A Cross-Domain Charge Interaction Governs the Activity of NO Synthase

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Running title: Interdomain electron transfer in NOS

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ABSTRACT

NO synthase (NOS) enzymes perform inter-domain electron transfer reactions during catalysis that may rely on complementary charge interactions at domain-domain interfaces. Guided by our previous results and a computer-generated domain docking model, we assessed the importance of cross-domain charge interactions in the FMN to heme electron transfer in neuronal NOS (nNOS). We reversed the charge of three residues (Glu-762, Glu-816, Glu-819) that form an electronegative triad on the FMN domain, and then individually reversed the charges of three electropositive residues (Lys-423, Lys-620, Lys-660) on the oxygenase domain (NOSoxy), to potentially restore a cross-domain charge interaction with the triad, but in reversed polarity. Charge reversal of the triad completely eliminated heme reduction and NO synthesis in nNOS. These functions were partly restored by the charge reversal at oxygenase residue Lys-423, but not at Lys-620 or Lys-660. Full recovery of heme reduction was likely muted by an accompanying change in FMN midpoint potential that made electron transfer to the heme thermodynamically unfavorable. Our results provide direct evidence that cross-domain charge pairing is required for the FMN to heme electron transfer in nNOS. The unique ability of charge reversal at position 423 to rescue function indicates that it participates in an essential cross-domain charge interaction with the FMN domain triad. This supports our domain docking model and suggests that it may depict a productive electron transfer complex formed during nNOS catalysis.

Nitric oxide (NO)1 is synthesized in mammals in a two-step oxidation of L-Arginine (Arg) that is catalyzed by three similar NO synthase isozymes (NOS, EC 1.14.13.39): inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) (1,2). Each NOS is comprised of an N-terminal oxygenase domain (NOSoxy) that is connected to a C-terminal flavoprotein domain by a central calmodulin
(CaM) binding sequence (3). Upon CaM binding, the NOS flavoprotein domain transfers NADPH-derived electrons to a heme located in the NOSoxy domain to enable heme-dependent oxygen activation for NO synthesis (4-6).

The NOS flavoprotein domain contains an FMN-binding domain that is attached by two flexible linkers to a NADPH- and FAD-binding domain (FNR domain) and to the NOSoxy domain (7-9) (Fig. 1A). During catalysis the FMN domain must undergo dynamic conformational switching to alternate between docking with the FNR to receive electrons, and docking with the NOSoxy domain for electron transfer (ET) to the heme (10-16) (Fig. 1A).

Evidence suggests that the rate of heme reduction in NOS enzymes is partly regulated through these large-scale conformational motions of the FMN domain (11-13,15,17-27). Electrostatic interactions are then proposed to direct short-range conformational sampling of the FMN domain that facilitate productive docking with its partner domains for ET (7,28). Indeed, NOS FMN domains all contain negatively-charged surface residues that are positioned to interact with complementary electropositive residues on the surface of their FNR domains (7). Such cross-domain charge pairing is thought to enable ET between the FAD and FMN groups. Moreover, several groups have proposed that the FMN domain could use some of the same electronegative surface residues to interact with the electropositive surface present on the NOSoxy domain, to enable ET from FMN to heme (Fig. 1A) (6,29-32). Indeed, substitutions that individually neutralized or reversed negatively-charged residues on the nNOS FMN domain partly inhibited electron transfer to the heme (32), with individual substitutions within an electronegative triad (Glu-762, Glu-816 and Glu-819) showing the greatest inhibition of heme reduction (12,32). Similarly, individual neutralization or reversal of positively-charged surface residues on the NOSoxy domain identified three (Lys-423, Lys-620, and Lys-660) as being important for enabling nNOS heme reduction (30). Subsequently, several groups have generated computer-based domain docking models that suggest complementary charge interactions may form between specific residues on the NOSoxy and FMN domains (30,31,33,34). The various model structures differ in several ways including which residue pairs form cross-domain charge pairing interactions, the distance between the FMN and heme, and whether NOS protein residues help bridge the ET reaction. In particular, our nNOS domain-docking model (30) predicts that a cross-domain salt-bridge interaction may form between Lys-423 of NOSoxy and Glu-762 of the FMN domain electronegative triad (Fig. 1B). However, the accuracy of the various FMN-NOSoxy domain-docking models and the importance of the proposed charge-pairing interactions have not yet been untested.

To address these issues, we reasoned that if cross-domain charge pairing is important for the FMN to heme ET in nNOS, then maintaining a charge pairing interaction should enable heme reduction, even when the charge polarity is reversed. We thus adopted a strategy of coordinated charge reversal, where we eliminated, and then restored in the opposite polarity, putative complementary charge pairing interactions as guided by our domain docking model. Specifically, we first reversed the charge of all three residues in the electronegative triad on the FMN domain, and then individually reversed each of the electropositive charges of the Lys-423, Lys-620, or Lys-660 residues on the NOSoxy surface (Fig. 2).
Each protein was characterized regarding its ET, NO synthesis, and thermodynamic profiles. Our results show that heme reduction in nNOS depends on the formation of a residue-specific but polarity-independent charge-pairing interaction, and thus help to define the geometry of a catalytically-productive FMN-NOSoxy domain docked complex.

RESULTS

Point mutations designed to eliminate and recover cross-domain complementary charge pairing—To eliminate the predicted complementary charge pairing between the nNOS FMN domain electronegative triad and the electropositive residues on the nNOSoxy domain, we reversed all three electronegative charges of the triad (Glu-762, Glu-816 and Glu-819) by substitution with Arg. We had previously incorporated this triple mutant into the nNOSr protein and found that it did not significantly diminish electron transfer through the FMN domain to cytochrome c (12). To then restore putative cross-domain complementary charge pairing, albeit with a reversed polarity, we added to this triple mutant individual nNOSoxy substitutions that reversed the charge at each of three electropositive residues (Lys-423, Lys-620 or Lys-660) (Fig. 2). All proteins were expressed in E. coli and their characterization showed that each purified protein had normal yields and spectroscopic features (data not shown), consistent with earlier reports showing that the individual mutations in nNOS, and the triple FMN domain mutation in nNOSr, were well-tolerated (12,30,32). We went on to compare the activities, heme reduction, and properties of the triple and the quadruple (Quad) nNOS mutants relative to wild-type nNOS.

Impact on nNOS heme reduction and NO synthesis—Our previous work had shown that reversing the individual charges at Glu-762, Glu-816 or Glu-819 on the nNOS FMN domain, or at Lys-423, Lys-620, and Lys-660 on the nNOSoxy surface, each partly inhibited the FMN to heme ET (12,30,32). Rates of heme reduction in the current proteins were determined under anaerobic conditions in a stopped-flow spectrophotometer as previously described (9,30). Reactions were initiated by mixing excess NADPH with each CaM-bound enzyme in the presence of CO, and heme reduction was followed by formation of the ferrous heme-CO complex at 444 nm. The triple mutant showed no detectable heme reduction, indicating that the combined charge reversal of the three electronegative triad residues was sufficient to prevent heme reduction in nNOS. Among the three Quad mutants, only the one containing the K423E substitution (Quad 1) recovered observable heme reduction, albeit at a lower magnitude and rate compared to wild-type nNOS (Fig. 3A, B). The absorbance traces fit well to a double exponential equation and the second phase gave rates for heme reduction that are listed in Table 1. Measurement of the NO synthesis activities (Table 1) showed that a mutant’s ability to generate NO from either Arg or the intermediate N⁶-hydroxy-L-arginine (NOHA) directly correlated with their capacity to support heme reduction.

Exploring the basis for diminished heme reduction in the Quad mutants—The driving force for heme reduction in nNOS is influenced by the relative midpoint potentials of the FMN and heme cofactors (29,32,35,36). The K423E and K660E substitutions were each previously found to increase the nNOS heme midpoint potential by 50 to 60 mV (30), but the effect of charge reversal at all three electronegative triad residues is unknown. We therefore measured the flavin midpoint potentials in the nNOS triple mutant to see if they were altered. A
potentiometric titration was performed on the fully-oxidized nNOSr version of the triple mutant (12) by adding graded amounts of dithionite and recording the absorbance trace and potential after each addition (13,37). Representative spectra recorded during the titration are shown in Fig. 4A, and the corresponding trace of the absorbance change at 457 nm vs change in potential (titration runs from right to left) is shown in Fig. 4B. The spectral changes followed the typical behavior seen for wild-type nNOSr and related enzymes during their reductive titrations (29), where an initial buildup of the flavin semiquinone is indicated by an absorbance increase around ~592 nm and a decrease of absorbance at 457 nm, followed by a decrease in flavin absorbance at both 592 nm and 457 nm as the titration progresses to drive the enzyme towards its fully reduced form. As previously observed (13,29), this generates a double-sigmoidal trace of the 457 nm absorbance vs potential curve (Fig. 4B), which reflects the sq/ox and hq/sq midpoint potentials of the two flavins. The midpoint potentials were determined from the trace by nonlinear fitting using the four-electron Nernst equation as a model, and the values derived for the triple mutant and wild-type nNOSr are reported in Table 2. The results indicate that the FMN hq/sq midpoint is more positive by ~100 mV in the triple mutant and its FMN sq/ox midpoint is more negative by ~100 mV relative to wild type nNOS. We then measured the flavin midpoint potentials for the E762R nNOSr mutant, and found that it had changes in its FMN couples that mirrored what we observed for the triple nNOSr mutant (Table 2). As illustrated in Fig. 4C, this change makes the FMN hq/sq midpoint potential in the triple mutant (-173 mV, Table 2) relatively more positive than the midpoint potential of the nNOS heme ferrous/ferric couple (-251 mV) (30), and slightly more positive than the ferrous/ferric heme couples reported for the K423E or K620E nNOS mutants (-190 and -199 mV, respectively) (30). Thus, a change in the FMN hq/sq midpoint potential that is caused by the triple mutation makes heme reduction become thermodynamically uphill in the triple nNOS mutant and in the Quad mutant enzymes.

To examine the influence of this thermodynamic effect, we measured steady-state rates of electron flux through the heme to O2 in the triple and Quad mutants relative to wild type nNOS, reasoning that all mutants should have lower rates of electron flux to O2 if unfavorable thermodynamics truly limit their heme reduction. In these experiments, the nNOS enzymes all had CaM bound to allow for heme reduction, and were given excess NADPH in the absence of Arg, or in the presence of L-arginine, which binds in the Arg binding site but is not oxidized by the enzyme (29,30). Under either condition, any NADPH-derived electrons that pass to the heme are used to quickly reduce O2 to superoxide or H2O2 instead of generating NO (38). The steady-state electron flux through the heme is thus related to the NADPH consumption rate. Results in Table 3 show that electron flux through the heme in the triple mutant and three Quad mutants was much lower than for wild type nNOS. These findings are consistent with a poor driving force for heme reduction in the triple and Quad mutants due to unfavorable thermodynamics.

**DISCUSSION**

The importance of charge pairing in protein-protein or domain-partner interactions is often invoked from results of point mutagenesis studies that neutralize or reverse the charge of individual surface residues in question. Here we performed a more rigorous test by examining if an
enzyme’s function could be rescued by restoring an inter-domain charge pairing interaction presumed to be important, but in a reversed polarity. Specifically, we generated an nNOS triple mutant that no longer displayed any heme reduction or NO synthesis due to a charge reversal of an electronegative triad on its FMN domain, which by design should antagonize an interaction of the FMN domain with an electropositive region on the NOSoxy domain that is presumed important for the FMN to heme ET. Importantly, the triple mutant supports a near-normal rate of electron flux through its FMN domain to cytochrome c (12), so we can assume its negative impact on nNOS heme reduction is primarily due to the triple mutation influencing the FMN domain interaction with the NOSoxy domain and the ET between the FMN and heme. We found that heme reduction and NO synthesis in the inactive triple mutant could be partly recovered by incorporating a K423E mutation onto the NOSoxy domain surface (to create the Quad 1 mutant). On its own, the K423E mutation is very deleterious to both heme reduction and NO synthesis in nNOS (30), but in the context of the Quad 1 mutant it was expected to potentially restore a cross-domain charge pairing interaction, albeit of opposite polarity, between the FMN and NOSoxy domains and thus aid ET between them. Our finding that the K423E mutation partly restored heme reduction and NO synthesis under this circumstance, and thus had a positive effect, provides compelling evidence that a cross-domain charge pairing interaction involving position 423 enables the FMN to heme ET in nNOS.

In contrast to the results achieved with the K423E Quad 1 mutant, creating individual charge reversals at either the Lys-620 or Lys-660 positions within the same triple mutant nNOS (to create the Quad 2 and 3 mutants) were both ineffective at rescue, although they created a similar opportunity for cross-domain charge pairing as did charge reversal at position 423. The unique ability of position 423 to rescue electron transfer, at least in part, under this circumstance is consistent with our domain docking model in Fig. 1, which has Lys-423 positioned close enough to the electronegative triad to form a cross-domain salt bridge interaction with Glu-762 in the FMN domain. In comparison, the model has the nNOSoxy Lys-620 and Lys-660 residues positioned at a greater distance from the FMN domain electronegative triad, consistent with charge reversal at these positions being ineffective in rescuing heme reduction and catalysis in Quad 2 and Quad 3 mutants. Perhaps Lys-620 or Lys-660 do not form charge pairing interactions with the electronegative triad, or do form interactions with it that are unproductive for supporting the inter-domain ET between FMN and heme. In any case, our experimental results support the formation of a domain-docked species like that shown in Fig. 1 in order to enable heme reduction in nNOS.

Although charge-reversal of the electronegative triad eliminated nNOS heme reduction, which was the intended effect, these mutations also complicated matters by causing the thermodynamics of nNOS heme reduction to become somewhat thermodynamically uphill in the triple and in all the Quad mutants. Because this effect diminished electron flux to the heme in the Quad 1 mutant, it likely blunted the positive impact of restoring the charge pairing interaction on the extent and rate of heme reduction and catalysis of NO synthesis. Our previous work had shown that neutralizing the charge at Glu-762 in nNOS (i.e., by an E762N mutation) caused, on its own, a similar change in the FMN midpoint potentials as what we observed here in the
triple mutant, and also diminished the extent and rate of heme reduction in nNOS (29,32). Also we report here that the E762R substitution on its own caused a similar change in FMN midpoint potentials, and this is most likely responsible for altering the FMN midpoint potentials in the triple mutant. In our previous work we also found that incorporating the K423E or K620E mutations into nNOS increased its heme midpoint potential by 50 or 60 mV, respectively (30), and thus incorporating these point mutations into the triple mutant improved the thermodynamics of heme reduction in the Quad 1 and Quad 2 mutants somewhat. However, despite the K423E and K620E mutations having similar thermodynamic effects, only the K423E substitution rescued heme reduction and NO synthesis. This downplays the importance of its positive thermodynamic effect, and suggests instead that its restoration of charge pairing is paramount.

As previously noted, our nNOS FMN-NOSoxy domain docking model differs somewhat from the computer-generated domain docking models of human or mouse iNOS (31,33,34). The iNOS models shift the docking site of the FMN domain relative to our nNOS model, and consequently predict that it involves different cross-domain charge pairing interactions, a greater FMN-to-heme distance, and invoke participation of a conserved aromatic residue (Trp-366 in iNOS) in mediating the FMN-to-heme ET. In particular, the residue that is equivalent to nNOS Glu-762 (Glu-546 in human iNOS FMN domain, Glu-540 in mouse iNOS) does not interact with the Lys-423 equivalent (Asn-208 in human iNOS, Asn-202 in mouse iNOS) in the iNOS models, and instead forms a salt bridge interaction with Arg-452 of the iNOSoxy domain. As in nNOS, mutagenesis confirmed that Glu-546 is important for iNOS FMN to heme ET (31), but the importance of its putative charge-pairing partner (Arg-452) was not tested. However, charge neutralization or reversal of the equivalent Arg residue in nNOS (Arg-667) is known to have little or no effect on the rate of heme reduction or NO synthesis (30). This suggests that there may be differences between iNOS and nNOS in the relative importance of charge pairing involving this position on the oxygenase domain. Regarding the longer FMN to heme distance in the iNOS docking models, it is consistent with a pulsed EPR distance estimate of 18 Å that was measured thus far only in iNOS (39). Trp-366 is well-positioned to mediate the FMN to heme ET in the iNOS models, and this possible role is supported by heme reduction being lost in the W366A mutant (31), although the mutation’s impact on iNOS dimeric structure, which must be maintained for heme reduction (23), was not considered. The importance of this conserved Trp has not yet been tested in nNOS or in other NOS enzymes. In any case, it is important to note that rapid conformational sampling likely occurs during NOS FMN-NOSoxy domain docking (40,41) to generate many transiently-docked species, several of which still place the FMN and heme close enough for ET between them. That said, there also may be docking preferences among the NOS enzymes that are driven by their structural differences, such as iNOS having fewer surface charges on its oxygenase and reductase domains relative to nNOS or eNOS (30). Further work may reveal if these differences enable docking preferences that in turn can help explain the order of magnitude difference in heme reduction rates that is observed between the three NOS enzymes (42).

Cross-domain charge pairing may be a common strategy used by the dual-flavin reductase family to enable docking of their
FMN domains with various acceptor heme proteins or heme domains. For example, a similar electronegative patch on the FMN domain of CPR enables it to interact with electropositive surfaces located on its heme oxygenase or cytochrome P450 redox partner proteins (43-45). Among the electronegative triad residues that we studied here, both previous (29,31,32) and the current work indicate that Glu-762 and its equivalent play a predominant role in enabling the FMN to heme ET in nNOS and iNOS. However, sequence comparisons show that Glu-762 is only conserved among NOS enzymes and is typically replaced by neutral residues in related flavoproteins (32). In comparison, the two other electronegative residues of the triad (Glu-816 and Glu-819) are either strongly or partly conserved across the broader flavoprotein family. These differences may relate to the fact that the NOS FMN domain must dock separately with partner FNR and NOSoxy domains during catalysis. The crystal structure of nNOSr shows that Glu-816 makes a salt bridge interaction with Arg-1229 of the FNR domain (7) that stabilizes a closed conformational state needed for ET from FAD to FMN (12). In contrast, Glu-762 makes no observable salt bridge or hydrogen-bonding contacts with the FNR domain in this conformation, and so likely plays a lesser role than does Glu-816 in FMN domain docking with its FNR partner in NOS. Thus, the electronegative triad residues may have distinct roles and levels of importance in assisting FMN docking to the FNR versus NOSoxy domains. This is consistent with a mass spectrometry study that showed the two FMN docking surfaces only partially overlap in iNOS (31). Among the larger flavoprotein family, the FMN domain residues that participate in charge pairing with the FNR domain may be more strongly conserved, while those participating in charge pairing with the different ET acceptor partners (NOSoxy, cytochrome P450, etc.) may be unique (i.e., Glu-762 and its homologs in NOS enzymes). This concept may help explain how dual-flavin reductases diverged to enable FMN domain docking to a variety of ET acceptors while preserving key electrostatic interactions that allow it to dock with the FNR domain.

**EXPERIMENTAL PROCEDURES**

**General Methods and Materials**— All reagents and materials were obtained from Sigma-Aldrich, Amersham Biosciences or other sources as previously reported (8,29,32). Absorption spectra and steady-state kinetic data were obtained using a Shimadzu UV-2401PC spectrophotometer. All plots and some additional curve-fitting were done using Origin® 8.0 (OriginLab, Northampton, MA). All experiments were repeated two or more times with at least two independently prepared batches of proteins to ensure consistent reproducibility of the results. Data were analyzed and are expressed as mean ± its standard deviation (S.D.).

**Preparation of nNOS Expression Plasmids**— Wild-type and mutant rat nNOS proteins containing a His$_6$ tag attached to their N-termini were overexpressed in *Escherichia coli* strain BL21(DE3) using a modified pCWori vector as described (9,32). Restriction digestions, cloning, and bacterial growth were performed using standard procedures. Transformations were performed using a TransformAid bacterial transformation kit (Fermentas-Thermo Scientific, Hanover, MD). Oligonucleotides used to construct mutants in nNOS were obtained from Integrated DNA Technologies (Coralville, IA). All mutated constructs were confirmed by DNA sequencing at the Cleveland Clinic Genomics Core.
Expression and Purification of Wild-Type type and Mutant Proteins—All proteins were purified in the presence of (6R)-tetrahydrobiopterin (H₄B) and L-Arg as described previously (9). The ferrous heme-CO adduct absorbing at 444 nm was used to measure heme protein content with an extinction coefficient of $\varepsilon_{444} = 74 \text{ mM}^{-1} \text{cm}^{-1} (A_{444}-A_{500})$ (9,46). Purity of each protein was assessed by SDS-PAGE and spectral analysis.

NO Synthesis, NADPH Oxidation, and Flux measurement—Steady-state activities of nNOS wild-type and mutant nNOS proteins were determined separately at 25 °C using the spectrophotometric oxyhemoglobin assay (8,9,29). Cuvettes contained 0.1 - 0.2 µM nNOS, 40 mM EPPS (pH 7.6), 150 mM NaCl, 0.3 mM dithiothreitol, 4 µM FAD, 4 µM FMN, 10 µM H₄B, 5 mM L-Arg, 1 mg/ml bovine serum albumin, 1.0 mM Ca²⁺, 0.2 mM EDTA, 1.0 µM CaM, 100 units/ml catalase, 40 units/ml superoxide dismutase, and 5 µM oxyhemoglobin. The reaction was initiated with 250 µM NADPH in a total reaction volume of 500 µl and was run for 2 min. 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 $\varepsilon_{401} = 38 \text{ mM}^{-1} \text{cm}^{-1}$. 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 $\varepsilon_{340} = 6.2 \text{ mM}^{-1} \text{cm}^{-1}$. For the electron flux measurement through the NOS heme during steady-state catalysis, we measured the rate of NADPH oxidation by each CaM-bound enzyme in the absence of L-Arginine or in the presence of 2 mM L-Agmatine (8,9,30).

Steady-state Cytochrome c reduction—Cytochrome c reductase activity was determined as previously reported (8,9,30) by following the absorbance change for the reduction of cytochrome c by nNOS at 550 nm using an extinction coefficient of 21 mM⁻¹ cm⁻¹. Reaction mixtures (total volume 500 µl) contained ≤ 0.01 µM nNOS, 6 µM FAD, 6 µM FMN, 0.1 mg/ml bovine serum albumin, 1 mM CaCl₂, 0.2 mM EDTA, 1.0 µM CaM, 100 units/ml catalase, 40 units/ml superoxide dismutase, 70 µM cytochrome c and 150 mM NaCl in 40 mM EPPS buffer, pH 7.6. The reaction was initiated by adding NADPH to a final concentration of 250 µM. In the assays without CaM, neither CaM nor CaCl₂ were added and EDTA concentration was 0.45 mM.

Anaerobic Heme Reduction Rate measurements—The kinetics of heme reduction were analyzed at 10 °C as described previously (8,9,30,47) using a stopped-flow apparatus and diode array detector (TGK Scientific KinetAsyst SF-61DX2) equipped for anaerobic analysis. Anaerobic samples were prepared in an air-tight cuvette using repeated cycles of vacuum followed by a positive pressure of catalyst-deoxygenated nitrogen gas. Ferric heme reduction was followed by formation of the ferrous heme-CO complex at 444 nm. Reactions were initiated by rapidly mixing an anaerobic, buffered, CO-saturated solution containing 100 µM NADPH with an anaerobic protein solution containing wild-type or mutant nNOS (8-10 µM), 100 mM EPPS pH 7.6, 100 mM NaCl, 20 µM H₄B, 5 mM L-Arg, 0.5 mM DTT, 50 µM CaM, 1 mM EDTA and 5 mM CaCl₂. Signal-to-noise ratios were improved by averaging 8-10 individual mixing experiments. The spectral traces were fit using software provided by the instrument manufacturer. The rate of heme reduction was determined by fitting the time course of the absorbance change increase at 444 nm to a double exponential equation using a
The initial decay in absorbance corresponds to the flavin reduction by NADPH, whereas the second step corresponds to the absorbance increase due to heme reduction and concomitant generation of the ferrous heme-CO complex.

Spectro-potentiometric titrations of the reductase domain of multiple mutants—All redox titrations were performed in an anaerobic glove box (Belle Technology, Weymouth, UK) under N₂ atmosphere with less than 2 ppm oxygen levels. Titrations were run in a custom-made small multi-armed glass cell in ~3 mL total volume. The titration cell was bathed in a water bath kept at 15°C. The cell housed an ORP electrode (Microelectrodes Inc., Bedford, NH) through one arm and a dip probe with an optical fiber through a second arm. The optical dip probe reports to a Cary 50 UV-Vis spectrophotometer used to monitor absorbance spectra during the titration. Aliquots of reductant (or oxidant) used to drive redox titration reactions were added through a third arm. Dithionite was used in the reduction branch of the titration and ferricyanide was used in the reverse oxidation branch. The potentials were measured with an MI-800/MI-710 microelectrode, a combination ORP electrode with an internal Ag/AgCl reference electrode in 3M KCl (Microelectrodes Inc.). Potentials recorded against this reference are converted to values versus standard hydrogen electrode (SHE) using a conversion factor of +214 mV for 15 °C. The concentration of all nNOSr proteins (WT and mutants) was in the ~40 µM range. All protein samples are first oxidized with ferricyanide. The oxidized protein is then separated from the mixture by size exclusion through a Sephadex G-25 column (PD-10, GE Healthcare). All measurements were performed in 40 mM EPPS (pH 7.6) with 10% glycerol and 150 mM NaCl without CaM (in the presence of ~2 mM EDTA).

The redox mediators used (0.5 µM final concentration) with their midpoint potentials versus SHE are as follows: phenazine methosulfate (+80 mV), indigo carmine (-125 mV), 2- hydroxy 1,4-naphthaquinone (-152 mV), anthraquinone-2,6-disulfide (-184 mV), phenosafranine (-252 mV), safranine O (-280 mV), benzylviologen (-374 mV), and methyl viologen (-443 mV). Prior to and after use in redox titrations, the potential response of the ORP microelectrode is checked against saturated quinhydrone solution in 100 mM phosphate (pH 7.0) (+301 mV at 22 °C) as well as in a solution of 5 mM ferricyanide/ferrocyanide redox couple in 100 mM phosphate (pH 7.0) (+425 mV at 22 °C). The response of the ORP microelectrode was in good agreement with listed values for the redox couples used (within ±5 mV). Absorbance changes at 457 nm (λ_max for oxidized flavin) are plotted against the measured potentials at each point of the titration. The data at 457 nm, which monitors the 4 sequential reductions for the two flavins (FMN and FAD) is best fitted to a derived four-electron Nernst equation relating the overall absorbance change to the relative absorbance contributions of the two flavins at a given system potential E (Equation 1):

\[
A = \frac{a10^{2(E-E_1)-E_2}/57 + b10^{(E-E_2)/57} + c}{1 + 10^{(E-E_1)-E_2)/57} + e10^{(E-E_1)/57} + 10^{(E-E_2)/57} + d10^{(E-E_2)/57}}
\]

(Eq.1)

The equation is derived by extension of combined single-electron Nernst equation and Beer-Lambert law to four sequential redox steps as described previously for similar systems (13,37,48). A is the overall absorbance at a wavelength of interest recorded at each measured potential E in millivolts; a-e are the relative absorbance contributions of the two flavins.
values of the diflavin system in each of its 5 nondegenerate oxidation states. $E'_1$ through $E'_4$ are the four midpoint potentials, two for each flavin. Data at ~592 nm ($\lambda_{\text{max}}$ for the blue semiquinone form of the flavins) is also used for fitting and extraction of values of flavin midpotentials. The 592 nm data is fitted using an equation similar to Equation 1 but modified and adapted for two two-electron steps as described in previous reports (13,29,37,49). Origin software was used for graphing absorbance-potential data. Matlab software was used for nonlinear fitting of potentiometric titration data using the derived four-electron Nernst equation shown above as a model. Equations modeling absorbance change as a function of potential are entered into global fitting subroutines within Matlab using either the Levenberg-Marquardt or the “Trust-Region” minimization algorithms. The 4 potential values (2 for each flavin) for the best fit are reported in each case.

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**Author contributions:** MMH, MB and CTK conducted the experiments. DJS and MMH conceived the idea for the project. DJS, MMH and JT analyzed the results and wrote the paper.
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**FOOTNOTES**

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† The abbreviations used are: CaM, Calmodulin; CPR, Cytochrome P450 reductase; Em, Equilibrium midpoint potential; ET, Electron transfer; NO, nitric oxide; NOHA, Nω-hydroxy-L-arginine; NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; nNOSoxy, nNOS oxygenase domain; nNOSr, nNOS reductase domain; Quad 1 mutant, K423E/E762R/E816R/E819R nNOS; Quad 2 mutant, K620E/E762R/E816R/E819R nNOS; Quad 3 mutant, K660E/E762R/E816R/E819R nNOS; Triple mutant: E762R/E816R/E819R nNOS.
Table 1. Rates of heme reduction and NO synthesis activities of the nNOS enzymes
Rates of heme reduction and NO synthesis were measured at 10 °C and 23 °C, respectively, as described under “Experimental Procedures”, and are the means ± S.D. of seven to ten or three individual reactions, respectively, representative of two enzyme preparations.

| nNOS Enzyme                     | Heme reduction | NO synthesis |
|--------------------------------|----------------|--------------|
|                               | s⁻¹            | Arg          | NOHAᵃ       |
| Wild type                     | 5.1 ± 0.4      | 48 ± 4       | 95 ± 7      |
| Triple (E762R/E816R/E819R)    | 0              | 0            | 0           |
| Quad 1 (K423E/E762R/E816R/E819R) | 1.6 ± 0.1     | 14 ± 0.5     | 25.5 ± 1.0  |
| Quad 2 (K620E/E762R/E816R/E819R) | 0             | 0            | 0           |
| Quad 3 (K660E/E762R/E816R/E819R) | 0             | 0            | 0           |

ᵃ NOHA: N⁰-hydroxy-L-arginine, the intermediate formed from Arg in NOS during NO synthesis.

Table 2. Flavin equilibrium midpoint potentials (Em) of nNOSr proteins
Midpoint potentials of each flavin couple (ox/sq and sq/hq) were determined versus a standard hydrogen electrode for the CaM-free enzymes by potentiometric titration at 15 °C as described under "Experimental Procedures" and are the mean ± SD of two independent titrations.

| Enzyme                        | FMN Potentials | FAD Potentials |
|-------------------------------|----------------|----------------|
|                               | ox/sq          | sq/hq          | ox/sq          | sq/hq          |
|                               | mv             |                | mv             |                |
| Wild type nNOSr               | -50 ± 6        | -276 ± 14      | -260 ± 9       | -280 ± 13      |
| E762R nNOSr                   | -30 ± 5        | -147 ± 15      | -264 ± 19      | -314 ± 21      |
| Triple mutant (E762R/E816R/E819R) nNOSr | -146 ± 11      | -173 ± 9       | -229 ± 10      | -291 ± 16      |
Table 3. Comparative electron flux through the heme in nNOS or mutant proteins during their steady-state catalysis of NADPH oxidation. NADPH oxidation rates (min$^{-1}$) were measured for each CaM-bound enzyme in the absence of L-Arg or in the presence of 2 mM L-Agmatine. Values are the mean ± SD of three measurements each, and are representative of two different enzyme preparations.

| Protein                  | NADPH oxidation |  |
|--------------------------|-----------------|---|
|                          | -Arg            | +Agmatine     |   |
| Wild type                | 460 ± 26        | 512 ± 36      |   |
| Double (K423E/E762R)     | 32 ± 2          | 34 ± 2.5      |   |
| Triple (E762R/E816R/E819R)| 38 ± 4          | 40 ± 3.2      |   |
| Quad 1 (K423E/E762R/E816R/E819R) | 33 ± 2      | 38 ± 3          |   |
| Quad 2 (K620E/E762R/E816R/E819R) | 27 ± 1.3     | 19 ± 1.6       |   |
| Quad 3 (K660E/E762R/E816R/E819R) | 23 ± 1.5   | 29 ± 2        |   |
FIGURE 1. Models of FMN domain function and docking onto the oxygenase domain for ET to the heme. Panel A, Cartoon shows how the FMN domain must move away from the FNR domain to reversibly dock with the NOSoxy domain in order to transfer an electron to the heme in NOSoxy. Red and blue patches signify electronegative and electropositive patches, respectively, that are present on the domain surfaces and are either known (FNR-FMN) or presumed (FMN-NOSoxy) to form complementary electrostatic interactions when the domains interact. Panel B, Computer docking model of an energy-minimized FMN-NOSoxy domain interaction in nNOS. The residues that form an electronegative triad on the FMN domain (gold) and three electropositive residues on the NOSoxy domains (blue and green) are labeled and shown as stick models, along with the bound FMN (yellow) and heme (red) cofactors. The side-chain interaction distance of Lys-423 with each electronegative triad residue, as well as the FMN to heme edge distance, are noted.
FIGURE 2. Electrostatic surfaces on the nNOS FNR, FMN and oxygenase domains and changes caused by targeted charge reversal. Upper left panel shows the surface of the FNR domain indicating the location of the FAD cofactor and the Arg-1229 residue. Upper right panels show the locations of three residues that create the electronegative triad and the associated change in surface charge. Lower panels show the locations of three electropositive residues in the heme domain that surround the exposed heme edge and the associated change in surface charge. The lower left panel indicates the location of the mutated residues in the wild-type heme domain surface and the lower right panels are close ups of the noted region for the native enzyme and the mutants. The red and blue colors indicate the electrostatic potentials on the solvent accessible surface calculated via the Poisson-Boltzmann equation as calculated by the APBS software (50). Figures were drawn with the PyMol software, v0.99rc6 (51).
FIGURE 3. Kinetics and extent of heme reduction in nNOS and the Quad 1 mutant. Each ferric CaM-bound enzyme was mixed at 10°C with excess NADPH in a stopped-flow spectrophotometer and spectra recorded on a diode array detector. The rate of heme reduction was determined from the increase in 444 nm absorbance which indicates the rate of heme-CO complex formation. Panels A and B, Kinetic traces of wild-type and Quad 1 nNOS, respectively. The initial drop in 444 nm absorbance is due to the initial flavin reduction step, and the red line in each kinetic trace is a biphasic fit of the data. Insets show the ferrous-CO minus ferric nNOS difference spectra obtained using the traces recorded at the final and first timepoints, respectively.
FIGURE 4. Spectro-electrochemical titration of nNOSr triple mutant to determine flavin midpoint potentials. *Left panel*, representative visible spectra recorded during a potentiometric titration of the oxidized, CaM-free triple nNOSr mutant with sodium dithionite. *Right panel*, plots of absorbance at 457 nm versus the electrochemical potential (mV) along with the line of best fit (red) as calculated using the four-electron Nernst equation described in "Experimental Procedures." SHE, standard hydrogen electrode. Data are representative of two experiments.
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