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Intraprotein Electron Transfer in Inducible Nitric Oxide Synthase Holoenzyme

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Abstract

Intraprotein 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 provided a direct determination of the kinetics of the FMN–heme IET in a truncated oxyFMN construct of murine inducible NOS (iNOS), in which only the oxygenase and FMN domains along with the calmodulin (CaM) binding site are present [Feng et al. (2006) *J. Am. Chem. Soc.* 128, 3808–3811]. Here we report the kinetics of the IET in a human iNOS oxyFMN construct, a human iNOS holoenzyme and a murine iNOS holoenzyme, using CO photolysis in comparative studies on partially reduced NOS and a NOS oxygenase construct that lacks the FMN domain. The IET rate constants for the human and murine iNOS holoenzymes are $34 \pm 5 \text{ s}^{-1}$ and $35 \pm 3 \text{ s}^{-1}$, respectively, thereby providing a direct measurement of this IET between the catalytically significant redox couples of FMN and heme in the iNOS holoenzyme. These values are approximately an order of magnitude smaller than that in the corresponding iNOS oxyFMN construct, suggesting that in the holoenzyme the rate-limiting step in the IET is the conversion of the shielded electron-accepting (input) state to a new electron-donating (output) state. The fact that there is no rapid IET component in the kinetic traces obtained with the iNOS holoenzyme implies that the enzyme remains mainly in the input state. The IET rate constant value for the iNOS holoenzyme is similar to that obtained for a CaM-bound neuronal NOS (nNOS) holoenzyme, suggesting that CaM activation effectively removes the inhibitory effect of the unique autoregulatory insert in nNOS.

Keywords

Electron transfer; nitric oxide synthase; laser flash photolysis; heme; flavin

Introduction

Nitric oxide (NO)¹, 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]. There is still much that is

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unknown about how NO production by nitric oxide synthase (NOS) is tightly regulated [3-10]. This is of biomedical importance because unregulated NO production by NOS has been implicated in an increasing number of diseases lacking effective treatments, including stroke, septic shock and cancer [2,11].

Mammalian NOS is a homodimeric flavo-hemoprotein that catalyzes the conversion of L-arginine (Arg) to NO with NADPH and O₂ as co-substrates [3,12]. There are three mammalian NOS isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). 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. The FMN-binding domain and oxygenase domain are linked by a calmodulin (CaM) binding region. The substrate, L-Arg, and a cofactor, (6R)-5,6,7,8 tetrahydrobiopterin (H₄B), both bind near the heme center in the oxygenase domain [6].

Knowledge of the catalytic mechanism is incomplete [5,6,8,9,13,14], but it is well established that intraprotein electron transfer (IET) is important in NO synthesis through coupling reactions between the flavin and heme domains [4,12,15]. In particular, the IET from the FMN to heme is essential in the delivery of electrons required for O₂ activation in the heme domain and the subsequent NO synthesis by NOS, and is thus under extraordinary control. The kinetics of this IET process between the catalytically significant redox couples of FMN and heme is the focus of this study, and the results will be compared with our previous studies [16-19].

The eNOS and nNOS isoforms are signal generators that are tightly controlled by Ca²⁺/CaM and other factors: CaM-binding triggers the IET reaction from the FMN hydroquinone (FMNH₂) to the catalytic heme iron in the oxygenase domain of another subunit [20]. It is generally accepted that CaM-binding has little effect on the thermodynamics of redox processes in NOS [21-23], implying dynamic CaM regulation of IET via redox-linked conformational changes. iNOS, in contrast, synthesizes NO at all physiological Ca²⁺ concentrations. The major difference between the CaM regulated isoforms of nNOS and eNOS and the Ca²⁺ insensitive isoform iNOS is the presence of internal control elements [3], such as a unique autoregulatory (AR) insert within the FMN domain [24] and the C-terminal tail [25].

An “FMN-domain tethered shuttle” (Figure 1) was originally proposed by us [7] and strongly supported by recent IET kinetic studies [16-19,26,27], that involves the swinging of the FMN domain from its original electron-accepting (input) state to a new electron-donating (output) state; CaM binding unlocks the FMN domain from the input state, thereby enabling this domain to shuttle between the two states. Crystal structure study on an intact nNOS reductase domain in the CaM-free state [28] reveals the input state of FMN for electrons from NADPH through FAD (top panel of Figure 1), but not the output state. Based upon the available structures of nNOS reductase [28] and oxygenase [29] constructs, FMN cannot be docked to within tunneling distance of the heme, and thus electrons cannot be transferred to the heme in this input state. Electron transfer from FMN to heme requires unlocking the FMN domain via CaM binding, and reorientation to a heme accessible position. The putative output state (bottom panel of Figure 1) is a complex between the oxygenase and FMN domains, which favors electron output from FMN to heme, and hence activates NO production. The output state structure has not yet been elucidated.

¹Abbreviations: NOS: nitric oxide synthase; iNOS, inducible NOS; nNOS: neuronal NOS; CaM, calmodulin; oxyFMN: two-domain NOS construct in which only oxygenase and FMN domains along with the CaM binding site are present; iNOS_{oxy}, iNOS oxygenase construct; IET, intraprotein electron transfer; dRF and dRFH^{*}: 5-deazariboflavin and 5-deazariboflavin semiquinone, respectively; H₄B, (6R)-5,6,7,8 tetrahydrobiopterin; sq, semiquinone; hq, hydroquinone.

To favor observation of the output state of the shuttle mechanism, we designed truncated two-domain oxyFMN constructs of all three NOS isoforms, in which only the heme-containing oxygenase and FMN domains, along with the CaM-binding region, are present so as to enhance the interaction of the FMN binding domain with the oxygenase domain over interactions within the reductase unit [30]. The IET kinetics between the FMN and heme domains in the oxyFMN constructs of nNOS and iNOS demonstrated that the oxyFMN constructs are valid models of the NOS output state [18,19], and provide the first direct observation of CaM control of IET via facilitation of the FMN/heme interactions in the output state [18]. We have also further extended our CO photolysis approach to measure the IET kinetics in a rat nNOS holoenzyme [17].

In the present work, we have investigated the kinetics of the IET between the FMN and heme domains in the murine and human iNOS holoenzymes. This allows us to address important open questions on the mechanism of the IET between the FMN and heme domains in NOS isoforms, and to extend and evaluate important ideas concerning the relationship between NO synthesis and electron transfer rates in mammalian NOS isoforms. Importantly, our results suggest that in the iNOS holoenzyme, the FMN domain shuttles between input and output states, and the protein exists primarily in the input state.

Materials and Methods

Expression and purification of murine and human iNOS holoenzymes

The DNA encoding murine and human iNOS holoenzyme was a kind gift from Dr. John Parkinson (Berlex Bioscience, USA), and was cloned in *Escherichia coli* expression vector pCWori+ by RT-PCR in the same way as described before [31-33]. iNOS was expressed in a protease deficient *E. coli* strain BL21DE, and purified using a combination of ammonium sulfate precipitation, nickel sulfate affinity column and gel filtration chromatography, followed by 2',5'-ADP Sepharose column chromatography [32]. The iNOS activity was measured by spectrophotometric oxyhemoglobin assay [32], and found to be between 1100-1300 nmol/min/mg protein. The purified iNOS holoenzyme contained between 0.8 and 1.0 heme/mol, and the flavin content measured after extraction from the protein was in all cases at least 90% of the heme content.

Cloning and Protein Purification of human iNOS oxyFMN

The human iNOS oxyFMN that carries a deletion of the first 70 amino acids and an N-terminal polyhistidine consist of residues 71-723 of the human iNOS enzyme. The construct was generated by PCR amplification of the human iNOS cDNA and the product was subcloned into pCWori+. The expression vector was co-expressed with wild-type CaM in *E. coli* BL21 (DE3), as in the previous studies [31,34]. Purification of iNOS oxyFMN co-expressed with CaM proteins involved the removal of other proteins using a 30% ammonium sulfate cut followed by a 70% ammonium sulfate precipitation. The precipitated protein was resuspended in pellet buffer (40 mM Tris-HCl, pH 7.5, 1 mM L-Arginine, 250 mM NaCl, 1mM PMSF) and then purified using metal chelation chromatography. After washing the column with pellet buffer containing 50mM imidazole, the protein was eluted using buffer containing 200 mM imidazole. The samples were dialysed as previously described [35], followed by the use of a Vivaspin 15 ultrafiltration spin column (Sartorius AG Biotechnology, Goettingen, Germany) to concentrate the protein. The FMN content of the protein was determined using the fluorescence method developed by Faeder and Siegel [36]. The FMN content of the purified human iNOS oxyFMN was greater than 93%.

Laser flash photolysis

CO photolysis experiments were performed at room temperature, and the sample was kept in ice between flashes to stabilize the protein, as previously described [17-19]. Data from ~ 30 laser flashes were averaged, and transient absorbance changes were analyzed using program SIFIT, obtained from OLIS Inc. (Jefferson, GA). The laser apparatus and associated visible absorbance detection system have been extensively described [37]. Briefly, a N₂ laser (Photochemical Research Associates (PRA), London, Ontario, Canada) was used to pump a dye laser (450 nm wavelength maximum; PRA), which was focused onto the sample cell, and used to trigger the reactions.

A solution containing 20 μ M 5-deazariboflavin (dRF) and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol) was well degassed in a laser photolysis cell by a mixture of Ar and CO (with a mole ratio of ~ 3:1). L-Arg was added to keep oxidized heme in a high spin state. Aliquots of concentrated iNOS protein (150 μ M) were subsequently injected through a septum, and the solution was kept in ice and further purged by passing the Ar/CO mixture over the surface for 40 min to remove minor oxygen before being subjected to illumination.

The iNOS solution was then illuminated for an appropriate period of time (~ 90 seconds) to obtain a partially reduced form of [Fe(II)-CO][FMNH[•]], a process that was followed by characteristic absorptions of Fe(II)-CO and FMNH[•] at 446 nm and 580 nm, 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 FMNH₂ and Fe³⁺. This latter process was followed by the loss of absorbance of FMNH[•] at 580 nm, and the loss of absorbance of Fe(II) at 465 and 430 nm. Since the reduced protein has a strong characteristic absorption of Fe(II)-CO at 450 nm relative to that of the FMN species, the energy of the 450 nm laser flash was predominately absorbed by Fe(II)-CO. The absorbance of dRF at this wavelength is nearly zero. As a control, solutions containing dRF alone in CO-degassed buffer were flashed with 450 nm laser excitation, and signals of much smaller magnitude were observed, as expected due to the minimal absorption of dRF at 450 nm.

Results

Photochemical reduction of the oxidized murine and human iNOS holoenzymes by deazariboflavin semiquinone

The iNOS holoenzymes were partially reduced by illumination of the proteins in the presence of dRF. The basic photochemical process by which 5-deazariboflavin semiquinone (dRFH[•]) is generated and used as the exogenous reductant to reduce redox-active proteins has been extensively described [38,39]. Since we observed similar photoreduction and IET kinetics for the murine and human iNOS holoenzymes, we have only reported here the results for murine iNOS holoenzyme in detail (see below).

The oxidized murine iNOS holoenzyme in the presence of dRF and CO was exposed to steady white light illumination for certain period of time to photoreduce the protein (Figure 2a). This steady-state difference spectrum has the characteristic peak of Fe(II)-CO at 446 nm and the broad band of flavin semiquinone around 580 nm. The UV-vis spectrum of the photo-reduced iNOS also possesses the characteristic absorption band of oxidized flavin at 480 nm (Figure 2b), although with reduced intensity due to reduction of flavins. Similar spectra were obtained for photo-reduced nNOS holoenzyme in the presence of dRF and CO [17]. Based upon the midpoint potentials of the FMN/FMNH[•] and FAD/FADH[•] couples in the iNOS holoenzyme

(-105 mV and -240 mV, respectively) [22], we assigned the absorption peaks to [Fe(II)–CO][FMNH[•]], using the same published rationale [17].

Electron transfer between the heme and FMN domains in the partially reduced iNOS holoenzymes

The [Fe(II)–CO][FMNH[•]] 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 S1 in Supporting Information shows the 450 nm laser flash-induced difference spectra between 400 nm and 480 nm for the partially reduced iNOS holoenzyme (panel a) and iNOS oxygenase (iNOSoxy) construct containing no flavin cofactors (panel b), respectively. The similarities in the absorption changes between these two NOS proteins indicate the prompt formation of free Fe(II). This is followed by a subsequent slow rebinding of CO to Fe(II) to regenerate the Fe(II)–CO complex. The rate constant of CO rebinding ($1.8 \pm 0.2 \text{ s}^{-1}$) was obtained from the traces at 455 nm for the iNOS holoenzyme, where Fe(II) and Fe(II)–CO dominate the absorption.

CO dissociation by photolysis of the [Fe(II)–CO][FMNH[•]] form of the iNOS holoenzyme results in a decrease of the midpoint potential of the Fe(III)/Fe(II) couple to around -270 mV [22], and thereby electron transfer from the transient CO-free Fe(II) (formed by the 450 nm laser) to FMNH[•] may proceed. Figure S2 shows the 450 nm laser flash-induced difference spectra between 530 nm and 600 nm for the photochemically reduced iNOS holoenzyme (Figure S2a) and for the iNOSoxy construct (Figure S2b). Note the significant difference in the transient traces between 580 and 600 nm (highlighted in the dashed box in Figure S2a) between the iNOS holoenzyme and iNOSoxy construct. The absorption of the iNOS holoenzyme at 580 nm rapidly decays below the baseline with a rate constant of $35 \pm 3 \text{ s}^{-1}$ (Figure 3a), followed by a slow recovery to the baseline at longer time with a rate constant of $1.1 \pm 0.3 \text{ s}^{-1}$ (Figure 3b), similar to that of the CO rebinding rate ($1.8 \pm 0.1 \text{ s}^{-1}$, see above). In other words, a “transition” *i.e.* a reversal in direction of absorption changes over time, exists in the 580 nm trace for the iNOS holoenzyme (Figure 3b). In contrast, the 580 nm absorption of the iNOSoxy construct (which does not contain FMN) stays above the baseline (inset of Figure 3a). Note that FMNH[•] dominates the absorption in the range of 580 nm [40]. More importantly, the rate constant of the absorption change at 580 nm for the iNOS holoenzyme is independent of signal amplitude (data not shown), indicating an intraprotein process. Our previous studies show that CO photolysis of the [Fe(II)–CO][FMNH[•]] form of murine iNOS oxyFMN [19], CaM-bound rat nNOS oxyFMN [18], or CaM-bound rat nNOS holoenzyme [17] give a similar decay followed by a slower CO rebinding at 580 – 600 nm. Based on these observations, we have assigned the rapid absorbance decay at 580 nm (Figure 3a) to the following IET process between the catalytically significant redox couples of FMN and heme in the iNOS holoenzymes:

Because Fe(III)/Fe(II) and FMNH[•]/FMNH₂ are nearly iso-potential [22], 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. Since the FMN–heme IET reaction is an equilibrium process, we are in fact measuring it in both directions; however the CO photolysis technique follows the IET process in the reverse direction of the enzymatic turnover. Thus, both k_r (*i.e.* heme reduction) and k_f in the murine iNOS holoenzyme at pH 7.6 are approximately equal to $18 \pm 2 \text{ s}^{-1}$. The observed IET rate constants are listed in Table 1. Previous stopped flow studies on heme reduction in the iNOS holoenzyme gave rate constants of $0.9\text{--}1.5 \text{ s}^{-1}$ [41]; these experiments were conducted at 10 °C. Note that our experiments were at room temperature;

the difference between the reported rate constants might be due to different temperatures, which could cause the reported slower rates in the stopped flow experiments.

The “transition” feature in the 580 nm trace (Figure 3b) also exists in the kinetic traces at 465 nm and 430 nm for the murine iNOS holoenzyme (Figure 4b); however, such a “transition” was absent in the 465 nm/430 nm traces for the iNOSoxy construct (data not shown). Moreover, this transition feature does not clearly exist in other traces between 410 and 480 nm (Figure S3). The rapid decay in the 465 nm and 430 nm traces can be well fitted by a single exponential model, giving a rate constant of $36 \pm 2 \text{ s}^{-1}$ and $34 \pm 1 \text{ s}^{-1}$, respectively (Figure 4a), which is similar to the value obtained from the 580 nm trace ($35 \pm 3 \text{ s}^{-1}$, Figure 3a). The subsequent slow recovery at 465 and 430 nm (Figure 4b) also gave a rate constant value (0.9 ± 0.4 and $0.8 \pm 0.4 \text{ s}^{-1}$, respectively), comparable to that for the CO rebinding process ($1.8 \pm 0.1 \text{ s}^{-1}$). These results strongly indicate that the rapid decay in the 465 and 430 nm traces (Figure 4a) is also due to the IET between the FMN and heme domains (eq 1).

Upon CO dissociation by photolysis of the $[\text{Fe(II)}\text{--CO}][\text{FMNH}^*]$, three processes take place after the 450 nm laser flash, which may contribute to the absorption changes at 465 and 430 nm:

Because the rate constant for the decay ($\sim 35 \text{ s}^{-1}$) was much faster than that of the CO rebinding (1.8 s^{-1}), and more importantly, a transition exists in the traces (Figure 4b), the rapid decay can not be due to process (i), i.e. the CO rebinding process.

Note also that the amplitudes of the 430 nm and 465 nm trace were ~ 3 times and ~ 2 times that of the 580 nm trace for the same sample, respectively (Figure S4). It is very unlikely for process (iii) to give rise to the rapid decay at 465/430 nm, because the absorbance change due to this reaction (*i.e.* the FMNsq/red transition) is approximately isosbestic at 430 nm, and maximal around 580 nm, with an intermediate amplitude at 465 nm [42]. This leaves process (ii) as most likely responsible for the rapid decay at 430/465 nm.

Indeed, given the facts that (i) heme reduction in iNOSoxy gives positive absorption changes at 430/465 nm (Figure S5), and (ii) 430 and 465 nm are isosbestic points in the flash induced difference spectra of the $[\text{Fe(II)}\text{--CO}]$ form of iNOSoxy (Figure S6), we assigned the rapid decay at 430/465 nm (Figure 4a) to the heme oxidation process, i.e. process (ii), in the FMN–heme IET (eq 1). The larger amplitude of the 430/465 nm trace (compared to the 580 nm trace, Figs. 3a and 4a) is due to much larger absorption coefficients of the heme species than the flavin species in NOS. Therefore we can follow the IET kinetics in the murine iNOS holoenzyme either by net reduction of FMNH^* at 580 nm, or by net oxidation of heme at 430/465 nm. At other wavelengths, the $\text{Fe(II)}\text{--CO}$ and Fe(II) species dominate the absorption, thus masking observation of the heme oxidation (process (ii)) (Figure S3).

A similar trace with a “transition” at 460 nm, rather than 465 nm, was also observed for the CaM bound nNOS holoenzyme [16]. In agreement with the rationale described above for iNOS, the isosbestic point of CO rebinding in the nNOS oxygenase construct is around 460 nm, rather than at 465 nm. These results with nNOS further validate our assignment of the rapid decay in the 465 nm trace to the FMN–heme IET in iNOS. The isosbestic point for the CO rebinding process gives only a narrow spectral window at these wavelengths to observe the heme

oxidation in the full length NOS proteins. At other wavelengths, the heme oxidation signal is overlapped by the contributions from the CO rebinding process which gives much larger absorbance changes.

Scheme 1 summarizes the processes that take place after photolysis of the Fe(II)–CO complex in the partially reduced iNOS holoenzyme. [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 1). 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^{*} (reaction 3). In the iNOS holoenzyme, CO rebinding (reaction 2) is a poor competitor for the efficient IET (reaction 3). Also note that in these experiments, we chose to use a CO/Ar mixture in order to slow down CO rebinding, and thus favor IET from Fe(II) to FMNH^{*}, making loss of FMNH^{*} and Fe²⁺ observable as a bleaching at 580 nm and 430/465 nm, respectively.

Electron transfer between the heme and FMN domains in the partially reduced human iNOS oxyFMN construct

The oxidized human iNOS oxyFMN in the presence of CO and dRF was exposed to steady light illumination for various periods of time to photoreduce the protein to the [Fe(II)–CO][FMNH^{*}] form, as was the case for murine iNOS oxyFMN [19]. As expected, CO dissociation by laser photolysis of the [Fe(II)–CO][FMNH^{*}] form results in a rapid decay at 580 nm below the pre-flash baseline with rate constant of $320 \pm 45 \text{ s}^{-1}$ (Figure 5a), followed by a slow recovery to the baseline at longer time with a rate constant of $1.1 \pm 0.1 \text{ s}^{-1}$ (Figure 5b), similar to that of the CO rebinding rate constant ($1.5 \pm 0.2 \text{ s}^{-1}$) obtained from the traces at 455 nm, where Fe(II) and Fe(II)–CO species dominate the absorption. The rate constant of the rapid decay at 580 nm for the human iNOS oxyFMN construct is independent of signal amplitude (data not shown), indicating an intraprotein process. The transition feature in the traces at 580 nm also clearly exists in the traces at 430 and 465 nm (data not shown), as was the case for the murine and human iNOS holoenzymes (see above).

Discussion

The kinetics reported here for the IET in the iNOS holoenzymes (Table 1) are about an order of magnitude slower than the kinetics in the corresponding iNOS oxyFMN constructs [19]. Measurement of the IET in the nNOS holoenzyme gave a rate constant of 36 s^{-1} , also roughly an order of magnitude smaller than the value obtained in experiments with the nNOS oxyFMN construct [17]. Note that the truncated oxyFMN constructs, in which only the heme-containing oxygenase domain and FMN domain, along with the CaM-binding region, are present, are designed to remove the constraints from the NADPH-FAD-binding domain [30]. The IET reaction scheme in the holoenzyme can be represented as follows:

where sq represents “semiquinone”, hq represents “hydroquinone”, red represents “reduced”, and ox represents “oxidized”.

The fact that the IET rate constant in the holoenzyme is approximately an order of magnitude smaller than that in the corresponding oxyFMN construct suggests that in the holoenzyme the rate-limiting step in the IET is the conversion of the input state to the output state (*i.e.* the first step in the above scheme, in which the FMN becomes accessible to the heme), and that the role of CaM is to allow this conversion to occur (Figure 1). Note that in the iNOS oxyFMN and nNOS oxyFMN (with added CaM) constructs, the IET rate constants are much larger and

not equal in the oxyFMN constructs, although they are similar in order of magnitude (850 ± 50 , 320 ± 45 and $262 \pm 40 \text{ s}^{-1}$, for oxyFMN constructs of murine iNOS [19], human iNOS, and rat nNOS [17], respectively). Therefore the limiting factor in the nNOS and iNOS holoenzyme is not the FMN-heme IET *per se*. As a preliminary interpretation, we propose that at least some of the interactions that constrain the holoenzyme to be in the input state are lost in these oxyFMN constructs, making it easier for the truncated protein to achieve the output state, thereby increasing the IET rate constant. Structural studies will be required in order to better understand this difference.

The fact that there is no rapid IET component in the traces obtained with the iNOS holoenzyme (Figures 3a and 4a) implies that the holoenzyme is mainly in the input state, despite having CaM bound to it; the iNOS holoenzyme is still constrained by the contacts that exist which control the rate constant for the IET. If there were a mixture of input and output states when the reaction was initiated by the laser flash, the decay would be biphasic, with rate constants and amplitudes determined by the characteristics of the two states; only the enzyme fraction initially in the 'input state' should display slow apparent IET, while enzyme already in the 'output state' should show the same rapid IET kinetics that was reported for the oxyFMN constructs.

CaM unlocks the input state, thereby enabling the FMN domain to shuttle between the two enzyme states, and thus make contacts with the heme domain (Figure 1); the CaM effect is primarily kinetic, although there may be a thermodynamic component as well [43]. CaM does not affect the kinetics of the rate-limiting step (i.e. conversion of the input state to output state, see above); the evidence for this is that the IET rate constant for the holoenzyme is smaller than that for the oxyFMN construct. Thus in the holoenzyme, the IET rate constant is still controlled by the conversion step, even when CaM is present. The IET in the oxyFMN constructs are faster because it has lost contacts with the FAD domain that constrain this motion.

The experiments reported here show that in the iNOS holoenzyme the rate constant for the IET between heme and FMN is indistinguishable from our previously reported rate for CaM activated nNOS holoenzyme [17]. This is a remarkable observation considering that in nNOS holoenzyme the IET in the absence of CaM is too slow to measure [17]. Control of the IET processes in eNOS/nNOS by CaM has been shown to mainly involve the CaM binding site [44], and the unique AR insert within the FMN binding domain [24,45]. The AR insert within the FMN domain, in the absence of CaM, locks the FMN binding domain to the reductase complex via a network of hydrogen bonds so as to obstruct CaM binding and enzyme activation [28]. When CaM binds to the linker between the FMN and oxygenase domains at high $[\text{Ca}^{2+}]$, the AR insert is proposed to be displaced so that the enzyme can be activated. Indeed, our results suggest that in nNOS holoenzyme CaM activation effectively removes the restraints imposed by the nNOS unique AR insert on the release of the FMN binding domain, at least in single turnover. Importantly, our recent study on an nNOS AR-deletion mutant demonstrated that the rate-limiting conversion of the input state to a new output state does not involve interactions with the AR insert [16]. Although the results presented here and those in the previous study [16] support each other, the AR-deletion mutant is an artificial construct whereas the iNOS holoenzyme is a native form.

Santolini *et al.* have proposed a 'global model' for NOS catalysis including feedback inhibition by geminate NO produced in the catalytic cycle [41,46]. In this model nNOS is ~80% inhibited in the steady state, while iNOS is much less inhibited (~15%) because of the more rapid reaction of oxygen with the ferrous NO complex; eNOS is essentially uninhibited because of much slower rates of heme reduction. Note that the bulk of the data used in their modeling were obtained at 10 °C. Some of the details of our observations differ from the parameters used in

their modeling; in particular, they assert that the rate of ferriheme reduction in nNOS is more rapid than in iNOS (at 10 °C) [41,46], while results presented here and in our previous paper [17] show that these rates are indistinguishable (at 25 °C). If the steady state rates of NO synthesis by iNOS and nNOS were determined purely by the rate of reduction of ferriheme, our IET results would suggest that these NO synthesis rates would be equal. However, the steady state rate of NO synthesis by iNOS at 25 °C is 2–3 fold faster than the rate of NO synthesis by nNOS (Ghosh, D.K. unpublished results). The global model is needed to account for the difference in NO synthesis rates: if the proportion of iNOS and nNOS in the inhibitory ferrous NO complex at 25 °C were the same as at 10 °C, the global model would predict that steady state NO production of iNOS would be 3–4 times greater than that of nNOS. Therefore, overall our results offer strong support for the basic ‘global model’ (at 10 °C), and extend it to higher temperatures (25 °C).

Two factors may complicate this assessment, however. On one hand, the degree of NO inhibition of both enzymes is likely to vary with temperature since it is determined by the interaction of several temperature dependent rate constants. In addition, the steady state rate of reduction of ferriheme is unlikely to correspond exactly to the single turnover rate we measure. More precisely, the steady state rate of heme reduction will be determined by the interaction of multiple processes in the catalytic cycle of the reductase unit. The single turnover IET rates measured in our experiments represent important features of this cycle and provide upper limits of the steady state rates, but assessment of the steady state rates require in addition partitioning of the system (e.g., by King-Altman kinetics). Understanding the kinetics and control of NOS will require an assessment of the interaction of kinetics models of the oxygenase unit with kinetics models of the reductase unit. We intend to present more detailed assessments, including King-Altman modeling of the reductase unit, in subsequent communications.

It is interesting that the reduction of oxygenase heme and of cytochrome (cyt) *c* both require release of the FMN domain from the input state, but that $V_{\max}$ for cyt *c* reduction in all mammalian isoforms [25] is an order of magnitude faster than the reduction of heme reported in this work. For cyt *c* reduction we can postulate a reaction first order in ferric cyt *c* and in accessible FMN binding domains; the rate of formation of the complex with cyt *c* is then the second order rate constant k_{cf} (cyt *c* complex formation) times the concentration of enzyme molecules with accessible FMN binding domains. The rapid rate of cyt *c* reduction could be accounted for by a large value of k_{cf} , but it is more informative to consider the ‘free’ FMN binding domain as partitioning into a range of conformational states. If a much large number of these states are accessible to cyt *c* than to the oxygenase domain, we would expect much more rapid reduction of cyt *c* under saturated cyt *c* concentration. This is a reasonable expectation given the small size of cyt *c* and the conformational constraints placed on the oxygenase/FMN complex by the dimeric structure of NOS and the connecting polypeptides linking the domains.

In conclusion, laser flash photolysis of an [Fe(II)–CO][FMNH*] form of iNOS holoenzyme has allowed us to directly observe the discrete IET step between the catalytically significant redox couples of FMN and heme in the holoenzyme. This work suggests that the FMN domain in the iNOS holoenzyme shuttles between a shielded electron-accepting (input) state and an electron-donating (output) state, and the enzyme remains mainly in the input state. The rate constant values are remarkably similar to those obtained in studies of the CaM-bound nNOS holoenzyme. These results demonstrate that the basic control mechanisms acting in these NOS homologs are similar, and that CaM activation appears to effectively remove the inhibitory effect of the unique autoregulatory insert in nNOS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Tethered shuttle model: FMN-binding domain shuttles between the NADPH-FAD-binding domain and the 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 its structure has not yet been elucidated. CaM binding unlocks the input state, thereby enabling the FMN domain to shuttle between the two enzyme states. In the holoenzyme the rate-limiting step in the IET is the conversion of input state to output state. The FMN domain in the iNOS holoenzyme exists predominantly in the input state, as demonstrated in this study.

Figure 2.

(a) Difference and (b) absorption spectra of murine iNOS holoenzyme in the presence of 20 μM dRF obtained after approximately 1 min of steady light illumination. In panel b, dashed line, oxidized iNOS; solid line, partially reduced iNOS; note the characteristic absorption of oxidized flavin at 480 nm, as indicated by an arrow. Anaerobic solutions contained 2.4 μM iNOS, $\sim 20 \mu\text{M}$ dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane,

400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺, and 10 % glycerol). The sample was well degassed by Ar/CO (3:1) before illumination.

Figure 3. Transient trace at 580 nm at (a) 0 – 0.2 s and (b) 0 – 5 s obtained for [Fe(II)–CO][FMNH*] form of the murine iNOS holoenzyme flashed by 450 nm laser excitation. Inset of panel a is of the [Fe(II)–CO] form of the iNOSoxy construct (at 0–1 s) flashed by 450 nm laser excitation; note that the trace remains above the pre-flash baseline. Anaerobic solutions contained 7.4

μM iNOS, $\sim 20 \mu\text{M}$ 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 H₄B, 1 mM Ca²⁺ and 10 % glycerol).

Figure 4. Transient trace at 465 nm (and 430 nm, inset) at (a) 0 – 0.2 s and (b) 0 – 1 s obtained for the [Fe(II)–CO][FMNH*] form of the murine iNOS holoenzyme flashed by 450 nm laser excitation. Note both traces possess a transition (*i.e.* a reversal in direction of absorption changes over time, panel b), as was the case for the 580 nm trace (Figure 3b). Experimental conditions were the same as Figure 3.

Figure 5. Transient traces at 580 nm at (a) 0 – 0.2 s and (b) 0 – 2 s obtained for the [Fe(II)–CO] [FMNH[•]] form of a human iNOS oxyFMN construct flashed by 450 nm laser excitation. Anaerobic solutions contained 8.2 μ M human iNOS oxyFMN, $\sim$ 20 μ M dRF and 5 mM fresh semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M

H₄B, 1 mM Ca²⁺ and 10 % glycerol). Note the significant difference in the time scale of the panel a and Figure 3a.

Scheme 1.

Summary of processes occurring upon CO photolysis in the partially reduced form [Fe(II)-CO][FMNH•] of iNOS holoenzymes. Species in the dashed boxes are CO-bound forms, whereas those in the solid boxes are CO-free and participate in the FMN-heme IET (reaction 3). This IET process can be followed at 580 nm (which is due to net reduction of FMNH•) and 465/430 nm (which is due to net oxidation of heme).

The rate constants (s^{-1}) of the FMN-heme IET reaction (k_{et}^{obs}) and CO rebinding process (k_{CO}) obtained for the iNOS holoenzymes ^a

Table 1

	580 nm trace		465 nm trace		430 nm trace		455 nm trace	
	k_{et}^{obs}	k_{CO}	k_{et}^{obs}	k_{CO}	k_{et}^{obs}	k_{CO}	k_{et}^{obs}	k_{CO}
Murine iNOS ^b	35 ± 3	1.1 ± 0.3	36 ± 2	0.9 ± 0.4	35 ± 2	0.8 ± 0.4	1.8 ± 0.1	
Human iNOS ^c	35 ± 5	0.9 ± 0.1	32 ± 3	0.8 ± 0.1	29 ± 2	0.7 ± 0.1	3.6 ± 0.6 (53%)	
							0.7 ± 0.2 (47%)	

^a Buffer: 40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10% glycerol, pH 7.6. 20 μ M 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 iNOS. k_{et}^{obs} values for murine and human iNOS oxyFMN constructs are 850 ± 50 s⁻¹ [19] and 320 ± 45 s⁻¹ (this study), respectively.

^b Experiments were repeated with 4.6 and 7.4 μ M murine iNOS.

^c Experiments were repeated twice at 11 μ M human iNOS.