational Keq setpoint.

**Enzyme species distribution and FMNqh reactivity toward cytochrome c:** The simulations can predict how eNOSr would distribute among the four species involved in its catalytic cycle (Fig. 1; FMNsq open, FMNsq closed, FMNhq closed, and FMNhq open) during its reaction with excess cytochrome c, and can also predict how CaM binding or the S1179D substitution will influence the distribution. At time = 0, all of the eNOSr enzyme molecules are fully-reduced (i.e., all contain FMNqh) and are simply distributed according to their conformational Keq values between the open reactive or closed unreactive forms (species d & a in Fig. 1). As shown in Fig. 7, upper panel, once the reaction is initiated the open FMNhq species population immediately falls to near zero (black lines), because of its fast reaction with the excess cytochrome c. This
creates an equivalent population of reacted, conformationally-open molecules that contain FMNsq (red lines in Fig. 7) that then must conformationally close in order to keep moving through the catalytic cycle.

The simulations in Fig. 7 A-D (upper panel) show how the enzyme species distribution changes during the reaction, in relation to the time it takes to reduce one equivalent of cytochrome c is reduced (1.8 s). For the CaM-free eNOSr (Fig. 7A), achieving a steady-state distribution takes about 1 s and occurs well before the first equivalent of cytochrome c is reduced. In comparison, CaM-bound eNOSr, or S1179D eNOSr with or without CaM, achieve their steady state distributions about 5 to 10 times faster and closer to the time they require to reduce the first equivalent of cytochrome c. Their faster approach to a steady state distribution is related to their faster rates of conformational switching and interflavin ET. These facets also enable shorter times for reducing one equivalent of cytochrome c. However, this aspect is also aided by their having Keq conformational setpoints greater than unity, which means more than 50% of the reduced enzyme molecules would be in an open reactive conformational state at the time of mixing to quickly react with cytochrome c.

The predicted steady-state enzyme distributions (Fig. 7, lower panel) indicate that for eNOSr the two closed conformational species would predominate and represent 85% of the total enzyme population, with the majority being the closed FMNHq species that is waiting to open to react with cytochrome c. This situation arises because the conformational Keq heavily favors closed conformations and the interflavin ET rate is five times faster than the rate of conformational opening in eNOSr (see Table 4). In contrast, for the CaM-bound eNOSr it is the open oxidized (FMNsq) species that would be most populated (60% of total) during the steady state (Fig. 7, lower panel). This distribution shift is due to CaM changing the Keq of eNOSr to favor open conformational forms, while also speeding the transitions between the conformational and redox states. The simulation predicts that the S1179D eNOSr would have a steady state distribution that is generally similar to the CaM-bound eNOSr, again with its open FMNsq form predominating (Fig. 7, lower panel). Finally, for the CaM-bound S1179D eNOSr (Fig. 7, lower panel), the simulation shows there would be even less build up of conformationally-closed species during the steady state, due to its having somewhat faster rates of interflavin ET and conformational opening relative to either CaM-bound eNOSr or S1179D eNOSr in the absence of CaM.

The steady-state distributions also influence in turn what percentage of FMNsq versus FMNHq would be present in each enzyme during their reaction with excess cytochrome c (Fig. 8). For example, the simulations indicate that CaM-free eNOSr would still maintain ~76% FMNHq despite the considerable thermodynamic and kinetic driving forces that favor reaction of its FMNHq with cytochrome c. This is because the low Keq setpoint and slow conformational opening rate of eNOSr relative to its rate of interflavin ET cause accumulation of the conformationally-closed FMNHq species, which is unreactive toward cytochrome c. In contrast, the percentage of FMNHq in S1179D eNOSr or in CaM-bound eNOSr during their steady state reactions is predicted to be 25% or less of the total FMN. This is because their Keq setpoints favor open conformational states, and their conformational opening rates are faster and more similar to their rates of interflavin ET. Thus, the simulations show how protein conformational and dynamic changes in eNOSr caused by CaM binding or by the S1179D substitution would increase the availability of the FMNHq for reacting with cytochrome c in unit time, thus allowing a greater electron flux through eNOSr and an increased cytochrome c reductase activity.

**Influence of eNOSr conformational distribution on ET to the eNOS heme:** We applied a similar analysis to determine if the increased NO synthesis activity in S1179D eNOS could be attributed to the changes in reductase domain behavior that are caused by the S1179D substitution. In this analysis, the eNOS heme replaces cytochrome c as the external electron acceptor in the catalytic cycle ($k_4$ parameter in Fig. 1). Because the rate of eNOS heme reduction is much slower than is cytochrome c reduction, we set the $k_4$ parameter to range between 0.1 and 1 s$^{-1}$.
in the simulation for eNOS heme reduction. These heme reduction rates are at least 10 times slower than the rates of conformational switching and interflavin ET in CaM-bound eNOSr (see Table 4), and thus would allow build up of the conformationally-open FMNhq species (species a in Fig. 1) during the steady-state, which is the species that interacts with the eNOSoxy domain to transfer an electron to the heme for NO synthesis. Thus, anything that increases the “concentration” of the open FMNhq species should increase the probability of eNOS heme reduction, which in turn would increase the rate of NO synthesis.

To examine this concept, we ran simulations that incorporated the range of eNOS heme reduction rates noted above as \( k_4 \), and also incorporated the kinetic parameters that we derived for CaM-bound eNOSr and for CaM-bound S1179D eNOSr (from Table 4). The pie graphs in Fig. 9, upper panel show how the steady-state distributions of the reductase domain would compare between the two enzymes during steady state heme reduction in eNOS, when the eNOS heme reduction rate is set at 0.1 s\(^{-1}\). Note that there is a greater percentage of the conformationally-open FMNhq species in the CaM-bound S1179D eNOS compared to CaM-bound wild type. Fig. 9, lower left panel shows that the S1179D substitution in CaM-bound eNOS would increase the percentage of the conformationally-open FMNhq species that builds up during the steady state across the entire range of heme reduction rates, while Fig. 9 lower right panel shows that this effect in turn would support a greater flux of electrons entering into the eNOS heme across the entire range of heme reduction rates. Thus, the simulations support a mechanism whereby the changes in reductase domain behavior that are caused by the S1179D substitution are sufficient on their own to increase electron flux to the heme in CaM-bound eNOS, and this helps to explain how its NO synthesis activity is increased by the S1179D substitution.

**DISCUSSION**

Our study compared how CaM binding and the phosphomimetic S1179D substitution impact the ET and conformational behaviors of eNOSr, and examined if the changes attributed to S1179D could explain how it increases both the cytochrome c reductase and NO synthesis activities of eNOS.

Regarding CaM, its ability to speed flavin reduction and shift the conformational Keq of nNOS and eNOS is well established (20,34-40). In contrast, the impact of Ser\(^{1179}\) modification on the eNOS Keq has not been previously explored, nor has its impact on interflavin ET or protein conformational dynamics, which have so far only been studied for CaM-free eNOSr, CaM-free and bound nNOSr, some nNOSr point mutants, and for the related dual-flavin enzymes cytochrome P450 reductase and methionine synthase reductase (15-17,41-44).

The S1179D substitution had an almost identical impact on eNOSr as did CaM binding, because they both sped the rate of electron input (NADPH-dependent flavin reduction), shifted the conformational equilibrium, and increased the conformational transition rates of eNOSr to equivalent extents. At first glance the equivalence seems surprising, but it is consistent with CaM acting to disable repressive structural elements that are present in eNOSr, one of which is the C-terminal tail, which contains Ser\(^{1179}\) (3,8,29,45-47). Thus, substitution of Ser\(^{1179}\) with Asp, and by inference Ser\(^{1179}\) phosphorylation, appear to be equivalent to CaM binding in relieving the repressive effects of the C-terminal tail, which otherwise retards ET into the flavins, lowers the conformational Keq, and slows the conformational dynamics of eNOSr, thereby repressing its cytochrome c reductase activity.

Despite their equivalent impacts, the S1179D mutation did not mimic CaM in one important aspect- its ability to trigger eNOS heme reduction for NO synthesis. This is consistent with CaM playing an additional role to enable productive contact between the FMN and NOSoxy domains for ET and heme reduction (48-51). Our results clearly show that altering the conformational and ET behaviors of eNOSr, even to an extent equivalent to that caused by CaM binding, is not on its own sufficient to overcome the block that prevents heme reduction in eNOS. Deletion studies suggest that two repressive structural elements in the eNOS reductase domain (the C-terminal tail and the autoinhibitory insert) act
together to block heme reduction from occurring in the absence of CaM (45). This implies that the control elements block productive interaction between the FMN and NOSoxy domains, and CaM binding removes the blocking effect. In addition, recent evidence suggests that CaM binding may restrict the degrees of motion of the FMN domain, and in this way promote NOS heme reduction (40,42,52).

We found the S1179D substitution changed the conformational and ET behaviors of CaM-bound eNOSr, beyond what CaM binding caused on its own. Through simulations we explored if these additional changes could explain why the S1179D substitution (and presumably, phosphorylation at this Ser) bestows CaM-bound eNOS with a faster heme reduction rate and an increased NO synthesis activity. The simulations found that the changes caused by the S1179D substitution would, in effect, increase the concentration of the conformationally-open FMNhq domain during catalysis of heme reduction in eNOS, and that this change would support an increased electron flux into the eNOS heme. In this way, the conformational and ET changes within eNOSr that are caused by the S1179D substitution would essentially increase the probability of eNOS heme reduction, which is rate-limiting for NO synthesis. In sum, our study provides new mechanistic insight by showing how a phosphomimetic substitution at Ser1179 regulates the ET and conformational behaviors of the eNOS reductase domain, and how these effects in turn can increase both the cytochrome c reductase activity and the NO synthesis activity of eNOS.

**EXPERIMENTAL PROCEDURES**

**Molecular Biology:** Restriction digestions, cloning, bacterial growth, transformation, and isolation of DNA fragments were performed using standard techniques (9,28,53). The bacterial expression vector pCWori contained cDNA that coded for either bovine eNOSr (with its adjacent N-terminal CaM binding site, amino acids 445–1205) or full-length eNOS. Oligonucleotides for site-directed mutagenesis were obtained from Integrated DNA Technologies (Coralville, IA). The primers used to generate the S1179D phosphomimetic mutation in eNOS protein were:

- Sense: 5’-GTATACGTACCGAGGACTTTTCCCTGCAG-
- 3’;
- Antisense: 5’-GGCCTGATCCACGTGCACAGAC-
- 3’.

Site-directed mutagenesis was performed using the QuikChange XL mutagenesis kit (Agilent Technologies-Stratagene, La Jolla, CA). The sequences of mutations were confirmed by DNA sequencing at the Cleveland Clinic Genomics Core Facility at the Cleveland Clinic DNA sequencing facility, and DNA containing the desired mutation was transformed into *E. coli* BL21(DE3) cells for protein expression. Cells were also transformed with a pACYC plasmid containing human CaM and selected with chloramphenicol to co-express CaM with the eNOSr proteins.

**Expression and Purification of Wild type and Mutant Proteins:** The bovine eNOSr proteins (amino acids 445–1205) were purified by sequential chromatography on a 2′,5′-ADP-Sepharose affinity column and CaM-Sepharose resin as previously reported (15,20). Their concentration was determined using an extinction coefficient of 22,900 M⁻¹ cm⁻¹ at 457 nm for the fully oxidized form (15,20). All full-length eNOS proteins were overexpressed in *E. coli* strain BL21(DE3) and contained a His₆ tag at the N terminus to aid purification by sequential Ni-resin and CaM resin affinity chromatography (28,49,54). All full-length eNOS proteins were purified in the presence of H₂B and L-Arg as described previously (28,49,54), and their concentration was estimated by quantifying the heme protein content through the formation of the ferrous-CO adduct with an absorption maximum at 444 nm (28,49,54).

**Steady-state Assays:** Steady-state NO synthesis and NADPH oxidation activities of full-length eNOS proteins were determined at 10 or 25 °C using spectrophotometric assays as described previously (28,49,55,56). The NO-mediated conversion of oxyhemoglobin to methemoglobin was monitored at 401 nm and converted to a rate of NO synthesis using a difference extinction coefficient of ε₄₀₁ = 38 mM⁻¹ cm⁻¹. NADPH oxidation rates were similarly measured at 340 nm in the presence of oxyhemoglobin under identical conditions, and the rate of NADPH oxidation was calculated using an extinction coefficient of ε₃₄₀ = 6.2 mM⁻¹ cm⁻¹. The cytochrome c reductase
activities of eNOSr and full-length eNOS proteins were determined by monitoring the absorbance increase at 550 nm and using an extinction coefficient $\varepsilon_{550} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ as described previously (15,28,49).

**Anaerobic Heme Reduction Measurements:** The kinetics of heme reduction were analyzed at 10 °C as described previously (28,49,55) using a stopped-flow apparatus and diode array detector (Hi-Tech Scientific KinetAsyst SF-61DX2) equipped for anaerobic analysis. Ferric heme reduction was followed by formation of the ferrous heme-CO complex at 444 nm. The rate of heme reduction was determined by fitting the time course of the absorbance increase at 444 nm to a single exponential equation using a nonlinear least-squares method provided by Hi-Tech Ltd.

**Anaerobic Stopped-flow Flavin Reduction Kinetics in eNOSr Proteins:** The absorbance changes associated with eNOSr flavin reduction by NADPH were recorded by rapidly mixing a solution of oxidized eNOSr (8 - 10 µM) containing either EDTA (1 mM) or CaCl$_2$ (2 mM) + CaM (80 - 100 µM) with a solution of 100 µM NADPH at 10 °C in the stopped-flow instrument. The individual rate constants associated with absorbance changes at 457 nm were first estimated by analysis of experiments of varying lengths. The final reported values were obtained by fitting an experiment on a time scale capturing all three rate constants to a triple exponential function such that the residuals were minimized and contained little or no systematic deviation between the fit curve and the actual data (15,29).

**Reaction of Fully Reduced Proteins with Excess Cytochrome c:** The rate of reduction of excess cytochrome c by fully-reduced eNOSr proteins were measured in the stopped-flow instrument under anaerobic conditions at 10 °C as described previously in detail (15,16,20).

**Simulation of the Kinetic Traces of Cytochrome c Reduction:** We used the computer program Gepasi v.3.30 (57) to simulate the experimental electron flux to cytochrome c using the kinetic model as outlined in Fig. 1. Details of this type of simulations have been reported earlier (19). Briefly, cytochrome c is set to be in 100-fold molar excess relative to the eNOSr proteins in different conditions. Values for each of the four conformational rates ($k_1$, $k_{-1}$, $k_3$ and $k_{-3}$), the interflavin electron transfer rate ($k_2$), and ET to cytochrome c ($k_4$) were input into the software. Each simulated reaction began with 100% of the enzyme in the fully reduced state (represented by species d and a in Fig. 1). For simulations of reactions of the proteins used in this study, the initial concentrations of species d and a were determined based on the experimentally obtained conformational Keq values. Inputs that satisfy the observed conformational equilibrium constant are first used, and then refined in an iterative process using the time of first turnover and overall best fit of the kinetic trace as criteria to identify the best rates for FMN conformational motion and interflavin electron transfer. To simulate eNOSr behavior during electron flux to the eNOS heme, a range of slower heme reduction rates (0.1 to 1 s$^{-1}$) were used for $k_4$ in place of the cytochrome c reduction rate.

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**Conflict of interest**- The authors declare that they have no conflicts of interest with the contents of this article.

**Author Contributions**- M.M.H. designed the study, performed the experiments, analyzed data and prepared the manuscript; S.S.R. carried out the experiments and analyzed data; D.J.S. designed the study, analyzed data and prepared the manuscript. All authors performed critical reading of the manuscript prior to submission and approved the final version of the paper.
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FOOTNOTES

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3 Abbreviations used are: NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NOSr, reductase domain of NOS; eNOSr, reductase domain of eNOS; nNOSr, reductase domain of nNOS; CaM, Calmodulin; CPR, cytochrome P450 reductase; FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide; FMNsq, one-electron reduced (semiquinone) FMN; FMNhq two-electron reduced (hydroquinone) FMN; FNR, Ferredoxin-NADP’-Reductase-like domain; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; EPPS, 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid; Arg, L-arginine; DTT, dithiothreitol; H4B, (6R)-5, 6, 7, 8-tetrahydro-L-biopterin.

Reference List


**Figure Legends**

**Fig. 1: Kinetic model for electron flux through a dual-flavin enzyme.** The model involves four enzyme species and uses four kinetic rates: association \((k_1\) or \(k_3\)) and dissociation \((k_1\) or \(k_3\)) of the FMN and FNR domains; the interflavin ET (FMNH• reduction) rate \((k_2\)), and the cytochrome \(c\) reduction rate \((k_4\)). The fully-reduced enzyme in the open conformation (species a) reduces cytochrome \(c\) and generates species b, which then undergoes successive conformational closing, interflavin electron transfer, and conformational opening steps to complete the cycle. \(K_{sq}\) and \(K_{hq}\) are the conformational equilibrium settings for the partly oxidized and fully-reduced forms of the enzyme, respectively. We assumed \(K_{sq} = K_{hq}\) in our study.

**Fig. 2: Steady-state cytochrome c reductase activities of eNOSr and full-length eNOS proteins.** Activities are expressed as turnover numbers and were measured at 10 °C and 25 °C in the presence of SOD and either in the absence or presence of CaM. Values are mean ± SD of three measurements done under identical conditions, using two different protein preparations.

**Fig. 3: Kinetics of NADPH-dependent flavin reduction in S1179D eNOSr.** Stopped-flow traces were collected at 457 nm after rapidly mixing ~8 µM oxidized protein with a 10-fold excess of NADPH at 10 °C, as described under “Experimental Procedures.” The point of maximum absorbance prior to any reduction is indicated with arrows. Traces shown are the average of 7-10 individual collections and were fit to a tri-exponential equation, with the dotted lines of best fit also shown. The calculated rates of absorbance change are listed in Table 2.

**Fig. 4: Stopped-flow traces for the reactions of fully-reduced eNOSr proteins with excess cytochrome c.** Solutions of pre-reduced, CaM-free, or CaM-bound eNOSr proteins (~10 µM) containing 200 µM NADPH were rapidly mixed with cytochrome \(c\) (100 µM) in a stopped-flow instrument under anaerobic conditions at 10 °C, and kinetic traces were recorded at 550 nm to monitor reduction of cytochrome \(c\). Traces shown are an average of 6 to 7 individual mixings. The absorbance increase that was due to fast cytochrome \(c\) reduction that took place in the instrument mixing dead time is indicated by an unlined absorbance increase shown at time = 0, at the start of each trace. The absorbance change and elapsed time associated with transfer of the first electron equivalent to cytochrome \(c\) are indicated by the blue dotted lines in each panel.

**Fig. 5: Conformational motion and interflavin electron transfer rate settings that support the observed electron flux through eNOSr proteins to cytochrome c.** Data were obtained from simulations of the kinetic model in Fig. 1. For any given \(k_2\) value, rates of conformational motion were screened for a value that yielded the observed electron flux. For all panels, blue dotted lines indicate the lower boundary values for the rates of conformational motion (Y-intercept) and interflavin electron transfer (X-intercept). The main panels show the conformational opening rates \((k_1 = k_3)\), while Insets show the rates of conformational closing \((k_1 = k_3)\), all on the y axes. The blue boxed points in each panel indicate the best-fit rate pairs, which were derived from additional fitting as explained in the text.

**Fig. 6: Simulated best-fit traces for the reaction of fully-reduced eNOSr proteins with excess cytochrome c.** The best-fit traces were obtained by simulating the kinetic model in Fig. 1, using different rates of conformational motion and interflavin electron transfer \((k_2)\) until the closest fit to each experimental trace in Fig. 4 was reached, as described in the text.

**Fig. 7: Impact of CaM binding or the S1179D substitution on eNOSr species distribution during reaction with excess cytochrome c.** Panels A-D: Change in the populations of the four eNOSr species depicted in the inset during the reactions that are described by the product formation traces in Fig. 6.
Lines indicate the relative concentrations of the four enzyme species (color coded as in inset) versus time, with the total enzyme concentration being 1.0 and the concentration of fully-reduced (blue + black) enzyme species set equal to 1.0 at time = 0 in the simulations. The green dotted line in each panel marks the time required for the enzyme to reduce one equivalent of cytochrome c. Lower Panel- Pie graphs indicate the different enzyme species distributions that are achieved at the steady state in each simulated reaction. The kinetic values used in all the simulations are either listed in Supplemental Table S1 or in Methods.

**Fig. 8:** Impact of CaM binding or the S1179D substitution on the redox state of FMN in eNOSr during cytochrome c reduction. Bars indicate the relative percentage of FMN hydroquinone and FMN semiquinone that would be present in each enzyme during its simulated steady-state reaction with excess cytochrome c.

**Fig. 9:** Expected impact of the S1179D substitution on the distribution of eNOS reductase domain species and on electron flux to the eNOS heme during catalysis by CaM-bound eNOS. Simulations were run using the kinetic values in Supplemental Table S1 or as noted here for $k_4$. Upper- Pie graphs indicate the reductase domain species distributions that would be achieved during steady-state heme reduction in either enzyme, at a set heme reduction rate ($k_4$) of 0.1 s$^{-1}$. Lower left panel- The percentage of the conformationally-open FMNH$_2$ species that would be present in either enzyme during steady state heme reduction, as a function of the heme reduction rate ($k_4$). Lower right panel- The corresponding changes in the steady-state electron flux to the eNOS heme in either enzyme, as a function of the heme reduction rate.
Table 1: *Steady state Rates of wild-type and S1179D eNOS full-length proteins* - Rates were measured at 25 °C and 10 °C as described under “Experimental Procedures”. Values (min⁻¹) represent the mean ± S.D. of three independent measurements with two preparations of each enzyme. WT, wild-type.

<table>
<thead>
<tr>
<th>Protein</th>
<th>NO Synthesis</th>
<th>NADPH Oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+CaM</td>
</tr>
<tr>
<td><strong>Activities at 25 °C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT eNOS FL</td>
<td>10.5 ± 1</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>S1179D eNOS FL</td>
<td>22 ± 2</td>
<td>68 ± 3</td>
</tr>
<tr>
<td><strong>Activities at 10 °C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT eNOS FL</td>
<td>6.5 ± 0.6</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>S1179D eNOS FL</td>
<td>12 ± 1</td>
<td>42 ± 3</td>
</tr>
</tbody>
</table>
Table 2: Rates of anaerobic flavin reduction of eNOSr proteins by excess NADPH at 457 nm. Reductions were carried out in the stopped-flow instrument at 10 °C with a 10-fold excess of NADPH while monitoring the absorbance changes at 457 nm. Data were fit to a triple exponential function as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conditions</th>
<th>Dead timea (%)</th>
<th>$k_1^b$ (%)</th>
<th>$k_2^b$ (%)</th>
<th>$k_3^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-CaM</td>
<td>25</td>
<td>24 ± 2</td>
<td>1.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Wild type</td>
<td>+CaM</td>
<td>27</td>
<td>46 ± 4</td>
<td>5 ± 0.6</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>S1179D</td>
<td>-CaM</td>
<td>25</td>
<td>44 ± 3</td>
<td>5.6 ± 2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+CaM</td>
<td>21</td>
<td>68 ± 7</td>
<td>8 ± 2</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

- Percentage of the total absorbance change occurring in the instrument dead time.
- Individual rate constants are reported as follows: calculated rate ($s^{-1}$) (% of the total absorbance change for this process).
Table 3: Summary of results from reactions mixing eNOSr enzymes with excess cytochrome c- An excess of cytochrome c was mixed with fully reduced proteins in a stopped-flow instrument at 10 °C as described under “Experimental Procedures”.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
<th>Time (1st turnover)</th>
<th>Fraction Open</th>
<th>Fraction Closed</th>
<th>$K_{eq}$</th>
<th>Steady-state Electron flux (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT eNOSr</td>
<td>-CaM</td>
<td>1.85 s</td>
<td>0.13</td>
<td>0.87</td>
<td>0.15</td>
<td>27.6</td>
</tr>
<tr>
<td></td>
<td>+CaM</td>
<td>110 ms</td>
<td>0.60</td>
<td>0.40</td>
<td>1.5</td>
<td>186</td>
</tr>
<tr>
<td>S1179D eNOSr</td>
<td>-CaM</td>
<td>134 ms</td>
<td>0.60</td>
<td>0.40</td>
<td>1.5</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>+CaM</td>
<td>70 ms</td>
<td>0.71</td>
<td>0.29</td>
<td>2.4</td>
<td>228</td>
</tr>
</tbody>
</table>
Table 4: Kinetic Parameters derived from fitting the experimental traces according to the four-state kinetic model.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Condition</th>
<th>Measured $K_{eq}$</th>
<th>Best fit $k_1 = k_3$ (s$^{-1}$)</th>
<th>Best fit $k_{-1} = k_{-3}$ (s$^{-1}$)</th>
<th>Best fit $k_2$ (s$^{-1}$)</th>
<th>Lower boundary value $k_1$ or $k_3$ (s$^{-1}$)</th>
<th>Lower boundary value $k_{-1}$ or $k_{-3}$ (s$^{-1}$)</th>
<th>Lower boundary value $k_2$ (s$^{-1}$)</th>
<th>Fitted Steady state electron flux (s$^{-1}$) (Experimental value)</th>
<th>Fitted Time required for 1$^{st}$ turnover (Experimental value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT eNOSr</td>
<td>-CaM</td>
<td>0.15</td>
<td>4.2</td>
<td>0.63</td>
<td>5</td>
<td>3.2</td>
<td>0.55</td>
<td>0.8</td>
<td>0.46 (0.46)</td>
<td>1.85 (1.85) s</td>
</tr>
<tr>
<td></td>
<td>+CaM</td>
<td>1.5</td>
<td>8.8</td>
<td>13.2</td>
<td>17</td>
<td>5.0</td>
<td>7.7</td>
<td>9.9</td>
<td>3.0 (3.1)</td>
<td>110 (110) ms</td>
</tr>
<tr>
<td>S1179D eNOSr</td>
<td>-CaM</td>
<td>1.5</td>
<td>8</td>
<td>12</td>
<td>9</td>
<td>3.4</td>
<td>5.3</td>
<td>5.1</td>
<td>2.15 (2.2)</td>
<td>135 (134) ms</td>
</tr>
<tr>
<td></td>
<td>+CaM</td>
<td>2.4</td>
<td>8.5</td>
<td>20.4</td>
<td>32.4</td>
<td>5.2</td>
<td>12.7</td>
<td>13</td>
<td>3.8 (3.7)</td>
<td>70 (70) ms</td>
</tr>
</tbody>
</table>
Figure 1

Diagram representing a biochemical reaction pathway involving FNR, FMNH, and Cyt $c_{\text{red}}$ and $c_{\text{ox}}$. Reactions include $k_1$, $k_2$, $k_3$, and $k_4$ with equilibrium constants $K_{sq}$ and $K_{hq}$.
Figure 4

- **A**: WT eNOSr absorbance at 550 nm over time.
- **B**: WT eNOSr +CaM absorbance at 550 nm.
- **C**: S1179D eNOSr absorbance at 550 nm over time.
- **D**: S1179D eNOSr +CaM absorbance at 550 nm.

**Mol of Cyt c reduced/mol of eNOSred**

- **A**: WT eNOSr.
- **B**: WT eNOSr +CaM.
- **C**: S1179D eNOSr.
- **D**: S1179D eNOSr +CaM.
Figure 5

(A) WT eNOSr

(B) WT eNOSr + CaM

(C) S1179D eNOSr

(D) S1179D eNOSr + CaM

Rate of Conformational motion

(k₁ & k₋₁) (s⁻¹)

Interflavin ET (k₂) (s⁻¹)
Figure 8

The figure shows a bar graph representing the percentage of total FMN in different conditions. The graph compares the levels of FMNH₂ and FMNH⁺ in wild type (WT), wild type with CaM (WT +CaM), S1179D, and S1179D +CaM.
Figure 9
Phosphorylation Controls Endothelial Nitric Oxide Synthase by Regulating its Conformational Dynamics
Mohammad Mahfuzul Haque, Sougata Sinha Ray and Dennis J. Stuehr

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