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

Joseph Sempombe,[†] Bradley O. Elmore,[‡] Xi Sun,[‡] Andrea Dupont,[§] Dipak K. Ghosh,[||] J. Guy Guillemette,[§] Martin L. Kirk,*[,†] and Changjian Feng*[,‡]

Department of Chemistry and Chemical Biology and College of Pharmacy, University of New Mexico, Albuquerque, New Mexico 87131, Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada, and Department of Medicine, Duke University and VA Medical Center, Durham, North Carolina 27705

Received March 18, 2009; E-mail: mkirk@unm.edu; cfeng@salud.unm.edu

In mammals, nitric oxide (NO) is synthesized by nitric oxide synthase (NOS), a homodimeric flavohemoprotein that catalyzes the oxidation of L-arginine (L-Arg) to NO and L-citrulline with NADPH and O_2 as cosubstrates.[1] Each NOS subunit contains a C-terminal electron-supplying reductase unit with binding sites for NADPH (the 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. Three NOS isoforms, iNOS, eNOS, and nNOS, achieve their key biological functions via an intriguing regulation of interdomain electron transfer (IET) processes.[1] It is of current interest to elucidate control mechanisms for the catalytically relevant IET processes in the NOS holoenzymes.[2−5] In particular, the IET from FMN to heme is essential in the delivery of electrons required for O_2 activation in the heme domain and the subsequent NO synthesis.[6] Interdomain FMN−heme IET is facilitated by formation of the NOS output state, which is an electron-donating (output) state of the FMN domain. The structure of the functional output state for NO production has not yet been determined.

Bidomain NOS oxyFMN constructs, in which only the oxygenase domain, the FMN domain, and the CaM-binding region are expressed, were recently designed (Figure 1).[7] This was done to preclude FAD−FMN interactions and favor interactions between the FMN-binding domain and the heme domain. Biochemical, spectroscopic, and IET kinetic data have demonstrated that these homologous dimeric oxyFMN constructs are well-validated models of the NOS output state.[7−9] Emerging evidence further indicates that CaM modulates the FMN−heme IET in the NOS oxyFMN constructs by facilitating the interdomain FMN−heme interactions.[7,8] Therefore, experiments that focus on crucial interdomain interactions should provide key insight into regulation of the formation of the NOS output state. In this study, we used low-temperature magnetic circular dichroism (MCD) spectroscopy to probe how mutations in the adjacent FMN domain affect the heme center in a human iNOS oxyFMN construct.

Wild-type (wt) human iNOS oxyFMN construct was expressed and purified as described elsewhere.[10] Located at the edge of the FMN domain of human iNOS, E546 and E603 are charged surface residues that are conserved in all NOS isoforms (Figure S1 in the Supporting Information). In order to address the roles of these charged residues in interdomain FMN−heme interactions, E546N and E603N mutants were constructed, expressed, and purified (see the Supporting Information). The MCD samples were prepared using microvolumetric techniques, and temperature-dependent MCD spectra arising from the heme site in the as-isolated proteins were obtained at 5, 10, and 20 K in a 7 T applied magnetic field (Figure 2).[11] Importantly, the MCD spectrum of the wt protein is noticeably perturbed upon incubation with the L-Arg substrate (Figure 2a), whereas the E546N and E603N mutants have similar MCD spectra in both the presence and absence of L-Arg (Figure 2b and Figure S5 in the Supporting Information, respectively). Previous room-temperature MCD studies on wt nNOS holoenzyme also suggested a perturbation of the heme signal upon L-Arg binding,[12] consistent with our low-temperature observations for the wt iNOS oxyFMN construct (see below). This L-Arg perturbation in the nNOS holoenzyme has previously been attributed to the conversion of a mixed high-spin/low-spin state into an exclusively high-spin heme when L-Arg binds near the heme site.[12] We probed the heme spin states in the wt and mutant iNOS oxyFMN constructs using variable-temperature variable-field (VTVH) MCD and EPR spectroscopies. Here we observed that binding of L-Arg near the wt construct heme center results in its conversion to a predominantly high-spin form (Figure S6 in the Supporting Information). However, the MCD and EPR spectra for the L-Arg-bound mutants indicate that a greater percentage of low-spin heme is present. It should be noted that except for the L-Arg-bound wt oxyFMN (red trace in Figure 2a), all of the other MCD spectra (Figure 2) are reminiscent of low-spin ferric heme species.[13] The direct implication of these results is that proper FMN docking at the heme domain is necessary for the formation of a high-spin ferric heme in the presence of L-Arg.

Further support of this hypothesis is provided by the fact that the spectral perturbation induced by L-Arg binding in the wt protein (Figure 2a) is completely absent in the iNOS oxygenase construct (Figure 2c), which possesses only a heme domain and no FMN

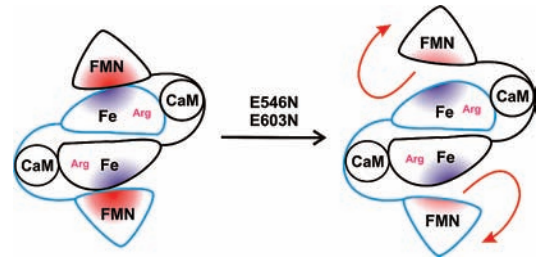

Figure 1. Schematic diagram of the truncated bidomain oxyFMN construct of human iNOS (left). Charge neutralization mutation at E546 or E603 of the FMN domain diminishes the L-Arg perturbation of the heme MCD spectrum, presumably by disrupting proper alignment of the FMN and heme domains (right).

[†] Department of Chemistry and Chemical Biology, University of New Mexico.
[‡] College of Pharmacy, University of New Mexico.
[§] University of Waterloo.
[||] Duke University and VA Medical Center.

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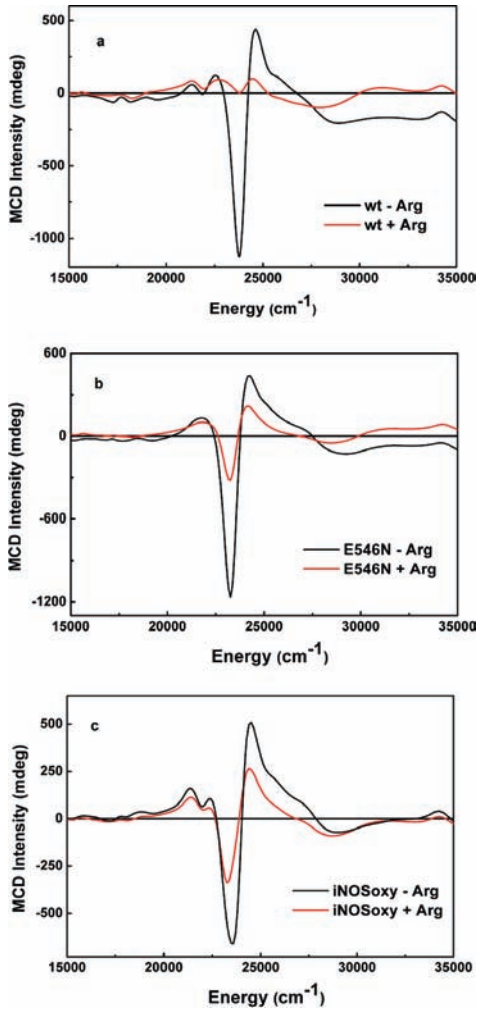

Figure 2. MCD spectra recorded at 5 K for the heme site in the as-isolated (a) wt and (b) E546N mutant of the human iNOS oxyFMN construct without (black traces) and with (red traces) 20 mM L-Arg. Panel (c) shows data for the iNOS oxygenase (iNOSoxy) construct. The samples were made in a pH 7.6 buffer (50 mM Tris, 200 mM NaCl, 1 mM DTT, 4 μM H_4B), and 42% (v/v) ethylene glycol was added to the protein samples as a cryoprotectant and glassing agent.

domain. Thus, the oxygenase construct provides an important control to assess the effects of a properly aligned FMN domain in the wt oxyFMN construct. Taken together, these data strongly suggest a correlation between the L-Arg perturbation of the heme MCD spectrum and the existence of a properly aligned FMN domain in the FMN−heme complex of the wt iNOS protein. Complementary evidence regarding the importance of these residues (E546, E603) in iNOS interdomain interactions derives from a recent mutational and kinetics study indicating the involvement of the equivalent FMN sites (E762, E819) in rat nNOS FMN/FAD alignment.[14]

Because of the lack of a crystal structure for the NOS FMN−heme complex, there is a clear need to probe the effects of interdomain FMN−heme interactions at the molecular level using alternative methods. Although EPR studies have suggested the presence of magnetic interactions between the paramagnetic centers of the FMN semiquinone radical and the heme iron in NOS holoenzymes,[15−17] another EPR study on an nNOS holoenzyme argued that the flavin and heme centers are not magnetically coupled.[18] The discrepancy in these EPR studies strongly indicates the need to utilize complementary spectroscopic approaches, such as low-temperature and VTVH MCD spectroscopies, in order to determine the nature of interdomain FMN−heme interactions with molecular-level resolution.[19]

In summary, this study has provided the first *direct* paramagnetic spectroscopic evidence to indicate that the docked FMN domain affects the nature of interactions between the L-Arg substrate and the catalytic heme center located in an adjacent domain in iNOS. Specifically, we propose that charge neutralization mutations at the E546 and E603 sites disrupt complementary electrostatic interdomain FMN−heme interactions, disturb the FMN−heme complex, and thereby diminish the L-Arg perturbation. We have shown that a combination of low-temperature MCD and EPR spectroscopies can serve as a promising site-selective probe of key interdomain FMN−heme interactions that modulate the formation of the NOS output state. This comparative MCD study of wt and mutant proteins clearly indicates that a properly docked FMN domain contributes to the observed L-Arg perturbation of heme MCD in the wt NOS protein and that the conserved surface residues in the FMN domain play key roles in facilitating a productive alignment of the FMN and heme domains in iNOS.

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Supporting Information Available: Experimental procedures and Figures S1−S6. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Roman, L. J.; Martasek, P.; Masters, B. S. S. *Chem. Rev.* **2002**, *102*, 1179–1189.
(2) Ilagan, R. P.; Tiso, M.; Konas, D. W.; Hemann, C.; Durra, D.; Hille, R.; Stuehr, D. J. *J. Biol. Chem.* **2008**, *283*, 19603–19615.
(3) (a) Feng, C. J.; Tollin, G.; Hazzard, J. T.; Nahm, N. J.; Guillemette, J. G.; Salerno, J. C.; Ghosh, D. K. *J. Am. Chem. Soc.* **2007**, *129*, 5621–5629. (b) Feng, C. J.; Roman, L. J.; Hazzard, J. T.; Ghosh, D. K.; Tollin, G.; Masters, B. S. S. *FEBS Lett.* **2008**, *582*, 2768–2772.
(4) Haque, M. M.; Panda, K.; Tejero, J.; Aulak, K. S.; Fadlalla, M. A.; Mustovich, A. T.; Stuehr, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 9254–9259.
(5) Jachymova, M.; Martasek, P.; Panda, S.; Roman, L. J.; Panda, M.; Shea, T. M.; Ishimura, Y.; Kim, J. J. P.; Masters, B. S. S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 15833–15838.
(6) Panda, K.; Ghosh, S.; Stuehr, D. J. *J. Biol. Chem.* **2001**, *276*, 23349–23356.
(7) 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.
(8) Feng, C. J.; Tollin, G.; Holliday, M. A.; Thomas, C.; Salerno, J. C.; Enemark, J. H.; Ghosh, D. K. *Biochemistry* **2006**, *45*, 6354–6362.
(9) Feng, C. J.; Thomas, C.; Holliday, M. A.; Tollin, G.; Salerno, J. C.; Ghosh, D. K.; Enemark, J. H. *J. Am. Chem. Soc.* **2006**, *128*, 3808–3811.
(10) 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.
(11) Jones, R. M.; Inscore, F. E.; Hille, R.; Kirk, M. L. *Inorg. Chem.* **1999**, *38*, 4963–4970.
(12) Sono, M.; Stuehr, D. J.; Ikedasaito, M.; Dawson, J. H. *J. Biol. Chem.* **1995**, *270*, 19943–19948.
(13) Berka, V.; Palmer, G.; Chen, P.-F.; Tsai, A.-L. *Biochemistry* **1998**, *37*, 6136–6144.
(14) Panda, K.; Haque, M. M.; Garcin-Hosfield, E. D.; Durra, D.; Getzoff, E. D.; Stuehr, D. J. *J. Biol. Chem.* **2006**, *281*, 36819–36827.
(15) Stuehr, D. J.; Ikedasaito, M. *J. Biol. Chem.* **1992**, *267*, 20547–20550.
(16) Galli, C.; MacArthur, R.; Abu-Soud, H. M.; Clark, P.; Stuehr, D. J.; Brudvig, G. W. *Biochemistry* **1996**, *35*, 2804–2810.
(17) Tsai, A. L.; Berka, V.; Chen, P. F.; Palmer, G. *J. Biol. Chem.* **1996**, *271*, 32563–32571.
(18) Perry, J. M.; Moon, N.; Zhao, Y.; Dunham, W. R.; Marletta, M. A. *Chem. Biol.* **1998**, *5*, 355–364.
(19) Feng, C. J.; Tollin, G. *Dalton Trans.* **2009**, in press.

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