Published in final edited form as:

Inorg Chem. 2011 August 1; 50(15): 6859–6861. doi:10.1021/ic200952c.

Mutation in the FMN Domain Modulates MCD Spectra of the iNOS Ferric Cyano Complex in a Substrate-Specific Manner

Joseph Sempombe[†], Mary Grace I. Galinato[‡], Bradley O. Elmore^{||}, Weihong Fan^{||}, J. Guy Guillemette[‡], Nicolai Lehnert[‡], Martin L. Kirk^{†,*}, and Changjian Feng^{||,*}

[†] Department of Chemistry and Chemical Biology, The University of New Mexico, NM 87131

[‡] Department of Chemistry, University of Michigan, MI 48109

^{||} College of Pharmacy, The University of New Mexico, NM 87131

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

Abstract

We have obtained low temperature MCD spectra for ferric cyano complexes of the wild type and E546N mutant of a human iNOS oxygenase/FMN construct. The mutation at the FMN domain has previously been shown to modulate the MCD spectra of the L-arginine-bound ferric iNOS heme [Sempombe et al., *J. Am. Chem. Soc.* 2009, 131, 6940–6941]. Addition of L-arginine to the wild type protein causes notable changes in the CN[−]-adduct MCD spectrum, while the E546N mutant spectrum is not perturbed. Moreover, the MCD spectral perturbation observed with L-arginine is absent in the CN[−] complexes incubated with N-hydroxy-L-arginine, which is the substrate for the second step of NOS catalysis. EPR spectroscopy also reveals a substrate dependency of the spectra. These results indicate that the interdomain FMN–heme interactions exert a long-range effect on key heme axial ligand-substrate interactions that determine substrate oxidation pathways of NOS.

In mammals, nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine (Arg) to L-citrulline and NO by a two-step process (Scheme 1).¹ In the first step, L-Arg is converted to N-hydroxy-L-arginine (NOHA), and in the second step, NOHA is converted to NO and L-citrulline.^{2, 3} Mechanistic details of the unique NOS oxidative chemistry remain to be fully elucidated.^{3–8} A central task is the determination of structures for the obligatory ferric-(hydro)peroxo intermediates because the two NOS reaction steps (Scheme 1) are distinguished by substrate-controlled delivery of protons to the ferric-heme-peroxo species that subsequently reacts with the substrate.^{6, 8, 9} It is challenging to probe the hydrogen bonding interaction of bound oxygen with the NOS substrate because of the highly reactive nature of the ternary dioxygen adduct intermediate. It is well-accepted to use alternative ligands such as CN[−], which form more stable heme complexes, as mimics of the obligatory ferric-(hydro)peroxo intermediate of P450 enzymes.^{10, 11} The crystal structure of the ferric cyano complex of inducible NOS (iNOS) indicates that the axial cyanide ligand H-bonds to a water molecule (Fig. 1) that could provide a proton required for O–O bond scission in the hydroxylation reaction of NOS.¹² In this study, we use the NOS CN[−] complexes as models of the key dioxygen intermediates in NOS catalysis.

* cfeng@salud.unm.edu, Tel: 505-925-4326 (CF); mkirk@unm.edu, Tel: 505-277-5992 (MK).

SUPPORTING INFORMATION. Measurements of dissociation constants of L-Arg and CN[−]; MCD spectra of iNOS oxygenase construct (Figs. S1 S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Mammalian NOS is a homodimeric flavo-hemoprotein. Each NOS subunit contains a C-terminal electron-supplying reductase unit with binding sites for NADPH (electron source), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and an N-terminal catalytic heme-containing oxygenase domain. The FMN and oxygenase domains are linked by a calmodulin (CaM) binding region. Bi-domain NOS oxyFMN constructs, which only consist of the oxygenase and FMN domains along with the CaM-binding linker, were recently designed.¹³ These homologous dimeric oxyFMN constructs are valid models of the electron-donating (output) state of the FMN domain.^{13–19}

A recent low-temperature magnetic circular dichroism (LT MCD) study by us showed that mutation at E546 in human iNOS FMN domain (Fig. 2) modulates the MCD spectra of the L-Arg-bound ferric heme.²⁰ In the present study, we have used LT MCD and EPR spectroscopies to further demonstrate that the E→N mutation at E546 in the FMN domain affects the key axial cyano ligand-substrate interaction at the heme active site in a human iNOS oxyFMN construct. Note that the ferric-cyano complex is a mimic of the obligatory heme-peroxo intermediates of NOS catalysis, and the nature of the axial ligand-substrate interaction in the intermediates is critical in determining the NOS substrate oxidation pathways (see above).^{6, 8}

Wild type (wt) and E546N human iNOS oxyFMN constructs were expressed and purified as described elsewhere.²⁰ The K_d of CN^- was determined to be 8.5 ± 0.9 mM (Fig. S1 in Supporting Information). Therefore 50 mM KCN was added to form CN^- complexes of the iNOS proteins. The MCD samples were prepared using microvolumetric techniques, and the temperature dependent MCD spectra, arising from the ferric-cyano center, were obtained at 5K, 10K and 20K in a 7T applied magnetic field (Fig. 3).²¹ All of the MCD spectra are reminiscent of low-spin ferric heme species.^{22, 23} However, addition of L-Arg resulted in notable changes in the MCD spectrum of the wt protein ferric- CN^- complex (the low-spin heme marker band is blue shifted by ~ 600 cm^{-1}), whereas the E546N mutant possesses similar MCD spectra in the presence and absence of L-Arg (Fig. 3). Importantly, the spectral perturbation induced by L-Arg binding in the wt- CN^- sample (Fig. 3) is absent in the iNOS oxygenase (iNOSoxy) construct (Fig. S2). The iNOSoxy construct only possesses a heme domain but no FMN domain, and thus provides an important control to assess the effects of a properly aligned FMN domain in the wt oxyFMN construct.

Interestingly, the ferric- CN^- complexes of wt and E546N proteins possess similar MCD spectra in the presence and absence of NOHA (Fig. 4), and the iNOSoxy ferric-cyano sample behaves similarly upon addition of NOHA (Fig. S2). The results show that perturbation of the ferric-cyano MCD spectrum is only observed for the wt iNOS oxyFMN sample in the presence of L-Arg (Fig. 3), but not for NOHA, and is thus substrate-specific. In light of our recent FMN...heme distance data,²⁴ these MCD results clearly indicate that interdomain FMN-heme interactions exert a *long-range* effect on heme axial ligand-substrate interactions, and that this effect is substrate-specific. This is important since these key interactions determine the NOS substrate oxidation pathway.⁸

We have also obtained continuous wave (CW) EPR spectra for the cyano complexes of the wt and E546N mutant (Fig. 5). We observed differential effects of L-Arg and NOHA binding on the wt CN^- complex; see different shifts in the turning points at g_2 and g_3 (red and green spectra, Fig. 5). The EPR results indicate that the substrate induces a perturbation at the heme site, and this effect is substrate-specific. Previous EPR studies of ferrous-NO forms of nNOS also indicated substrate-specific perturbation of NO adduct structure.²⁵ This is consistent with the fact that the active site structure of the iNOS ferric cyano complex¹² is similar to that of the NO complex of the neuronal NOS oxygenase domain,²⁶ in which the bound NO/ CN^- ligand interacts with the substrate.

Note that the EPR spectra of the E546N mutant are rather similar to those of the wt protein (Fig. 5). However, addition of L-Arg causes *notable* change in the ferric heme MCD spectrum of the wt protein CN⁻ complex (red spectrum in Fig. 3), while this change is absent in the E546N mutant (green spectrum in Fig. 3). This indicates that MCD detects different structural variations at the iNOS heme site (compared to EPR).

In order to confirm that the observed differences in the MCD spectra of the wt and E546 mutant (Fig. 3) are not due to a change in binding capability of L-Arg to the CN⁻ complexes, we determined the L-Arg dissociation constants (K_d) of the proteins by titrating with L-Arg in the absence and presence of 50 mM KCN (Figures S3–S5, Table 1). The K_d values of L-Arg in the CN⁻ complexes are greater than the CN⁻-free samples, indicating that the axial CN⁻ ligand affects L-Arg binding to the protein. This effect may be due to the fact that the guanidino group of L-Arg moves ~ 0.38 Å closer to the conserved substrate-binding residue E371 (to accommodate the cyanide ligand), resulting in a heme site with altered substrate binding properties.¹² Importantly, L-Arg K_d for the CN⁻ complex of the E546N mutant is similar to wt protein (Table 1). This is in contrast to the abolished L-Arg perturbation of the MCD spectra of the mutant (Fig. 3). Therefore, the absence of an L-Arg perturbation in the E546N CN⁻ complex MCD spectrum (Fig. 3) is not due to ineffectiveness of L-Arg binding to the mutant. These data further support a model where the E→N mutation in the FMN domain is responsible for the observed difference in the L-Arg-induced perturbation of the MCD spectra for the ferric-CN⁻ complexes.

E546 is a charged surface residue located at the edge of the human iNOS FMN domain (Fig. 2), and is conserved in the NOS isoforms. The full length NOS structure is not available. Nonetheless, in the computational docked structure,²⁴ the E546 residue is ~ 47 and 21 Å away from the heme center in the same subunit and the other subunit of the dimeric protein, respectively. This residue is thus not in close contact with the heme center, and is believed to be important in the iNOS interdomain FMN/heme interactions.²⁰ Also note that in the crystal structure of CaM-bound human iNOS FMN domain,²⁷ the only interactions between the FMN domain and CaM involve R536 of iNOS, which makes a salt bridge with E47 of CaM, and the E546 residue does not interact with the CaM-binding linker or the bound CaM (Fig. 2). Therefore, the CaM-binding region is not directly involved in the effect induced by the mutation at E546 site either.

It has been shown that the interdomain FMN/heme interactions are important in electron transfer between the FMN and heme domains in NOS.¹⁴ Importantly, emerging evidence further indicates that the FMN domain docking to the heme domain also regulates NOS catalysis by modulating reactivity^{28,29} and structure²⁰ of the heme active site. Based on the results in the present study, we propose that the charge neutralization mutation at the iNOS E546 site may disturb the interdomain FMN/heme complex (by interfering with the complementary interdomain electrostatic interactions), and thereby reduces the L-Arg-perturbation of the MCD spectra of the NOS ferric-CN⁻ complex.

In summary, this study provides spectroscopic evidence to indicate that the docked FMN domain affects the key interaction between the L-Arg substrate and the axial ligand (i.e. CN⁻) of the catalytic heme center located in an adjacent domain in iNOS, and that the effect is substrate-specific.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Institutes of Health (GM081811 and HL091280 to C.F.; GM057378 to M.L.K.), AHA Grant-in-Aid (09GRNT2220310 to C.F.), the National Science Foundation (CHE-0846235 to N.L.), and NSERC (183521 to J.G.G.). The project described was also supported by Grant Number P20RR016480 from the National Center for Research Resources, a component of the National Institutes of Health. CF also acknowledges support from UNM HSC RAC.

References

1. Roman LJ, Martasek P, Masters BSS. *Chem Rev.* 2002; 102:1179–1189. [PubMed: 11942792]
2. Alderton WK, Cooper CE, Knowles RG. *Biochem J.* 2001; 357:593–615. [PubMed: 11463332]
3. Zhu Y, Silverman RB. *Biochemistry.* 2008; 47:2231–2243. [PubMed: 18237198]
4. Santolini J. *J Inorg Biochem.* 2011; 105:127–141. [PubMed: 21194610]
5. Daff S. Nitric Oxide. 2010; 23:1–11. [PubMed: 20303412]
6. Davydov R, Sudhamsu J, Lees NS, Crane BR, Hoffman BM. *J Am Chem Soc.* 2009; 131:14493–14507. [PubMed: 19754116]
7. Woodward JJ, Nejaty Jahromy Y, Britt RD, Marletta MA. *J Am Chem Soc.* 2010; 132:5105–5113. [PubMed: 20307068]
8. Li D, Kabir M, Stuehr DJ, Rousseau DL, Yeh SR. *J Am Chem Soc.* 2007; 129:6943–6951. [PubMed: 17488012]
9. Giroud C, Moreau M, Mattioli TA, Balland V, Boucher JL, Xu-Li Y, Stuehr DJ, Santolini J. *J Biol Chem.* 2010; 285:7233–7245. [PubMed: 19951943]
10. Deng TJ, Macdonald IDG, Simianu MC, Sykora M, Kincaid JR, Sligar SG. *J Am Chem Soc.* 2001; 123:269–278. [PubMed: 11456513]
11. Yang TC, McNaughton RL, Clay MD, Jenney FE, Krishnan R, Kurtz DM, Adams MWW, Johnson MK, Hoffman BM. *J Am Chem Soc.* 2006; 128:16566–16578. [PubMed: 17177406]
12. Fedorov R, Ghosh DK, Schlichting I. *Arch Biochem Biophys.* 2003; 409:25–31. [PubMed: 12464241]
13. Ghosh DK, Holliday MA, Thomas C, Weinberg JB, Smith SME, Salerno JC. *J Biol Chem.* 2006; 281:14173–14183. [PubMed: 16461329]
14. Feng CJ, Tollin G, Holliday MA, Thomas C, Salerno JC, Enemark JH, Ghosh DK. *Biochemistry.* 2006; 45:6354–6362. [PubMed: 16700546]
15. Feng CJ, Thomas C, Holliday MA, Tollin G, Salerno JC, Ghosh DK, Enemark JH. *J Am Chem Soc.* 2006; 128:3808–3811. [PubMed: 16536556]
16. Feng CJ, Dupont A, Nahm N, Spratt D, Hazzard JT, Weinberg J, Guillemette J, Tollin G, Ghosh DK. *J Biol Inorg Chem.* 2009; 14:133–142. [PubMed: 18830722]
17. Ilagan RP, Tejero JS, Aulak KS, Ray SS, Hemann C, Wang ZQ, Gangoda M, Zweier JL, Stuehr DJ. *Biochemistry.* 2009; 48:3864–3876. [PubMed: 19290671]
18. Feng CJ, Tollin G. *Dalton Trans.* 2009; 6692–6700
19. Feng C, Fan W, Dupont A, Guy Guillemette J, Ghosh DK, Tollin G. *FEBS Lett.* 2010; 584:4335–4338. [PubMed: 20868689]
20. Sempombe J, Elmore BO, Sun X, Dupont A, Ghosh DK, Guillemette JG, Kirk ML, Feng C. *J Am Chem Soc.* 2009; 131:6940–6941. [PubMed: 19405537]
21. Jones RM, Inscore FE, Hille R, Kirk ML. *Inorg Chem.* 1999; 38:4963–4970. [PubMed: 11671238]
22. Berka V, Palmer G, Chen PF, Tsai AL. *Biochemistry.* 1998; 37:6136–6144. [PubMed: 9558353]
23. Galinato MGI, Spolitak T, Ballou DP, Lehnert N. *Biochemistry.* 2011; 50:1053–1069. [PubMed: 21158478]
24. Astashkin AV, Elmore BO, Fan W, Guillemette JG, Feng C. *J Am Chem Soc.* 2010; 132:12059–12067. [PubMed: 20695464]
25. Migita CT, Salerno JC, Masters BSS, Martasek P, McMillan K, IkedaSaito M. *Biochemistry.* 1997; 36:10987–10992. [PubMed: 9283090]

26. Li HY, Igarashi J, Jamal J, Yang WP, Poulos TL. J Biol Inorg Chem. 2006; 11:753–768. [PubMed: 16804678]
27. Xia C, Misra I, Iyanagi T, Kim JJP. J Biol Chem. 2009; 284:30708–30717. [PubMed: 19737939]
28. Haque MM, Fadlalla M, Wang ZQ, Ray SS, Panda K, Stuehr DJ. J Biol Chem. 2009; 284:19237–19247. [PubMed: 19473991]
29. Liu XD, Mazumdar T, Xu Y, Getzoff ED, Eissa NT. J Immunol. 2009; 183:5977–5982. [PubMed: 19828635]

Figure 1. Active site structure of the ferric cyano complex of the murine iNOS oxygenase domain (PDB 1N2N). Cyanide binds to the heme iron and interacts with L-Arg.

Figure 2.

Structure of a human iNOS FMN domain/CaM complex (PDB 3HR4). The iNOS FMN domain is gold, and the CaM-binding linker is yellow. CaM is green, and Ca²⁺ ions are grey spheres. The conserved E546 residue is at the edge of the FMN domain. The missing heme domain is at the other end of the CaM-binding linker.

Figure 3.

MCD spectra (C-term) recorded at 5 K, 7 Tesla for ferric-cyano complexes in wild type (wt) and the E546N mutant of the human iNOS oxyFMN construct without or with 20 mM L-Arg. The samples were made in a pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μ M H₄B); 42% (v/v) ethylene glycol was added to the protein samples as a glassing agent. Note that the MCD spectrum of the wt+CN+Arg sample (red trace) is significantly blue shifted relative to the other spectra.

Figure 4.

MCD spectra recorded at 5 K, 7 Tesla for ferric-cyano complexes in wt and E546N mutant of the human iNOS oxyFMN construct without or with 20 mM NOHA. The samples were made in a pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μ M H4B); 42% (v/v) ethylene glycol was added to the protein samples.

Figure 5.

X-band EPR spectra of ferric-cyano complexes in the human iNOS oxyFMN samples of (1) wt + CN, (2) wt + CN + Arg, (3) wt + CN + NOHA, (4) E546N + CN, and (5) E546N + CN + Arg. The samples were made in a pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μ M H₄B, 42% (v/v) ethylene glycol). The CW EPR spectra were acquired at $\sim$ 9.4 GHz using a microwave power of 20 dB and a modulation amplitude of 5 G.

Scheme 1.
NOS chemistry.

Table 1

Dissociation constant (K_d) of L-Arg for the wild type (wt) and E546N mutant human oxyFMN proteins in the presence of 42% ethylene glycol

Samples	[CN ⁻] (mM)	K_d (μ M)
wt	0	50.0 ± 3.6
wt	50	330 ± 81
E546N	0	94.4 ± 5.7
E546N	50	235 ± 61