Supporting Information

Mutations in the FMN Domain Modulate MCD Spectra of the Heme Site in the Oxygenase Domain of Inducible Nitric Oxide Synthase

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Materials and Methods

DNA Cloning. The human iNOS oxyFMN plasmid, pCHADB, is a pCWori+ derivative containing amino acid residues 71-723 of human iNOS, coding for the oxygenase and the FMN-binding domains. The coding sequence is inserted between the pCWori+ *Nde*I and *Hind*III restriction endonulease sites. The plasmid was constructed by using the published procedures.[1]

Site specific variants E546N and E603N of iNOS oxyFMN were constructed using the Stratagene QuickChange XL kit, following the manufacturer's instructions. Sequences were confirmed by DNA sequencing in both the sense and anti-sense directions. Residue numbering corresponds to the full length human iNOS isoform. The mutagenic oligos are as follows:

E546N1	5′-GAGTCACCATCCTCTTTGCGACA*AAC*ACAGGAAAATCAGA
E546N2	5′-TCTGATTTTCCTGT*GTT*TGTCGCAAAGAGGATGGTGACTC
E603N1	5′-GACTGCCCTGGCAATGGA*AAC*AAACTGAAGAAATCGCTC
E603N2	5′-GAGCGATTTCTTCAGTTT*GTT*TCCATTGCCAGGGCAGTC

Protein Expression. The iNOS oxyFMN (and variants) and p209 plasmids were co-transformed into competent BL21(DE3) cells by electroporation; p209 is a low copy number plasmid expression vector for rat CaM. Positive selection was then done by growth on LB agar plates in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol.

Fresh transformants were grown successively at 37°C in 1 ml, 50 ml and 4 liters of Terrific Broth in the presence of 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Sub-culturing was done in the mid to late log phase of growth. At an OD_{600} of 0.8–1.2, protein expression was induced by addition of 0.25 mM isopropyl β-D-thiogalactopyranoside. δ-aminolevulinic acid and riboflavin were added to final concentrations of 450 and 3 μM, respectively. Temperature was reduced to 25 °C, and the flasks were shaken in the dark at ~ 250 rpm. Cells were harvested by

centrifugation after ~ 40 hours post-induction. Cell pellet was frozen and stored at -80 °C until purification.

Protein Purification. Purification of the iNOS oxyFMN construct was conducted with modified procedures.[2] Briefly, the cells were lysed by sonification in a minimal volume of buffer (40 mM Tris-Cl, 10% glycerol, 200 mM NaCl, 10 μM tetrahydrobiopterin (H_4B), 1 mM L-Arginine, pH 7.6). The iNOS protein was then precipitated in the 30–70% ammonium sulfate fraction. The protein pellet was re-suspended in a minimal amount of buffer A (see below). Complete Protease Inhibitor Cocktail Tablets (Roche) were added throughout these processes.

Affinity chromatography (Clontech TALON® polyhistidine-tag purification resin) was performed on a GE FPLC by using a gradient from buffer A to buffer B.

Buffer A: 40 mM Tris-Cl, 250 mM NaCl, 10% glycerol, 1 mM L-Arginine, pH 7.6

Buffer B 40 mM Tris-Cl, 250 mM NaCl, 10% glycerol, 1 mM L-Arginine, 150 mM imidazole, pH 7.6

Fractions with $A_{280}/A_{400} < 3.0$ were pooled and concentrated. The pooled protein was then dialyzed into the storage buffer (40 mM Tris-Cl, 200 mM NaCl, 10% glycerol, 1 mM DTT, 4 μM H_4B, pH 7.6); concentrated protein (~ 200 μM) was aliquoted and stored at -80 °C.

MCD Experiments. The oxyFMN proteins used for the MCD experiments have a A_{280}/A_{400} of 2.1–2.4. An optically transparent glass is required for low temperature MCD measurements. This was achieved by preparing the protein samples in 42% v/v ethylene glycol. The sample was centrifuged to remove any precipitate, if necessary. The final concentration of the NOS protein in the MCD sample cell was about 22 μM; buffer: 50 mM Tris-Cl, 200 mM NaCl, 1 mM DTT, 2 μM H_4B, pH 7.6. Low temperature MCD spectra were measured in an applied magnetic field of

7 T using a JASCO J-810 spectropolarimeter that is interfaced to a computer and an Oxford Instruments Spectromag 4000-7 split-coil superconducting magnet system.

UV-vis Spectra. Absorption spectra of the protein samples under the same conditions as the MCD samples were obtained to confirm that the protein is sensitive to L-Arg binding, and that the integrity of the protein has been maintained with added ethylene glycol. The spectra were recorded using a Cary 50 spectrophotometer at room temperature.

(a)

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sp|P29475|NOS1_HUMAN        MGQAMAKRVKATILYATETGKSQAYAKTLCEIFKHAFDAKVMSMEEYDIV 799
sp|P29476|NOS1_RAT          MGQAMAKRVKATILYATETGKSQAYAKTLCEIFKHAFDAKAMSMEEYDIV 794
sp|P29474|NOS3_HUMAN        MGTVMAKRVKATILYGSETGRAQSYAQQLGRLFRKAFDPRVLCMDEYDVV 558
sp|P35228|NOS2A_HUMAN       MRKTMASRVRVTILFATETGKSEALAWDLGALFSCAFNPKVVCMDKYRLS 578
                            *  .**.**:.***:.:***:::: *  *  :*  **:.:.:.*::* :

sp|P29475|NOS1_HUMAN        HLEHETLVLVVTSTFGNGDPPENGEKFGCALMEMRHPNSVQ---EERKSY 846
sp|P29476|NOS1_RAT          HLEHEALVLVVTSTFGNGDPPENGEKFGCALMEMRHPNSVQ---EERKSY 841
sp|P29474|NOS3_HUMAN        SLEHETLVLVVTSTFGNGDPPENGESFAAALMEMSGPYNSSPRPEQHKSY 608
sp|P35228|NOS2A_HUMAN       CLEEERLLLVVTSTFGNGDCPGNGEKLKKSLFMLKELNNKF--------- 619
                             **.* *:*********** * ***.:  :*: :    .
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(b)

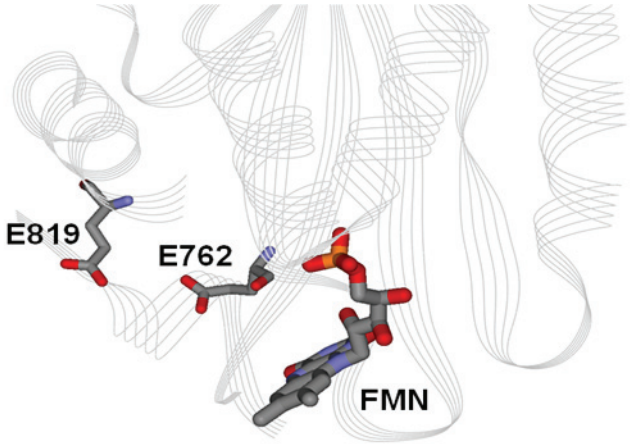


Figure S1. (a) Sequence alignment of nNOS (NOS1), iNOS (NOS2) and eNOS (NOS3); only residues near edge of the FMN domain are shown for clarity. Note that E546 and E603 in human iNOS (highlighted) are conserved in the three NOS isoforms. (b) E762 and E819 at the edge of the FMN domain in a rat nNOS reductase construct (pdb entry: 1TLL), the equivalent residues of E546 and E603 in human iNOS. These two charged residues were mutated to asparagine in this study.

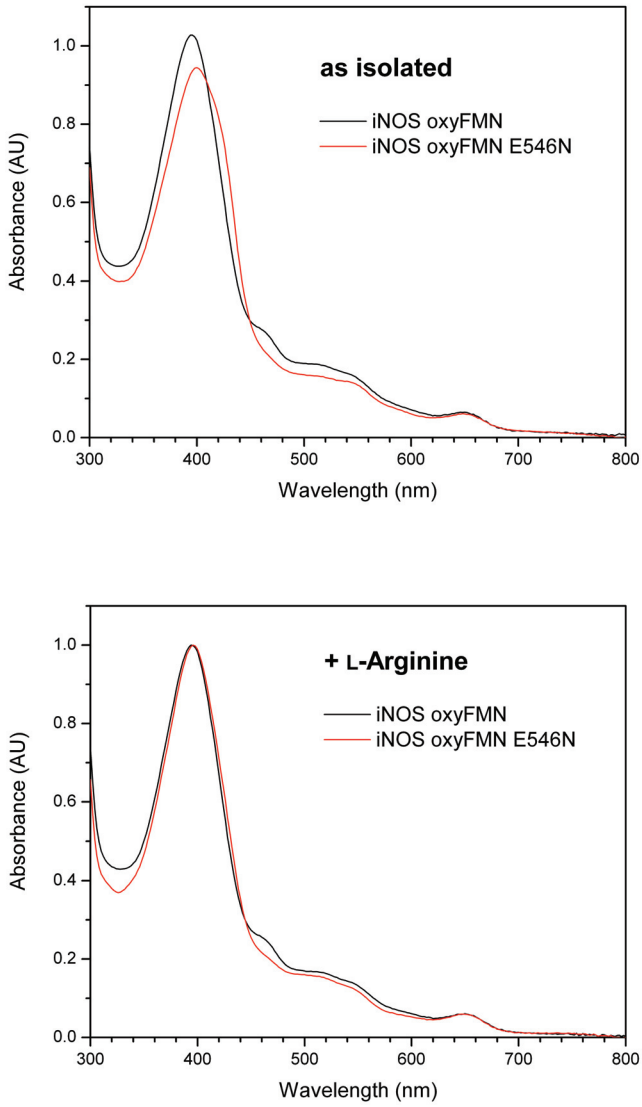


Figure S2. UV-vis spectra of wild type (wt) and E546N iNOS oxyFMN constructs without (top), and with 3 mM L-Arg (bottom). The spectra of E603N are similar to E546N (data not shown). Spectra are normalized so that the Soret peak of L-Arg-bound iNOS equals 1.0 (bottom panel). The proteins (~ 1 μM) were in a pH 7.6 buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, 4 μM H_4B). The Soret maximum at 396 nm in the presence of L-Arg (bottom panel) is characteristic of high-spin heme. These spectra are similar to those of oxyFMN constructs reported in the literature.[1]

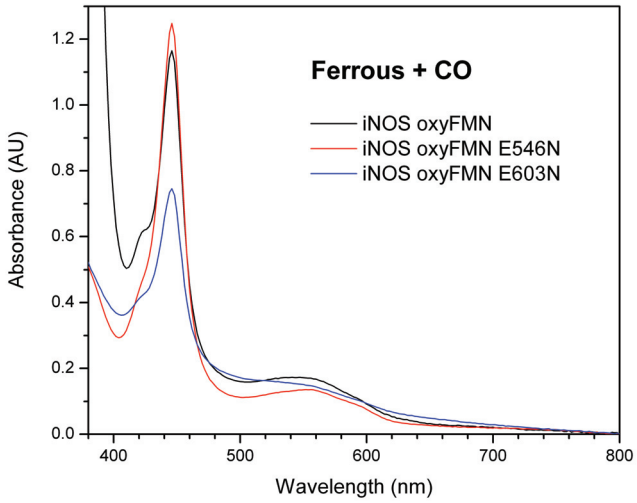


Figure S3. UV-vis spectra of the ferrous-CO form of the human iNOS oxyFMN proteins. The sample conditions were the same as those described in Fig. S2. The proteins were reduced by excess dithionite in the presence of CO. The peaks at 445 nm indicate that the proteins are in their native forms that have thiolate-bound heme centers.

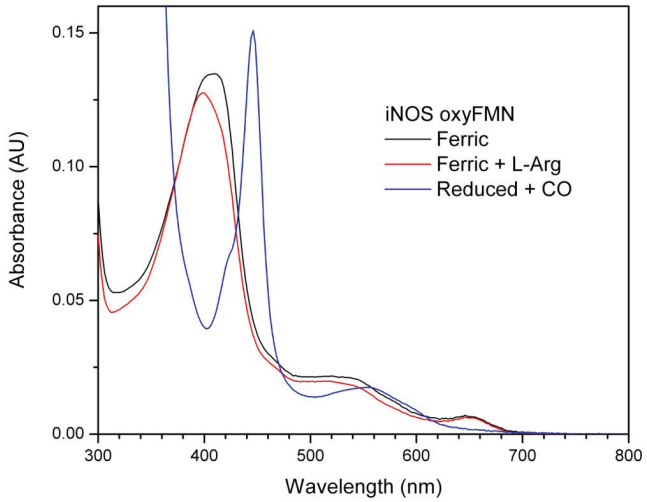


Figure S4. UV-vis spectra of wt human iNOS oxyFMN in the presence of 42% ethylene glycol. The spectra of the E546N and E603N mutants are similar to the wt (data not shown). The samples were made in a pH 7.6 buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, 4 μM H_4B, 42% ethylene glycol); the same buffer was used for the MCD samples. The protein responds to L-Arg treatment (red trace), indicating that it remains active with added ethylene glycol. The integrity of the protein in the presence of ethylene glycol was further confirmed by the lack of conversion to a P420 type species upon dithionite reduction in the presence of CO (blue trace).

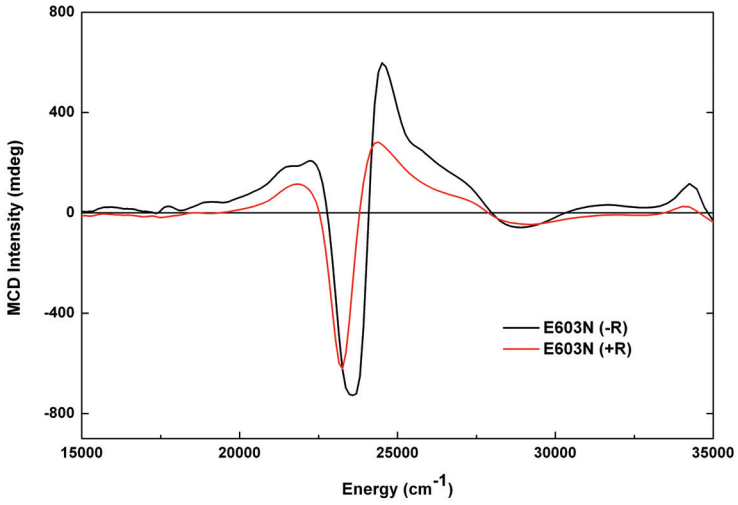
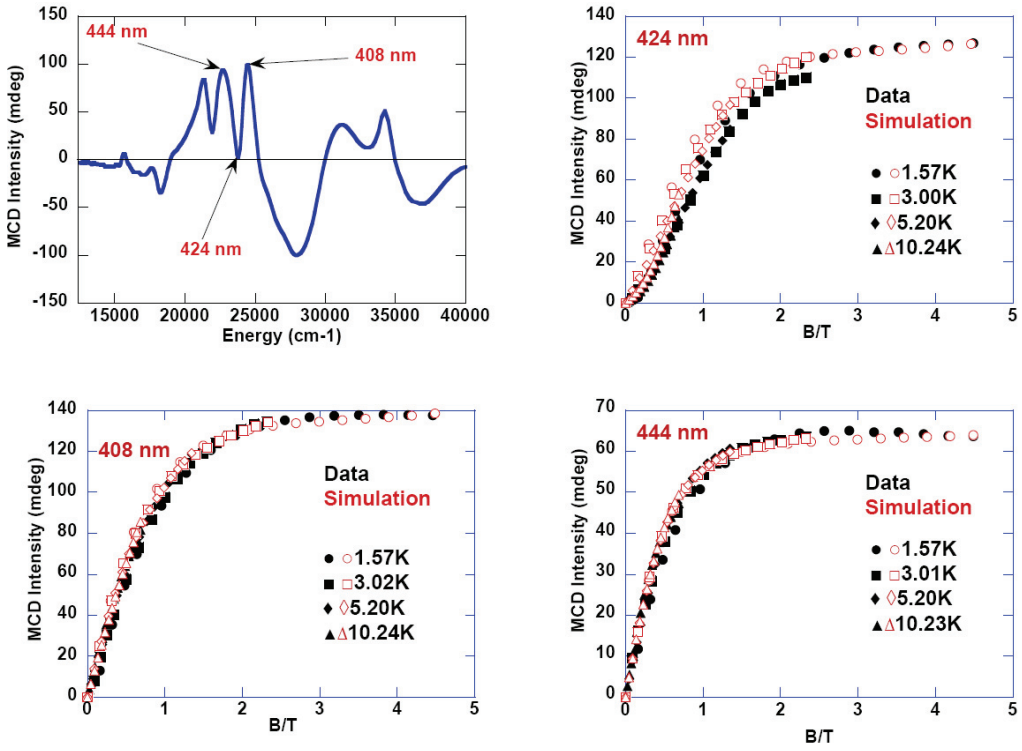


Figure S5. MCD spectra recorded at 5K for the heme site in the as-isolated E603N mutant of human iNOS oxyFMN construct without (black trace) and with 20 mM L-Arg (red trace). The samples were made in a pH 7.6 buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, 4 μM H_4B) in the presence of 42% (v/v) ethylene glycol.

(a)



(b)

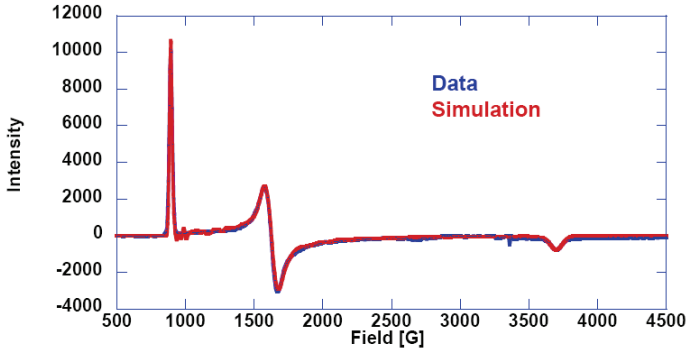


Figure S6. (a) MCD magnetization curves of the as-isolated wt human iNOS oxyFMN construct at 408, 424 and 444 nm with added L-Arg. Spectral simulation has been achieved with g_x = 2.020, g_y = 1.949, g_z = 2.024, S = 2.5, E/D = 0.08 cm^{-1}, $A_x = A_y = A_z = 0$, L_x = 90, L_y = 40, L_z = 50; polarizations are: xy = 1.000, xz = -0.010, yz = -0.100 at 424 nm, xy = 1.000, xz = 0.019, yz = -0.100 at 408 nm, and xy = 1.000, xz = 0.140, yz = -0.130 at 444 nm. (b) The simulation of the EPR spectrum has been accomplished by using these intrinsic parameters obtained from the VTVH data.

References

1. Ghosh, D. K.; Holliday, M. A.; Thomas, C.; Weinberg, J. B.; Smith, S. M. E.; Salerno, J. C. *J. Biol. Chem.* **2006**, *281*, 14173-14183.
2. Feng, C. J.; Dupont, A.; Nahm, N.; Spratt, D.; Hazzard, J. T.; Weinberg, J.; Guillemette, J.; Tollin, G.; Ghosh, D. K. *J. Biol. Inorg. Chem.* **2009**, *14*, 133-142.
3. Ledbetter, A. P.; McMillan, K.; Roman, L. J.; Masters, B. S. S.; Dawson, J. H.; Sono, M. *Biochemistry* **1999**, *38*, 8014-8021.