J|A|C|S
ARTICLES
Published on Web 04/11/2007

Direct Measurement by Laser Flash Photolysis of Intraprotein Electron Transfer in a Rat Neuronal Nitric Oxide Synthase

Changjian Feng,*,† Gordon Tollin,*,‡ James T. Hazzard,‡ Nickolas J. Nahm,§ J. Guy Guillemette,|| John C. Salerno,⊥ and Dipak K. Ghosh*,§

Contribution from the College of Pharmacy, University of New Mexico, Albuquerque, New Mexico 87131, Department of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, Arizona 85721, Department of Medicine, Duke University and VA Medical Centers, Durham, North Carolina 27705, Department of Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada, and Department of Biological and Physical Sciences, College of Science and Mathematics, Kennesaw State University, Kennesaw, Georgia 30144

Received December 4, 2006; E-mail: cfeng@salud.unm.edu; gtollin@u.arizona.edu; dgx@acpub.duke.edu

Abstract: Intraprotein interdomain electron transfer (IET) from flavin mononucleotide (FMN) to heme is essential in nitric oxide (NO) synthesis by NO synthase (NOS). Our previous laser flash photolysis studies have provided a direct determination of the kinetics of IET between the FMN and heme domains in truncated oxyFMN constructs of rat neuronal NOS (nNOS) and murine inducible NOS (iNOS), in which only the oxygenase and FMN domains along with the calmodulin (CaM) binding site are present [Feng, C. J.; Tollin, G.; Holliday, M. A.; Thomas, C.; Salerno, J. C.; Enemark, J. H.; Ghosh, D. K. *Biochemistry* **2006**, *45*, 6354−6362. 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]. Here, we report the kinetics of IET between the FMN and heme domains in a rat nNOS holoenzyme in the presence and absence of added CaM using laser flash photolysis of CO dissociation in comparative studies on partially reduced NOS and a single domain NOS oxygenase construct. The IET rate constant in the presence of CaM is 36 s^{-1}, whereas no IET was observed in the absence of CaM. The kinetics reported here are about an order of magnitude slower than the kinetics in a rat nNOS oxyFMN construct with added CaM (262 s^{-1}). We attribute the slower IET between FMN and heme in the holoenzyme to the additional step of dissociation of the FMN domain from the reductase complex before reassociation with the oxygenase domain to form the electron-transfer competent output state complex. This work provides the first direct measurement of CaM-controlled electron transfer between catalytically significant redox couples of FMN and heme in a nNOS holoenzyme.

Nitric oxide (NO), a ubiquitous cellular signaling molecule, is one of the most studied small molecules in biology due to its involvement in numerous biological processes such as vasodilation, neurotransmission, and immune response.[1,2] In mammals, NO is synthesized by nitric oxide synthase (NOS), a homodimeric flavo-hemoprotein that catalyzes the 5-electron oxidation of L-arginine (Arg) to NO and L-citrulline with NADPH and O_2 as cosubstrates.[3] There are three mammalian NOS isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Eukaryotic NOS evolved via a series of gene fusion events, resulting in a modular heme- and flavin-containing enzyme that produces NO by very intricately controlled redox processes.[4] Each subunit of all three mammalian isoforms 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; these components are linked by a calmodulin (CaM) binding region. The substrate, L-Arg, and a cofactor, (6*R*)-5,6,7,8 tetrahydrobiopterin (H_4B), both bind near the heme center in the oxygenase domain.[5]

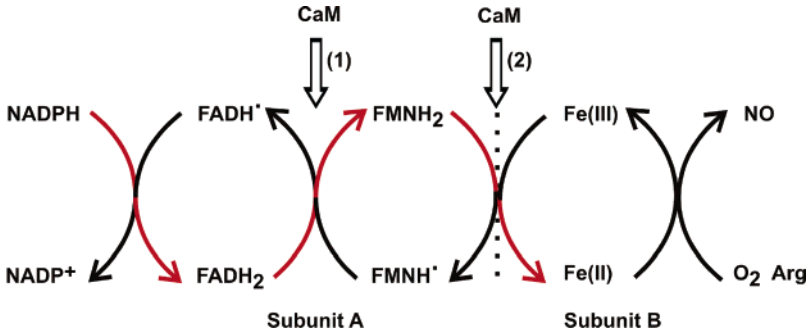
The structure of the holoenzyme is not yet available, although extensive crystallographic studies of the oxygenase domains of all three NOS isoforms have been performed;[6−9] these structures have provided considerable insight into the NOS catalytic mechanisms. Only a few crystal structures of partial[10] and

† University of New Mexico.
‡ University of Arizona.
§ Duke University and VA Medical Centers.
|| University of Waterloo.
⊥ Kennesaw State University.

(1) Schmidt, H.; Walter, U. *Cell* **1994**, *78*, 919−925.
(2) Moncada, S.; Higgs, E. A. *Br. J. Pharmacol.* **2006**, *147*, S193−S201.
(3) Alderton, W. K.; Cooper, C. E.; Knowles, R. G. *Biochem. J.* **2001**, *357*, 593−615.
(4) Roman, L.; Martasek, P.; Masters, B. S. *Chem. Rev.* **2002**, *102*, 1179−1190.
(5) Wei, C. C.; Crane, B. R.; Stuehr, D. J. *Chem. Rev.* **2003**, *103*, 2365−2383.
(6) Crane, B. R.; Arvai, A. S.; Ghosh, D. K.; Wu, C. Q.; Getzoff, E. D.; Stuehr, D. J.; Tainer, J. A. *Science* **1998**, *279*, 2121−2126.
(7) Raman, C. S.; Li, H. Y.; Martasek, P.; Kral, V.; Masters, B. S. S.; Poulos, T. L. *Cell* **1998**, *95*, 939−950.
(8) Li, H. Y.; Igarashi, J.; Jamal, J.; Yang, W. P.; Poulos, T. L. *J. Biol. Inorg. Chem.* **2006**, *11*, 753−768.
(9) Fedorov, R.; Vasan, R.; Ghosh, D. K.; Schlichting, I. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 5892−5897.
(10) Zhang, J.; Martasek, P.; Paschke, R.; Shea, T.; Masters, B. S. S.; Kim, J. J. P. *J. Biol. Chem.* **2001**, *276*, 37506−37513.

10.1021/ja068685b CCC: $37.00

Scheme 1. Electron Flow in the Dimeric NOS Goes from NADPH through FAD and FMN in the Reductase Domain of One Subunit to the Heme Iron in the Oxygenase Domain of Another Subunit[a]



[a] The red arrows indicate the direction of electron flow. CaM binding facilitates two IET reactions in eNOS and nNOS: (1) IET between FAD and FMN within the reductase domain, and (2) inter-subunit IET between FMN and heme. Reaction 2 is physiologically essential in NO synthesis, and is of particular interest in this study.

intact[11] nNOS reductase domains and homologues,[12] as well as CaM bound to a peptide corresponding to the CaM-binding sequence in human eNOS,[13] have been reported. These crystal structures have provided a structural basis for understanding the molecular mechanisms of controlling NO synthesis by NOS. The combination of kinetics and equilibrium titration methods with site-directed mutagenesis allows critical determination of the roles of specific amino acid residues in regulating NOS function.[14−20]

Although many details of the novel NOS catalytic mechanism remain to be elucidated,[5,21,22] it is well-established that inter-domain electron-transfer (IET) processes are key steps in NO synthesis through coupling reactions between the flavin and heme domains (Scheme 1).[3,23−25] It is thus of current interest to investigate the thermodynamics[26−28] and kinetics[29−31] of IET in NOS.

Unlike iNOS, eNOS and nNOS synthesize NO in a Ca^{2+}/CaM-dependent manner (Scheme 1): CaM binding triggers IET reactions from the FAD hydroquinone ($FADH_2$) to FMN semiquinone ($FMNH^{\bullet}$) within subunit A (reaction 1),[17] and the FMN hydroquinone ($FMNH_2$) to the catalytic heme iron in the oxygenase domain of subunit B (reaction 2).[16,32−34] CaM binding has little or no effect on the thermodynamics of redox processes in NOS,[28,35,36] indicating that the regulation by CaM is accomplished dynamically through controlling redox-linked conformational changes required for effective IET.

The major difference between the CaM regulated isoforms eNOS and nNOS and the inducible isoform iNOS, which binds CaM at all physiological Ca^{2+} concentrations, is the presence of internal control elements.[4,37] Identified originally from sequence alignments and modeling and postulated to restrict alignment of the FMN binding domain with the oxygenase and/or FAD binding domains,[38] the FMN domain autoinhibitory element pins the FMN binding domain to the reductase complex via a network of hydrogen bonds.[11]

We recently proposed an "FMN-domain tethered shuttle" (Figure 1),[39] supported by our IET studies,[40,41] that involves the swinging of the FMN domain from its original electron-accepting (input) state to a new electron-donating (output) state. The putative output state is a complex between the FMN binding and oxygenase domains, thus facilitating efficient IET between the FMN and the catalytic heme in the oxygenase domain. Crystal structure study on an intact nNOS reductase domain in the CaM-free state[11] reveals the input state of FMN for electrons from NADPH through FAD, in which FMN is in close contact

(11) Garcin, E. D.; Bruns, C. M.; Lloyd, S. J.; Hosfield, D. J.; Tiso, M.; Gachhui, R.; Stuehr, D. J.; Tainer, J. A.; Getzoff, E. D. *J. Biol. Chem.* **2004**, *279*, 37918−37927.
(12) Wang, M.; Roberts, D. L.; Paschke, R.; Shea, T. M.; Masters, B. S. S.; Kim, J. J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 8411−8416.
(13) Aoyagi, M.; Arvai, A. S.; Tainer, J. A.; Getzoff, E. D. *EMBO J.* **2003**, *22*, 766−775.
(14) Panda, S. P.; Gao, Y. T.; Roman, L. J.; Martasek, P.; Salerno, J. C.; Masters, B. S. S. *J. Biol. Chem.* **2006**, *281*, 34246−34257.
(15) Konas, D. W.; Takaya, N.; Sharma, M.; Stuehr, D. J. *Biochemistry* **2006**, *45*, 12596−12609.
(16) Tiso, M.; Konas, D. W.; Panda, K.; Garcin, E. D.; Sharma, M.; Getzoff, E. D.; Stuehr, D. J. *J. Biol. Chem.* **2005**, *280*, 39208−39219.
(17) Panda, K.; Adak, S.; Konas, D.; Sharma, M.; Stuehr, D. J. *J. Biol. Chem.* **2004**, *279*, 18323−18333.
(18) Konas, D. W.; Zhu, K.; Sharma, M.; Aulak, K. S.; Brudvig, G. W.; Stuehr, D. J. *J. Biol. Chem.* **2004**, *279*, 35412−35425.
(19) Wang, Z. Q.; Wei, C. C.; Stuehr, D. J. *J. Biol. Chem.* **2002**, *277*, 12830−12837.
(20) Panda, K.; Haque, M. M.; Garcin-Hosfield, E. D.; Durra, D.; Getzoff, E. D.; Stuehr, D. J. *J. Biol. Chem.* **2006**, *281*, 36819−36827.
(21) Stuehr, D. J.; Santolini, J.; Wang, Z. Q.; Wei, C. C.; Adak, S. *J. Biol. Chem.* **2004**, *279*, 36167−36170.
(22) Rosen, G. M.; Tsai, P.; Pou, S. *Chem. Rev.* **2002**, *102*, 1191−1199.
(23) Li, H. Y.; Poulos, T. L. *J. Inorg. Biochem.* **2005**, *99*, 293−305.
(24) 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.
(25) Sagami, I.; Sato, Y.; Noguchi, T.; Miyajima, M.; Rozhkova, E.; Daff, S.; Shimizu, T. *Coord. Chem. Rev.* **2002**, *226*, 179−186.
(26) Udit, A. K.; Belliston-Bittner, W.; Glazer, E. C.; Le Nguyen, Y. H.; Gillan, J. M.; Hill, M. G.; Marletta, M. A.; Goodin, D. B.; Gray, H. B. *J. Am. Chem. Soc.* **2005**, *127*, 11212−11213.
(27) Bayachou, M.; Boutros, J. A. *J. Am. Chem. Soc.* **2004**, *126*, 12722−12723.
(28) Gao, Y. T.; Smith, S. M. E.; Weinberg, J. B.; Montgomery, H. J.; Newman, E.; Guillemette, J. G.; Ghosh, D. K.; Roman, L. J.; Martasek, P.; Salerno, J. C. *J. Biol. Chem.* **2004**, *279*, 18759−18766.
(29) Zemojtel, T.; Scheele, J. S.; Martasek, P.; Masters, B. S. S.; Sharma, V. S.; Magde, D. *Biochemistry* **2003**, *42*, 6500−6506.
(30) Gribovskaja, I.; Brownlow, K. C.; Dennis, S. J.; Rosko, A. J.; Marletta, M. A.; Stevens-Truss, R. *Biochemistry* **2005**, *44*, 7593−7601.
(31) Guan, Z. W.; Kamatani, D.; Kimura, S.; Iyanagi, T. *J. Biol. Chem.* **2003**, *278*, 30859−30868.
(32) Panda, K.; Ghosh, S.; Stuehr, D. J. *J. Biol. Chem.* **2001**, *276*, 23349−23356.
(33) Abusoud, H. M.; Stuehr, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10769−10772.
(34) Abusoud, H. M.; Yoho, L. L.; Stuehr, D. J. *J. Biol. Chem.* **1994**, *269*, 32047−32050.
(35) Noble, M. A.; Munro, A. W.; Rivers, S. L.; Robledo, L.; Daff, S. N.; Yellowlees, L. J.; Shimizu, T.; Sagami, I.; Guillemette, J. G.; Chapman, S. K. *Biochemistry* **1999**, *38*, 16413−16418.
(36) Daff, S.; Noble, M. A.; Craig, D. H.; Rivers, S. L.; Chapman, S. K.; Munro, A. W.; Fujiwara, S.; Rozhkova, E.; Sagami, I.; Shimizu, T. *Biochem. Soc. Trans.* **2001**, *29*, 147−152.
(37) Roman, L. J.; Masters, B. S. S. *J. Biol. Chem.* **2006**, *281*, 23111−23118.
(38) Salerno, J. C.; Harris, D. E.; Irizarry, K.; Patel, B.; Morales, A. J.; Smith, S. M. E.; Martasek, P.; Roman, L. J.; Masters, B. S. S.; Jones, C. L.; Weissman, B. A.; Lane, P.; Liu, Q.; Gross, S. S. *J. Biol. Chem.* **1997**, *272*, 29769−29777.
(39) Ghosh, D. K.; Salerno, J. C. *Front. Biosci.* **2003**, *8*, D193−D209.
(40) Feng, C. J.; Tollin, G.; Holliday, M. A.; Thomas, C.; Salerno, J. C.; Enemark, J. H.; Ghosh, D. K. *Biochemistry* **2006**, *45*, 6354−6362.
(41) 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.

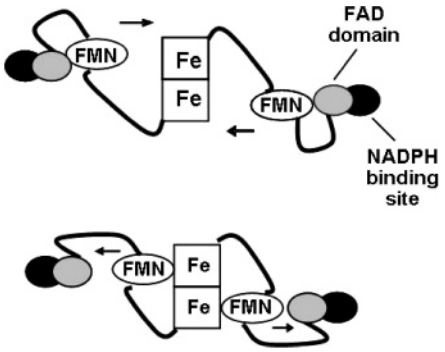


Figure 1. Tethered shuttle model: FMN binding domain shuttles between the "NADPH dehydrogenase" unit (that contains the FAD domain and NADPH binding site) and heme-containing oxygenase domain. Top, putative input state; bottom, putative output state. The putative output state is envisioned as a complex between oxygenase and FMN binding domains, and the structure of the output state has not yet been elucidated. The FMN motion is controlled by CaM binding to and dissociation from NOS.

with FAD (top panel of Figure 1), but not as it exists in the output state to the heme in the oxygenase domain. Based upon the available crystal structures of nNOS reductase[11] and oxygenase[42] constructs, FMN cannot be docked to within tunneling distance of the heme, and thus electrons cannot be transferred to the heme in this state. This restrained state of FMN is locked through a concerted interplay of control elements such as the C-terminal tail and autoinhibitory insert.[11,43] According to the tethered shuttle model, for electrons to be transferred from the FMN to heme for activation of NO production, the FMN domain must be unlocked via CaM binding and must move to another position such that it is accessible to the heme. The putative output state (bottom panel of Figure 1) is a complex between the oxygenase and FMN domains, which favors electron output from the FMN to heme, and hence activates NO production. Therefore, the tethered shuttle model involves an input state corresponding to the existing reductase structures, and an output state in which the FMN binding domain associates with the oxygenase domain. The structure of this latter state has not yet been elucidated.

To favor observation of the output state of the shuttle mechanism, we designed a series of truncated NOS oxyFMN constructs in which only the oxygenase and FMN domains, along with the CaM-binding sequence, were expressed to favor the interaction of the FMN binding domain with the oxygenase domain over interactions within the reductase unit.[44] The absence of the two-domain "NADPH dehydrogenase" unit (containing the FAD and NADPH binding domains) removes the dominant input state complex from the conformational repertoire of the construct. This has provided us with a greatly enhanced opportunity to observe the putative output state, in which the FMN binding site is closely associated with the oxygenase active site rather than with the dehydrogenase unit. We have directly determined the IET kinetics between the FMN and heme domains in the NOS output state using CO photolysis in comparative studies on the oxyFMN and single domain NOS oxygenase constructs.[40,41] Furthermore, our results provided the first direct observation of the effect of CaM on controlling IET between FMN and heme domains through facilitation of the FMN/heme interactions in the NOS output state.[40]

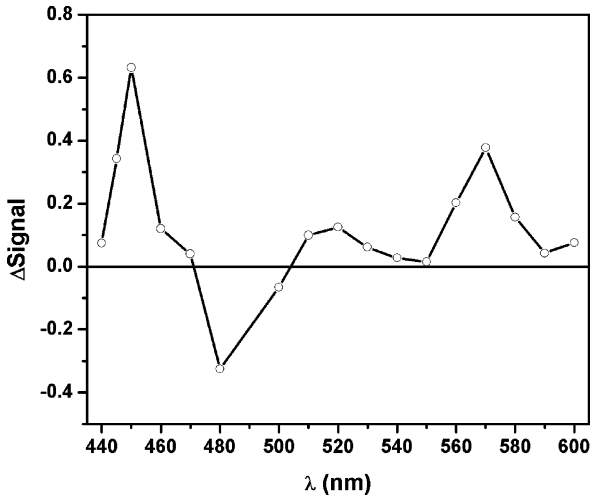


Figure 2. The 400 nm laser flash-induced difference spectrum of the oxidized nNOS holoenzyme in the presence of added CaM obtained 630 ms after the laser pulse. Anaerobic solutions contained 18 μM nNOS, 46 μM CaM, ~20 μM dRF, and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B, 1 mM Ca^{2+}, and 10% glycerol). The 400 nm flash-induced difference spectra of nNOS in the absence of added CaM are similar to those in the presence of added CaM.

In the present work, as part of a systematic study of the role of critical IET processes in the regulation of NOS activity, we have used laser flash photolysis to investigate the kinetics of the IET between the FMN and heme domains in the output state of a rat nNOS holoenzyme. Previous studies by others on heme reduction in NOS isoforms, indirectly probed by formation of the Fe(II)−CO complex using a stopped-flow technique,[45] gave rate constants of 3−4 s^{-1} for the nNOS holoenzyme;[32] the observed rate constants may involve sequential reactions. As shown below, direct measurements of the IET rate constants in the nNOS holoenzyme yield very different results. This work has further validated our laser flash photolysis approaches in directly determining the discrete FMN-heme IET kinetics in the NOS holoenzyme and the influence of CaM binding on this process.

Results

Photochemical Reduction of the Oxidized nNOS Holoenzyme by Deazariboflavin Semiquinone. The nNOS holoenzyme was partially reduced by illumination of the protein in the presence of deazariboflavin (dRF). The basic photochemical process by which 5-deazariboflavin semiquinone (dRFH$^{\bullet}$), which has a midpoint potential of −630 mV, is generated and used as the exogenous reductant to reduce redox-active proteins has been extensively described.[46]

A 400 nm laser flash-induced difference spectrum of the holoenzyme in the presence of CO (Figure 2) shows that the absorption changes at 446 and 480 nm under these conditions are due to formation of Fe(II)−CO complex and flavin reduction, respectively, through reaction with dRFH$^{\bullet}$.[40,41] In addition, a net positive absorbance change around 580 nm (Figure 2) indicates the formation of the blue neutral flavin

(42) Li, H. Y.; Shimizu, H.; Flinspach, M.; Jamal, J.; Yang, W. P.; Xian, M.; Cai, T. W.; Wen, E. Z.; Jia, Q. A.; Wang, P. G.; Poulos, T. L. *Biochemistry* **2002**, *41*, 13868−13875.
(43) Craig, D. H.; Chapman, S. K.; Daff, S. *J. Biol. Chem.* **2002**, *277*, 33987−33994.
(44) 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.
(45) Abu-Soud, H. M.; Feldman, P. L.; Clark, P.; Stuehr, D. J. *J. Biol. Chem.* **1994**, *269*, 32318−32326.
(46) Tollin, G. *Electron Transfer Chem.* **2001**, *IV*, 202−231.

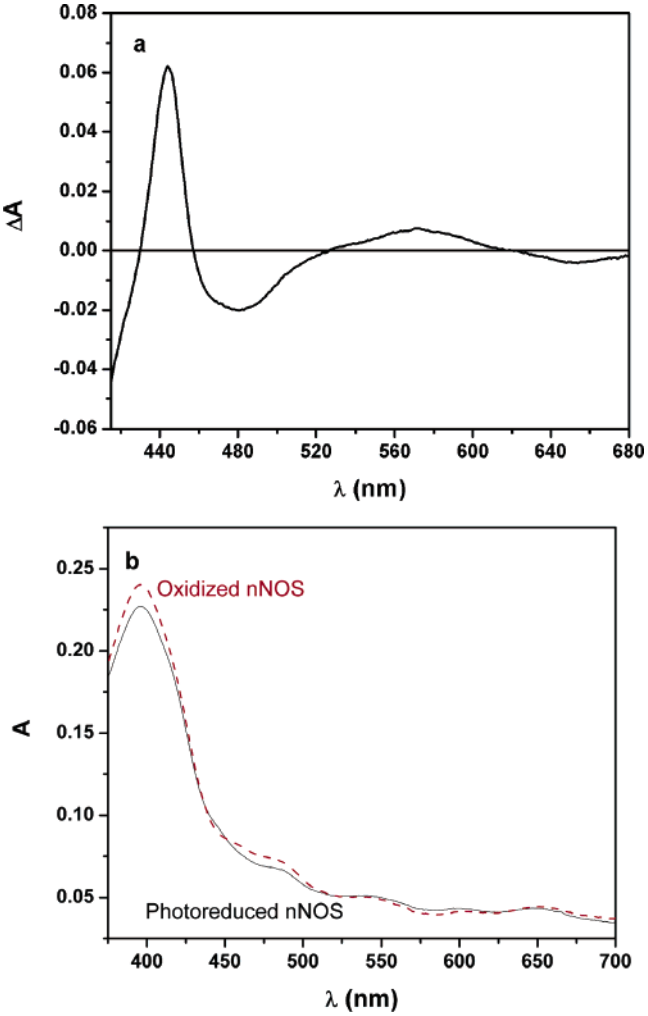


Figure 3. (a) Difference and (b) absorption spectra of nNOS holoenzyme in the presence of 20 μM dRF obtained after approximately 1 min of steady light illumination. In panel b, dashed line, oxidized nNOS; solid line, photoreduced nNOS. Anaerobic solutions contained 6 μM nNOS, 22 μM CaM, ~20 μM dRF, and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B, 1 mM Ca^{2+}, and 10% glycerol). Steady-state illumination of nNOS in the absence of added CaM yields similar difference and absorption spectra.

semiquinone.[35,47] These data clearly indicate that dRFH$^\bullet$ can readily reduce both flavin and heme in the nNOS holoenzyme, as expected.

The oxidized nNOS holoenzyme in the presence of dRF and CO was exposed to steady white light illumination for various periods of time to photoreduce the protein (Figure 3a). This steady-state difference spectrum closely resembles the flash-induced spectrum (Figure 2) and has the characteristic peak of Fe(II)−CO at 446 nm and the broad band of flavin semiquinone around 580 nm. The UV−vis spectra of the photoreduced nNOS also possess the characteristic absorption band of oxidized flavin at 480 nm (Figure 3b), although with reduced intensity. It is unlikely for the reduced protein to have oxidized FMN in the presence of FADH$^\bullet$ because the midpoint potentials of the FMN/FMNH$^\bullet$ and FAD/FADH$^\bullet$ couples in the nNOS holoenzyme have been determined to be −120 and −250 mV, respectively.[28] We thus assigned the 480 nm band in Figure 3b to oxidized FAD. Taken together, the broad band at 580 nm in Figure 3a

(47) Garnaud, P. E.; Koetsier, M.; Ost, T. W. B.; Daff, S. *Biochemistry* **2004**, *43*, 11035−11044.

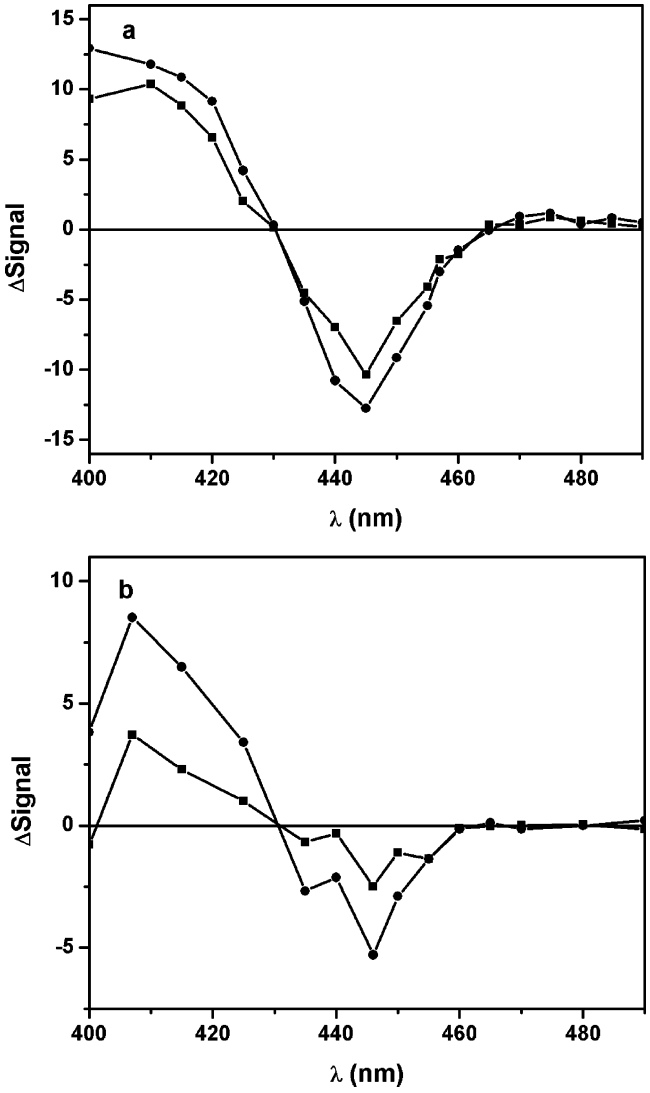


Figure 4. The 450 nm laser flash-induced difference spectra (400−490 nm) of (a) the partially reduced nNOS holoenzyme and (b) reduced nNOSoxy constructs obtained 274 ms (●) and 353 ms (■) after the laser pulse. Experimental conditions were the same as those in Figure 3.

has been ascribed to absorption of FMNH$^\bullet$; that is, the nNOS holoenzyme was partially reduced to [Fe(II)−CO][FMNH$^\bullet$] as the predominant form.

The midpoint potential of the FMNH$^\bullet$/$FMNH_2$ couple of the nNOS holoenzyme has been determined to be approximately −220 mV.[28] The apparent midpoint potential of the Fe(III)/Fe(II)−CO couple, as determined using potentiometric titration in the presence of CO, is −120 mV (Salerno, J. C., unpublished data). Therefore, electron transfer from Fe(II)−CO to FMNH$^\bullet$ is disfavored, thus allowing formation of the partially reduced [Fe(II)−CO][FMNH$^\bullet$] species in high yield.

Electron Transfer between the Heme and FMN Domains in the Partially Reduced nNOS Holoenzyme. The [Fe(II)−CO][FMNH$^\bullet$] form was then flashed by a 450 nm laser excitation to dissociate CO from the Fe(II)−CO complex and form a transient Fe(II) species. Figure 4 shows the 450 nm laser flash-induced difference spectra between 400 and 480 nm for the partially reduced nNOS holoenzyme (Figure 4a) and nNOSoxy construct containing no flavin cofactors (Figure 4b), respectively. The similarities in the absorption changes at 446 and 407 nm between these two NOS constructs indicate the

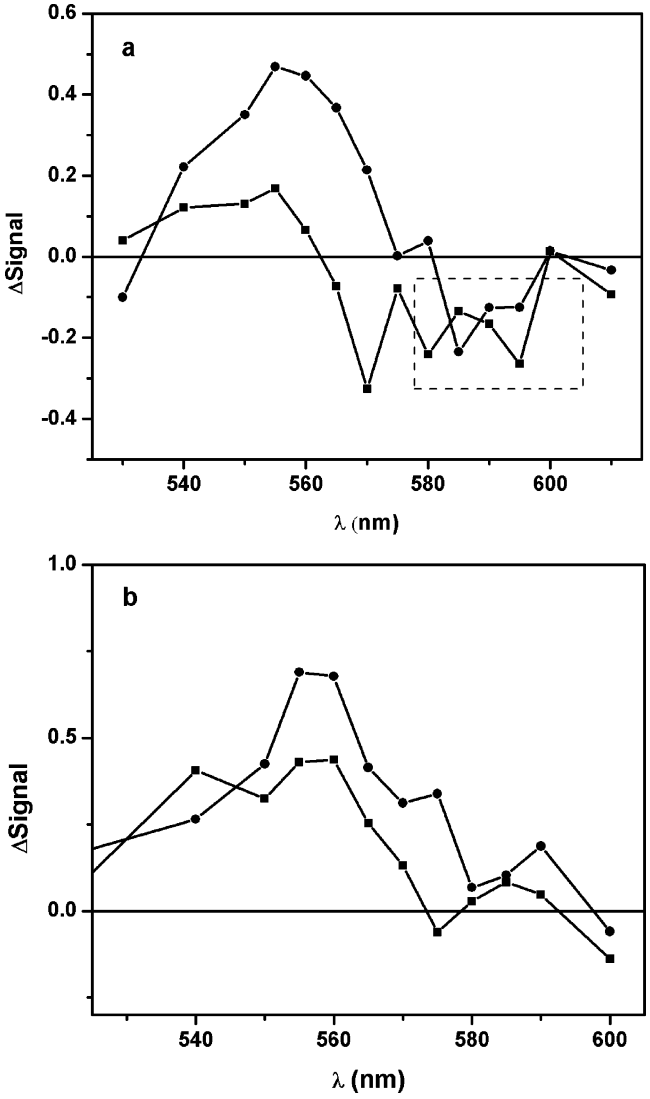


Figure 5. The 450 nm laser flash-induced difference spectra (520−600 nm) of (a) partially reduced nNOS holoenzyme obtained 142 ms (●) and 296 ms (■), and (b) reduced nNOSoxy construct obtained 150 ms (●) and 230 ms (■) after the laser pulse. Note the region highlighted in the dashed box in Figure 5a, which has a contribution from electron transfer between Fe(II) and FMNH• (eq 1).

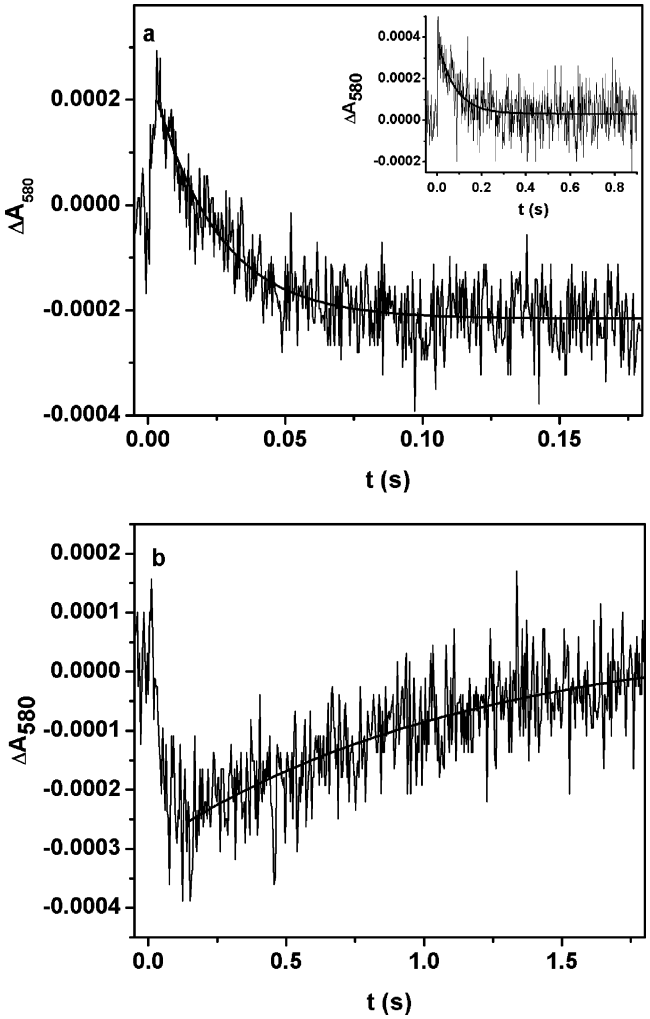


Figure 6. Transient trace at 580 nm at (a) 0−0.2 s and (b) 0−2 s obtained for [Fe(II)−CO][FMNH•] form of a rat nNOS holoenzyme with added CaM flashed by 450 nm laser excitation. Anaerobic solutions contained 18 μM nNOS, 46 μM CaM, ∼20 μM dRF, and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B, 1 mM Ca^{2+}, and 10% glycerol). Inset of Figure 6a is of [Fe(II)−CO] form of nNOSoxy construct (at 0−1 s) flashed by 450 nm laser excitation; note that the trace remains above the pre-flash baseline.

prompt formation of free Fe(II). This is followed by a subsequent slow recombination of CO to Fe(II) to regenerate the Fe(II)−CO complex. The rate constant of CO recombination (3.3 ± 0.3 s^{-1}) is similar to that for the formation of an Fe(II)−CO complex obtained from flashing oxidized protein in the presence of dRF and CO to reduce Fe(III) to Fe(II) (4.9 ± 0.6 s^{-1}), suggesting that this is due to rebinding of CO to Fe(II).

CO dissociation by photolysis of the [Fe(II)−CO][FMNH•] form of nNOS holoenzyme results in a decrease of the midpoint potential of the Fe(III)/Fe(II) couple to around −250 mV,[28] and thereby electron transfer from the transient CO-free Fe(II) (formed by the 450 nm laser) to FMNH• may proceed. Figure 5 shows the 450 nm laser flash-induced difference spectra between 520 and 600 nm for the photochemically reduced nNOS holoenzyme in the presence of CaM (Figure 5a) and for the nNOS oxygenase (nNOSoxy) construct (Figure 5b), which has no FMN cofactor. Note the significant difference in the transient traces between 580 and 600 nm (highlighted in the dashed box in Figure 5a) between the nNOS holoenzyme and nNOSoxy construct. In particular, the absorption of the nNOS holoenzyme at 580 nm rapidly decays below the baseline with rate constant of 36 ± 4 s^{-1} (Figure 6a), followed by a slow recovery to the baseline at longer times with a rate constant of 1.8 ± 1.2 s^{-1} (Figure 6b), similar to that of the CO rebinding rate (3.3 ± 0.3 s^{-1}, see above) obtained from the traces at 440−455 nm for the nNOS holoenzyme, where Fe(II) and Fe(II)−CO dominate the absorption. In contrast, the 580 nm absorption of the nNOSoxy construct (which does not contain FMN) stays above the baseline (inset of Figure 6a). Note that FMNH• dominates the absorption in the range of 580−600 nm (Figure 3a). More importantly, the rate constant of the absorption change at 580−600 nm for nNOS holoenzyme is independent of signal amplitude, that is, concentration of reduced protein (data not shown), indicating an intraprotein process. Our previous studies show that CO photolysis of the [Fe(II)−CO][FMNH•] form of rat nNOS oxyFMN[40] or murine iNOS oxyFMN[41] constructs gives a similar rapid decay followed by a slower CO rebinding at 580−600 nm. On the basis of these observations, we have

Table 1. Rate Constants of the IET (k_{et}^{obs}) and CO Rebinding (k_{CO}) Reactions of a Rat nNOS Holoenzyme[a] and a Rat nNOS oxyFMN Construct with Added CaM

	580 nm trace		455 nm trace
	k_{et}^{obs} (s^{-1})	k_{CO} (s^{-1})	k_{CO} (s^{-1})
nNOS + CaM[b]	36 ± 4	1.8 ± 1.2	3.3 ± 0.3
nNOS oxyFMN + CaM[c]	262 ± 40	3.8 ± 0.3	10.5 ± 1.0 (74%) 3.7 ± 0.5 (26%)

[a] Buffer: 40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B, 1 mM Ca^{2+}, and 10% glycerol, pH 7.6. 20 μM dRF solutions with 5 mM fresh semicarbazide in the buffer were used. A mixture of CO/Ar (1:3) was used to degas the dRF solution and purge the concentrated nNOS. [b] Excess CaM (Sigma-Aldrich) added. Experiments were repeated at 18 μM nNOS with 46 μM CaM, and 16 μM nNOS with 34 μM CaM, respectively. [c] Data taken from previous studies.[40] The same buffer was used. A mixture of CO/Ar (1:2) was used to degas 20 μM dRF solutions with 5 mM fresh semicarbazide in the buffer.

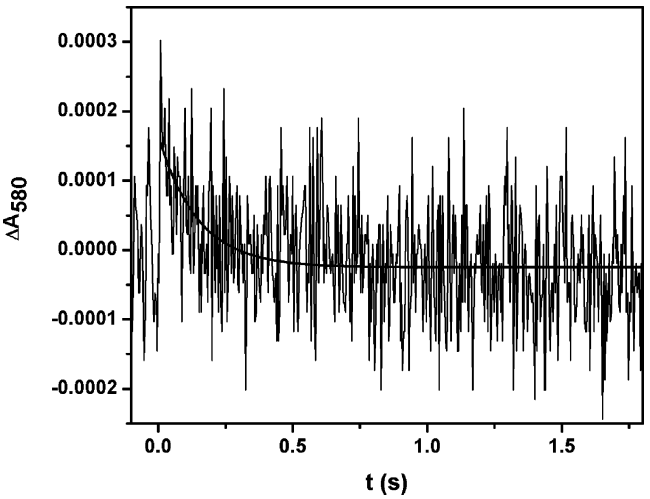


Figure 7. Transient trace at 580 nm at 0−2 s for [Fe(II)−CO][FMNH•] form of nNOS without added CaM flashed by 450 nm laser excitation. Note that the trace stays above the baseline, similar to that of nNOSoxy (inset of Figure 6a). Experimental conditions were the same as those in Figure 6.

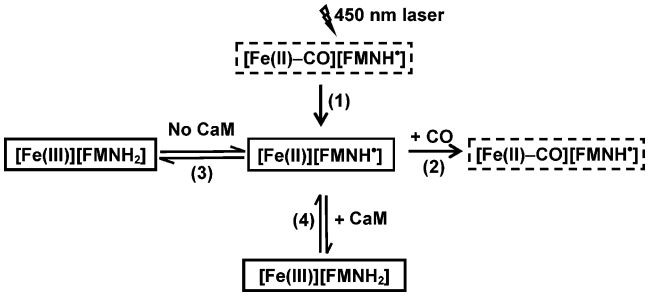
assigned the rapid absorbance decay at 580 nm (Figure 6a) in the nNOS holoenzyme in the presence of CaM to the following IET process (eq 1):

$$[\mathrm{Fe(II)}][\mathrm{FMNH^{\bullet}}] + \mathrm{H^{+}} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} [\mathrm{Fe(III)}][\mathrm{FMNH_2}] \qquad (1)$$

Because Fe(III)/Fe(II) and $FMNH^{\bullet}/FMNH_2$ are nearly isopotential,[28] the electron transfer between Fe(II) and FMNH• is reversible and the observed rate constant is the sum of the forward (k_f) and reverse (k_r) electron-transfer steps. Thus, both k_r (i.e., heme reduction) and k_f in the nNOS holoenzyme at pH 7.6 are approximately equal to 18 ± 2 s^{-1}. The observed IET rate constant is listed in Table 1.

CaM Effect on the Electron Transfer between the Heme and FMN Domains in the nNOS Holoenzyme. Figure 7 shows the transient trace at 580 nm obtained by flashing the partially reduced nNOS holoenzyme in the absence of CaM with 450 nm laser excitation. Note the absence of bleach, similar to that of nNOSoxy construct (inset of Figure 6a), indicating the absence of FMNH• consumption, that is, no FMN−heme IET. Therefore, IET between the FMN and heme domains in the nNOS holoenzyme was only observed in the presence of added CaM.

Scheme 2. Summary of Processes Occurring upon CO Photolysis[a]



[a] Species in dashed boxes are CO-bound forms, whereas those in solid boxes are CO-free and participate in the FMN-heme IET.

Scheme 2 summarizes the processes that take place after photolysis of the Fe(II)−CO complex in the reduced nNOS holoenzyme. The partially reduced form [Fe(II)−CO][FMNH•] is flashed with a 450 nm laser excitation to dissociate CO from the Fe(II)−CO complex with the formation of a transient free Fe(II) species (reaction 1 in Scheme 2). The laser-induced CO dissociation results in a drop of the midpoint potential of the heme and rapidly converts a good electron acceptor (the Fe(II)−CO complex) into an electron donor (the free Fe(II) species), favoring electron transfer from Fe(II) to FMNH•. In the absence of added CaM, CO rebinding to Fe(II) (reaction 2) competes well with the slow IET between the heme and FMN domains (reaction 3), whereas in the presence of CaM, CO rebinding is a poor competitor for IET (reaction 4), and CO binds only to a fraction of the reduced heme. Note that, in these experiments, we chose to use a CO/Ar mixture to slow CO rebinding (reaction 2), and thus favor IET from Fe(II) to FMNH•, making loss of FMNH• observable as a bleach between 580 and 600 nm (i.e., loss of absorbance).

Discussion

In the laser flash photolysis experiments, we use a 450 nm laser to flash CO off the prereduced Fe(II)−CO complex and to trigger the FMN−heme IET. We can individually time-resolve consumption of FMNH• (due to the FMN-heme IET, eq 1) and CO rebinding, and thereby reliably measure the rapid FMN−heme IET (Figure 6a) followed by a much slower CO rebinding process (Figure 6b) in the nNOS holoenzyme, and in NOS oxyFMN constructs as well.[40,41] The stopped-flow approach by others was unable to distinguish these two reactions as only formation of the Fe(II)−CO complex was observed.[32,45] Thus, our CO photolysis approach offers clear advantages because it allows us to observe both processes directly.

Previous stopped-flow studies on heme reduction in the nNOS holoenzyme gave rate constants of 3−4 s^{-1} for the FMN−heme IET.[32] In these stopped-flow experiments, a solution of NADPH was mixed with a CO-saturated solution of NOS to trigger the electron flow from NADPH through FAD and FMN to the heme, and the absorbance changes at 444 nm due to formation of the Fe(II)−CO complex were followed to give the observed rates. Because the rate of CO binding observed during NADPH reduction was 2−4-fold slower than the rate of CO binding to fully reduced enzyme measured as a control (by dithionite reduction prior to mixing with CO-saturated buffer), these investigators concluded that the slower rate of CO binding observed in the NADPH mixing experiment demonstrated that

electron transfer was slower than CO binding and that observation of the formation of the CO adduct in their experiments could therefore be used to measure the rate of heme reduction by NADPH.[45] In the absence of detailed concentration dependence data, this was a possible explanation for their observed results, but other assumptions could also account for the data. The rate of CO binding to the heme is proportional to the ferroheme concentration. If during the first turnover FMN and heme are in rapid equilibrium on the time scale of CO binding, the initial formation of the Fe(II)−CO adduct will be observed at the rate of CO binding to fully reduced heme multiplied by the fraction of the heme present in the ferrous state. Because the system is partitioned between $FMNH_2$/Fe(III) and FMNH•/Fe(II), the rate of CO binding will be slower than the rate of CO binding in the fully reduced system, as observed in their experiments, and will depend upon the effective difference in the midpoint potentials of FMN and heme (which determines the equilibrium between $FMNH_2$/Fe(III) and FMNH•/Fe(II), i.e., the fraction of heme being reduced). In this case, the observed rate of CO binding will not depend on the rate of heme reduction. In fact, we observed a very similar effect on the rate of CO rebinding to heme in the nNOS oxyFMN construct, where FMN/heme electron transfer is much faster, based on the fraction of the heme in the ferrous state ($\sim^1/_3$) during CO rebinding after photolysis.[40] Therefore, we believe that the CO binding stopped-flow data may provide information on FMN/heme redox equilibria during electron transfer, but not on electron transfer rates. In mutants having profoundly inhibited electron transfer, very slow rates of internal electron transfer result in conditions in which CO binding is limited by electron transfer, which may account for the observation of a range of nNOS heme reduction rates that correlate with NO synthesis activities when using a series of CaM proteins in wild-type nNOS and the nNOS S1412D mutant.[48]

More importantly, in view of NOS catalysis, it is clearly impossible that the heme reduction rates in the holoenzyme are as slow as the rates reported by others using formation of the Fe(II)−CO complex as a measure of heme reduction in the stopped-flow experiments. Active nNOS preparations have turnover numbers of about 2 s^{-1} for NO production, requiring a net electron-transfer rate of 6 s^{-1}, which is about double that reported for the rate of heme reduction via the CO binding kinetics,[32] even if electron transfer is assumed to be completely coupled to NO production and NO inhibition can be ignored. For iNOS, the net electron-transfer rate needed to account for NO synthesis is about 12 s^{-1}, and the rate of ferriheme reduction via CO binding is an order of magnitude slower.[49] The present study shows that the observed heme reduction rate in the nNOS holoenzyme with added CaM is 36 s^{-1} (Figure 6a), which is consistent with the NOS catalytic activity, again demonstrating that our laser flash photolysis approach is reliable for studying the discrete FMN−heme IET step.

It is important to note that we conducted similar CO photolysis experiments on the nNOSoxy construct, which does not contain the FMN and FAD domains. Therefore, by carefully comparing the absorption changes of NOS holoenzyme and oxygenase constructs occurring upon a 450 nm laser pulse, we can distinguish the process of FMN−heme IET from rebinding of CO to Fe(II). In Figure 6a, the 580 nm trace of nNOS holoenzyme at shorter time scale is a bleach (i.e., loss of absorbance), whereas the trace of nNOSoxy stays above the baseline (inset of Figure 6a). This comparison gives us definitive evidence that the bleach is due to consumption of FMNH• triggered by prompt formation of free Fe(II), that is, the FMN−heme IET (eq 1, reaction 4 in Scheme 2).

We always collect traces with different signal amplitudes, that is, different reduced protein concentrations. This is due to differing laser intensities and to different sample absorbances. If the traces give similar rates (which is the case in this study), the electron transfer process is of an intra-protein nature. This is important because other inter-protein processes may confound the absorption changes, and, if so, the observed rate would depend upon protein concentration. Therefore, the kinetics must be determined at various protein concentrations to be sure that we are studying the intra-protein FMN−heme IET. In all cases, we have confirmed that the IET kinetics are independent of signal amplitude over a 2-fold range of concentration (0.0002−0.0004 absorbance units); that is, the IET is an intra-protein process.

Masters reported a heme reduction rate of 49 s^{-1} in a rat nNOS holoenzyme under similar conditions by following the absorption change of heme at 397 nm.[50] Note that the so-called heme reduction rate in their study is in fact a sum of forward and reverse IET reactions between the FMN and heme domains (eq 1) because the redox couples of the Fe(III)/Fe(II) and FMN/FMNH• couples are nearly iso-potential. The heme reduction rate so obtained is similar to the value we obtained in this study (36 s^{-1}).

Measurements of the IET from the heme to FMN domains in a rat nNOS holoenzyme were performed using pulsed radiolysis.[51] The observed rate ($\sim$1200 s^{-1}) is that of a different IET reaction: Fe(II) + FMN $\rightleftharpoons$ Fe(III) + FMNH•, which is not physiologically relevant. The IET step we measured by laser flash photolysis (eq 1, reaction 2 in Scheme 1) is physiologically essential for delivery of electron to the heme for activation of oxygen and the subsequent NO production by NOS. These investigators were well aware of this, and their estimates of the probable rates with the couples involved in NOS catalysis are in fact in reasonable agreement with our measurements.[51]

The kinetics reported here are about an order of magnitude slower than the kinetics in a rat nNOS oxyFMN construct with added CaM (262 s^{-1}).[40] We expected to observe slower IET between the FMN and heme domains in the nNOS holoenzyme because in the holoenzyme most of the FMN binding domain is not instantaneously available for electron-transfer reactions with the heme, even in the presence of CaM. In the absence of CaM, the FMN binding domain is apparently closely associated with the "NADPH dehydrogenase" unit as in the previously reported structures of NADPH P450 reductase[11] and the three-domain nNOS reductase construct;[12] the IET between the FMN and heme domains is disfavored, as no IET was observed in the absence of CaM (Figure 7). In the presence of CaM, the FMN binding domain is free to shuttle between the "NADPH

(48) Adak, S.; Santolini, J.; Tikunova, S.; Wang, Q.; Johnson, J. D.; Stuehr, D. J. *J. Biol. Chem.* **2001**, *276*, 1244−1252.
(49) Santolini, J.; Meade, A. L.; Stuehr, D. J. *J. Biol. Chem.* **2001**, *276*, 48887−48898.
(50) Miller, R. T.; Martasek, P.; Omura, T.; Masters, B. S. S. *Biochem. Biophys. Res. Commun.* **1999**, *265*, 184−188.
(51) Kobayashi, K.; Tagawa, S.; Daff, S.; Sagami, I.; Shimizu, T. *J. Biol. Chem.* **2001**, *276*, 39864−39871.

dehydrogenase" unit and the oxygenase domain, alternatively existing as input and output electron transfer complexes. The IET between the FMN and heme domains in the holoenzyme cannot be more rapid than that in the output state (as observed in the nNOS oxyFMN construct in the presence of CaM[40,41]) multiplied by the fraction of the FMN binding domain in the output state conformation. The observed rates would suggest that about 85% of the FMN binding domain is sequestered under our experimental conditions. We can thus attribute the slower observed rate to competition for the FMN binding domain between the "NADPH dehydrogenase" unit and the oxygenase domain. A secondary factor that prevents much faster rates than that in the truncated oxyFMN construct may be the limitations placed on electron transfer by the structures of the FMN and oxygenase domains. We presume that the presence of the "NADPH dehydrogenase" unit in the holoenzyme would affect the docking of the FMN domain to the oxygenase domain in the holoenzyme output state and produce the slower rate that we observed in this study. We note that the holoenzyme rate is still faster than the lower limits established by NO synthesis. This indicates that the IET between the FMN and heme domains per se is not the rate-limiting step in CaM activated NOS catalysis.

The IET in the nNOS holoenzyme was activated upon adding CaM (Figures 6a and 7). Thus, this work provides a direct measurement of the CaM controlled electron transfer between the FMN and heme domains in a nNOS holoenzyme. Note that CaM has little effect on the thermodynamics of redox processes in the holoenzyme,[28] indicating that the regulation by CaM is accomplished kinetically through controlling redox-linked conformational changes required for effective IET. In our tethered shuttle model (Figure 1), FMN domain realignment is controlled by CaM binding/dissociation. During NOS catalysis, the FMN domain has to undergo dissociation from the input state complex and reassociation with the oxygenase domain before rapid electron transfer can occur (Figure 1). In the absence of CaM, most of the NOS holoenzyme is in the input state, facilitating the transfer of electrons from FAD to FMN (top panel of Figure 1). In this state, FMN is buried in the reductase complex and distant from the heme edge, preventing rapid IET between FMN and heme domains. Indeed, in all of the reported crystal structures of flavoenzymes of NOS[11] or P450 reductase,[10] the edge of the FMN isoalloxazine ring is within 5 Å of the FAD isoalloxazine system, well positioned for FAD−FMN but not FMN−heme electron transfer. Upon CaM binding to NOS, considerable domain motion to bring the FMN close to the heme in an electron-donating position (i.e., the NOS output state) is required for efficient IET between the heme and FMN domains (bottom panel of Figure 1). This motion may be the rate-limiting step in the electron transfer process. Measurement of midpoint potential and IET kinetics as a function of temperature could shed additional light on the nature of this conformation-gated process.[52]

This work clearly indicates CaM-dependence outside of the reductase domain in the nNOS holoenzyme. Previously, CaM control of NOS electron transfer was widely believed to be localized within the reductase portion of the enzyme.[53,54] Recent evidence for multiple steps of CaM control was attributed to separate CaM modulation of FMN reduction and heme reduction.[34,55] A study on CaM mutants suggests that the primary control of electron transfer in eNOS and nNOS is through CaM activation of the release of the FMN binding domain from the reductase complex input state, making FMN accessible to exogenous acceptors such as cytochrome *c*; the requirement of CaM for iNOS, in contrast, is apparently associated with alignment of FMN and heme.[56] It was recently shown that CaM-free iNOS constitutively reduces cytochrome *c* and that iNOS requires only the two N terminal Ca^{2+}-binding motifs of CaM for NO synthesis.[57] Taken together, we conclude that the formation of the output state involves two primary steps: (i) dissociation of the FMN domain from its reductase binding site, triggered by CaM binding, and (ii) the subsequent reassociation of FMN with the oxygenase domain (Figure 1). The NOS state formed at step (i) is competent to reduce cytochrome *c*, which requires accessibility of FMN to cytochrome *c*. This is necessary but not sufficient for NO production by NOS, which in addition requires CaM-dependent promotion of the oxygenase−FMN domain interactions. Further study of the IET in the NOS isoforms with added CaM chimera/mutants is underway and may provide new mechanistic information on differential activation of the output state of NOS isoforms by CaM.

Our previous kinetic studies provide strong evidence that the FMN/heme interactions are important in controlling electron transfer between the FMN and heme domains in the NOS output state.[40,41] Interestingly, the recently reported structure of the nNOS reductase supports plausible domain movements triggered by CaM binding, which may be primarily driven by electrostatic interactions between the charged surface residues on the heme and FMN domains.[11] The contacts between the heme and FMN domains of NOS must be highly specific because if the FMN domain of nNOS is replaced by its counterpart from the cytochrome P450 reductase, the resultant chimeric enzymes no longer support electron transfer from FMN to heme, despite their similar ET functions.[58] Further laser flash photolysis studies on mutants of residues related to the possible docking between the FMN and heme domains in the NOS holoenzymes are underway and should provide important insight into the role of specific electrostatic interactions in the coupling of IET and NO synthesis in NOS isoforms.

The work presented here establishes a role for CaM in the formation of the nNOS holoenzyme output state. This may be the original role of CaM in the evolution of NOS and could predate the evolution of the modern control elements. It will be of interest to ascertain whether the effects of these control elements are limited to input state stabilization, or if they also affect output state formation in the NOS holoenzyme. Such information may lead to a better understanding of mechanism of formation of the NOS output state in a synergistic mechanism.

(52) Davidson, V. L. *Biochemistry* **1996**, *35*, 14035−14039.
(53) Gachhui, R.; Presta, A.; Bentley, D. F.; AbuSoud, H. M.; McArthur, R.; Brudvig, G.; Ghosh, D. K.; Stuehr, D. J. *J. Biol. Chem.* **1996**, *271*, 20594−20602.
(54) Sheta, E. A.; McMillan, K.; Masters, B. S. S. *J. Biol. Chem.* **1994**, *269*, 15147−15153.
(55) Matsuda, H.; Iyanagi, T. *Biochim. Biophys. Acta* **1999**, *1473*, 345−355.
(56) Newman, E.; Spratt, D. E.; Mosher, J.; Cheyne, B.; Montgomery, H. J.; Wilson, D. L.; Weinberg, J. B.; Smith, S. M. E.; Salerno, J. C.; Ghosh, D. K.; Guillemette, J. G. *J. Biol. Chem.* **2004**, *279*, 33547−33557.
(57) Spratt, D. E.; Newman, E.; Mosher, J.; Ghosh, D. K.; Salerno, J. C.; Guillemette, J. G. *FEBS J.* **2006**, *273*, 1759−1771.
(58) Roman, L. J.; McLain, J.; Masters, B. S. S. *J. Biol. Chem.* **2003**, *278*, 25700−25707.

Concluding Remarks

In conclusion, laser flash photolysis of an [Fe(II)−CO]-[FMNH•] form of a rat nNOS holoenzyme has allowed us to directly observe the discrete IET step between the FMN and heme domains in the holoenzyme. This work provides the first direct measurement of CaM-controlled electron transfer between catalytically significant redox couples of FMN and heme in the nNOS holoenzyme.

Experimental Section

Expression and Purification of Rat nNOS Holoenzyme. The DNA encoding rat nNOS holoenzyme was a kind gift from Dr. S. Snyder (John Hopkins, MD) and was cloned in *Escherichia coli* expression vector pCWori+ by RT-PCR in the same way as described before.[9,44] Rat nNOS was expressed in a protease-deficient *E. coli* strain BL21DE and was purified using a combination of ammonium sulfate precipitation and gel filtration chromatography, followed by 2′,5′-ADP Sepharose column chromatography.[9,28,44,56] The NOS activity was measured by spectrophotometric oxyhemoglobin assay and was found to be between 500 and 700 nmol/min/mg protein.[28,56] The purified nNOS holoenzyme contained between 0.8 and 1.0 heme/mol (based on the CO difference spectra), and the flavin content measured after extraction from the protein was in all cases at least 90% of the heme content.

Laser Flash Photolysis. A solution containing 20 μM dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B, 1 mM Ca^{2+}, and 10% glycerol) was well degassed by a mixture of Ar and CO (with a ratio of ∼3:1). L-Arg was added to keep oxidized heme in a high spin state, and Ca^{2+} was present in all of the experiments involving subsequent addition of CaM. Aliquots of concentrated rat nNOS holoenzyme (200 μM) were subsequently injected, and the solution was further purged by passing the Ar/CO mixture over the surface to remove added oxygen before being subjected to laser flash photolysis. The oxidized protein solution in the presence of dRF was flashed by 400 nm laser excitation to follow reactions between the protein and dRFH•.

The nNOS solution was then illuminated for an appropriate period of time (∼30 s) to obtain a mixture of the [Fe(II)−CO][FMNH•] and [Fe(II)−CO] forms of the protein, followed by characteristic absorptions of Fe(II)−CO and FMNH• at 446 and 580 nm in the difference spectra, respectively. The reduced protein was subsequently flashed with 450 nm laser excitation to dissociate CO from Fe(II)−CO and generate a transient Fe(II) species that is able to intramolecularly transfer one electron to the FMNH• to produce fully reduced FMN. This latter process was followed by the loss of absorbance of FMNH• at 580 nm. Because the reduced protein has a strong characteristic absorption of Fe(II)−CO at 450 nm relative to that of FMN, the 450 nm laser flash was predominantly absorbed by Fe(II)−CO (the absorbance of dRF at this wavelength is minimal). As a control, solutions containing dRF alone in CO-degassed buffer were flashed with 450 nm laser excitation, and negligible signals of much smaller magnitude were observed, as expected due to the weak absorption of dRF at 450 nm.

All of the experiments were performed at room temperature, and the sample was kept in ice between flashes. Generally, data from 20 laser flashes were averaged. Transient absorbance changes were analyzed using program SIFIT, obtained from OLIS Inc. (Jefferson, GA).

Acknowledgment. C.F. acknowledges the support of the PhRMA Foundation and UNM HSC RAC grant. We thank Prof. John H. Enemark for helpful discussions.

JA068685B