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Mutation in the Flavin Mononucleotide Domain Modulates Magnetic Circular Dichroism Spectra of the iNOS Ferric Cyano Complex in a Substrate-Specific Manner

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Supporting Information

ABSTRACT: We have obtained low-temperature magnetic circular dichroism (MCD) spectra for ferric cyano complexes of the wild type and E546N mutant of a human inducible nitric oxide synthase (iNOS) oxygenase/flavin mononucleotide (oxyFMN) 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, J.; et al. *J. Am. Chem. Soc.* **2009**, *131*, 6940−6941). The 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. These results indicate that 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 (L-Arg) to L-citrulline and NO by a two-step process.[1] In the first step, Arg is converted to *N*-hydroxy-L-arginine (NOHA), and in the second step, NOHA is converted to NO and citrulline.[2,3] However, the mechanistic details of the oxidative chemistry remain to be elucidated.[3−8] A central task is the determination of structures for the obligatory ferric (hydro)peroxo intermediates because the two NOS reaction steps are distinguished by the substrate-controlled delivery of protons to the ferric−heme−peroxo species that subsequently reacts with substrate.[6,8,9] It is challenging to probe the hydrogen-bonding interaction of bound dioxygen with the NOS substrate because of the highly reactive nature of the ternary dioxygen adduct intermediate. It is well-accepted that alternative ligands such as CN^-, which form more stable heme complexes, may be used 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 CN^- ligand hydrogen-bonds to a water molecule (Figure S1 in the Supporting Information) that could provide a proton required for O−O bond scission in the hydroxylation reaction.[12] Here, we use NOS CN^- complexes as models of key dioxygen intermediates in NOS catalysis.

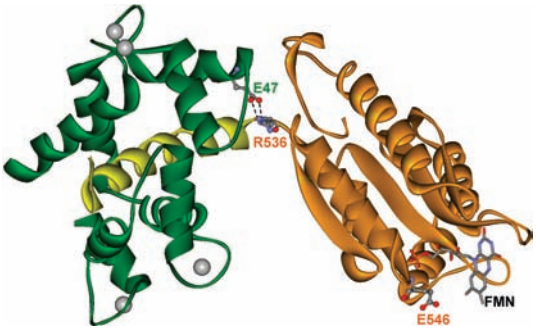

Figure 1. Structure of a human iNOS FMN domain/CaM complex (PDB 3HR4). The iNOS FMN domain is gold, and the CaM-binding linker is yellow. CaM is green, and Ca^{2+} ions are gray spheres. The conserved E546 residue is at the edge of the FMN domain. The missing heme domain is at the other end of the CaM-binding linker.

Bidomain NOS oxyFMN constructs, which consist of the oxygenase and flavin mononucleotide (FMN) domains along with the calmodulin (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 of the NOS oxyFMN proteins showed that mutation at E546 in the human iNOS FMN domain (Figure 1) modulates the MCD spectra of the L-Arg-bound ferric heme.[20] In the present study, we used LT MCD and electron paramagnetic resonance (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.

Wild-type (wt) and E546N human iNOS oxyFMN constructs were expressed and purified as described.[20] K_d of CN^- was

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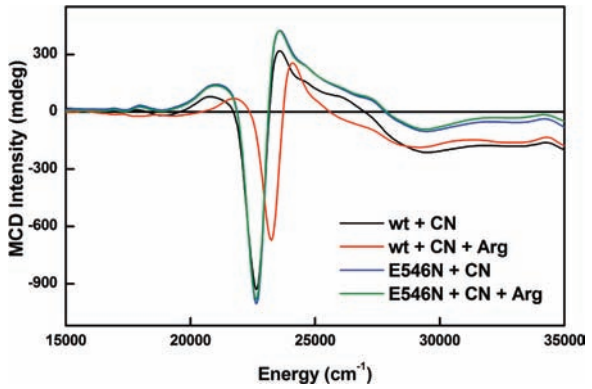

Figure 2. MCD spectra (C-term) recorded at 5 K and 7 T for ferric CN^- complexes of the wt and E546N mutant of the human iNOS oxyFMN construct without or with 20 mM L-Arg. The samples were prepared in pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μM H_4B); 42% (v/v) ethylene glycol was added to the samples as a glassing agent. Note that the MCD spectrum of the wt + CN + L-Arg sample (red trace) is significantly blue-shifted relative to the other spectra.

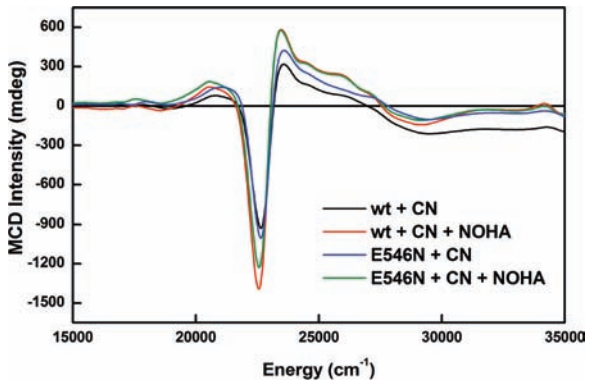

Figure 3. MCD spectra recorded at 5 K and 7 T for ferric CN^- complexes of the wt and E546N mutant of the human iNOS oxyFMN construct without or with 20 mM NOHA. The samples were prepared in pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μM H_4B); 42% (v/v) ethylene glycol was added to the protein samples.

determined to be 8.5 ± 0.9 mM (Figure S2 in the Supporting Information). Therefore, 50 mM KCN was added to form CN^- complexes of the iNOS proteins. MCD samples were prepared using microvolumetric techniques,[21] and the temperature-dependent MCD spectra, arising from the ferric cyano center, were obtained at 5, 10, and 20 K in a 7 T applied magnetic field (Figure 2). All of the MCD spectra are reminiscent of low-spin ferric heme species.[22,23] However, the addition of L-Arg resulted in notable changes in the MCD spectrum of the wt 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 (Figure 2). Importantly, the spectral perturbation induced by L-Arg binding in the wt CN^- sample (Figure 2) is absent in the iNOS oxygenase (iNOSoxy) construct (Figure S3 in the Supporting Information). 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.

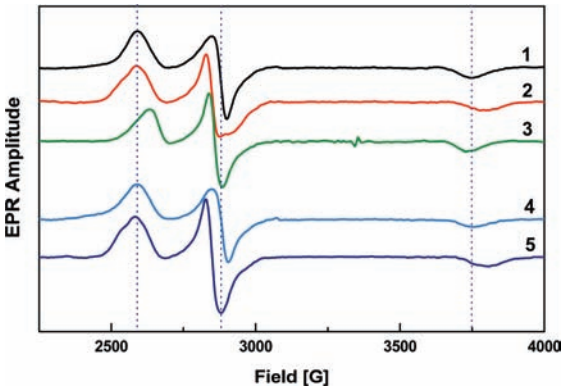

Figure 4. X-band EPR spectra of ferric CN^- complexes in the human iNOS oxyFMN samples of (1) wt + CN, (2) wt + CN + L-Arg, (3) wt + CN + NOHA, (4) E546N + CN, and (5) E546N + CN + L-Arg. The samples were prepared in pH 7.6 buffer (100 mM Bis-Tris propane, 200 mM NaCl, 1 mM DTT, 4 μM H_4B, 42% (v/v) ethylene glycol). The CW EPR spectra were acquired at ~9.4 GHz using a microwave power of 20 dB and a modulation amplitude of 5 G.

Interestingly, the ferric CN^- complexes of wt and E546N proteins possess similar MCD spectra in the presence and absence of NOHA (Figure 3), and the iNOSoxy ferric cyano sample behaves similarly upon the addition of NOHA (Figure S3 in the Supporting Information). 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 (Figure 2) 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 because these key interactions determine the NOS substrate oxidation pathway.[8]

We have also obtained EPR spectra for the CN^- complexes of the wt and E546N mutant (Figure 4) that show differential effects of L-Arg and NOHA binding on the wt CN^- complex; see shifts in the turning points at g_2 and g_3 (Figure 4). 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 neuronal NOS (nNOS) also indicated substrate-specific perturbation of the NO adduct structure.[25] This is consistent with the fact that the active-site structure of the iNOS ferric cyano complex[12] is similar to that of the NO complex of the nNOS oxygenase domain,[26] in which the bound CN^-/NO ligand interacts with the substrate. Note that the EPR spectra of the E546N mutant are similar to those of the wt protein (Figure 4). This indicates that MCD detects different structural variations at the iNOS heme site.

In order to confirm that the observed differences in the MCD spectra of the wt and E546 mutant (Figure 2) are not due to a change in the binding capability of L-Arg to the CN^- complexes, we determined the L-Arg dissociation constants (K_d) by titrating with L-Arg in the absence and presence of 50 mM KCN (Figures S4−S6 and Table S1). The K_d values of L-Arg for the CN^- complexes are greater than those of 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

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ligand), resulting in a heme site with altered substrate-binding properties.[12] Importantly, the L-Arg K_d for the CN^- complex of the E546N mutant is similar to that of the wt protein (Table S1). This is in contrast to the abolished L-Arg perturbation of the MCD spectra of the mutant (Figure 2). Therefore, the absence of an L-Arg perturbation in the E546N CN^- MCD spectrum (Figure 2) is not due to the ineffectiveness of L-Arg binding to the mutant.

E546 is a charged surface residue located at the edge of the human iNOS FMN domain (Figure 1) and is conserved in NOS isoforms. The full-length NOS structure is not available. Nonetheless, in the computationally 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. Thus, this residue is not in close contact with the heme center and is believed to be important in iNOS interdomain FMN−heme interactions.[20] Also note that, in the crystal structure of the 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. The E546 residue does not interact with the CaM-binding linker or the bound CaM (Figure 1). Therefore, the CaM-binding region is not directly involved in the effect induced by the mutation at the 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 FMN domain docking to the heme domain also regulates NOS catalysis by modulating the reactivity[28,29] and structure[20] of the heme active site. On the basis of 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 reduce 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.

■ ASSOCIATED CONTENT

Supporting Information. Active site structure of iNOS ferric cyano complex, measurements of the dissociation constants of L-Arg and CN^- and MCD spectra of iNOS oxygenase constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

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