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Direct Measurement by Laser Flash Photolysis of Intramolecular Electron Transfer in a Two-Domain Construct of Murine Inducible Nitric Oxide Synthase

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Abstract: Intersubunit intramolecular electron transfer (IET) from FMN to heme is essential in the delivery of electrons required for O_2 activation in the heme domain and the subsequent nitric oxide (NO) synthesis by NO synthase (NOS). Previous crystal structures and functional studies primarily concerned an enzyme conformation that serves as the input state for reduction of FMN by electrons from NADPH and FAD in the reductase domain. To favor formation of the output state for the subsequent IET from FMN to heme in the oxygenase domain, a novel truncated two-domain oxyFMN construct murine inducible nitric oxide synthase (iNOS), in which only the FMN and heme domains were present, was designed and expressed. The kinetics of the IET between the FMN and heme domains in this construct was directly determined using laser flash photolysis of CO dissociation in comparative studies on partially reduced oxyFMN and single domain heme oxygenase constructs.

Introduction

In mammals, nitric oxide (NO) is synthesized by nitric oxide synthase (NOS), a homodimeric flavo-hemoprotein that catalyzes the oxidation of L-arginine (Arg) to NO with NADPH and O_2 as cosubstrates.[1,2] Each subunit of NOS contains a C-terminal electron-supplying reductase unit with binding sites for NADPH, FAD, and FMN and an N-terminal catalytic heme-containing oxygenase domain; these components are linked by a calmodulin (CaM) binding region.[2,3] The substrate, L-arginine, and a cofactor, (6R)-5,6,7,8 tetrahydrobiopterin (H_4B), both bind near the heme center in the oxygenase domain.[1] The structure of the holoenzyme is not yet available, though extensive crystallographic studies of the oxygenase domain have been performed.[4−7] Only a few crystal structures of nNOS reductase domains[8,9] and homologues [10] have been reported, and these have provided new insights into the function of these enzymes.

Although many details of the novel NOS mechanism remain to be elucidated,[3] it is well established that electron-transfer processes are key steps in NO synthesis,[2,11,12] and therefore, elucidating the mechanism of electron transfer in NOS is important for understanding the regulation of NO synthesis. Recent investigations have been carried out on the thermodynamics[13−15] and kinetics[16−19] of electron-transfer processes

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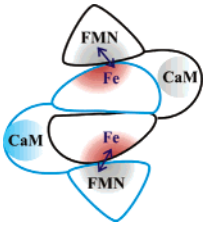

Figure 1. Schematic diagram of the truncated two-domain iNOS oxyFMN construct. The two subunits are shown in black and blue, respectively. The IET between heme and FMN domains (marked by an arrow) is of particular interest in this work.

in NOS. In particular, intersubunit intramolecular electron transfer (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,[1,2] and the kinetics of this process have been indirectly probed by CO binding.[20−22] The observed rate constants involve sequential reactions, in which the IET step may not be rate limiting, and the rate constants obtained in this way may in fact be the CO binding rates. As shown below, direct measurements of the IET rate constants in NOS yield very different results.

A major obstacle in IET kinetics studies in NOS is that there is significant overlap in the absorption spectra of the FAD and FMN moieties in the holoenzyme, making direct observation of the discrete IET between heme and FMN domains difficult. This makes the study of subsets of the various enzyme domains important. In this work, as part of a systematic study of the role of IET processes in the regulation of NOS, we have used laser flash photolysis to directly investigate the kinetics of the IET between the FMN and the heme domains in a novel two-domain murine inducible NOS (iNOS) oxyFMN construct in which only the FMN and oxygenase domains are expressed (Figure 1).

Results

Biochemical Characterization of the Murine iNOS Construct. The murine iNOS oxyFMN construct has been expressed and purified as a homogeneous protein with activity.[23] The protein binds cofactors nearly stoichiometrically, has native catalytic sites by spectroscopic criteria, and does not contain significant amounts of denatured material.[23]

Photochemical Reduction of Oxidized nNOS oxyFMN by Deazariboflavin Semiquinone. The iNOS constructs were partially reduced by illumination of the proteins in the presence of CO and deazariboflavin (dRF). The basic photochemical process by which 5-deazariboflavin semiquinone (dRFH•), which has a redox potential of −630 mV, is generated and used as the exogenous reductant to reduce redox-active proteins has been extensively described.[24] The flash-induced difference spectrum of the construct (Figure 2) indicates that flash-generated dRFH• can readily reduce FMN (to FMNH•) and heme simultaneously.

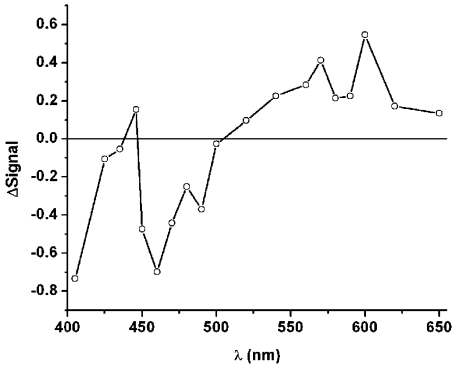

Figure 2. Flash-induced difference spectra of oxidized iNOS oxyFMN obtained 958 ms after a pulse of 400-nm laser excitation. The solution contained 6.9 μM iNOS oxyFMN, ~20 μM dRF, and 5 mM freshly prepared semicarbazide in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-arginine, 1 mM Ca^{2+}, and 10% glycerol) degassed by a mixture of CO and Ar. The absorption changes at 446 and 480 nm are due to formation of an Fe(II)−CO complex and FMN reduction, respectively, via reaction of the protein with flash-generated dRFH•. In addition, a net positive absorbance change around 580 nm indicates the formation of FMNH•.

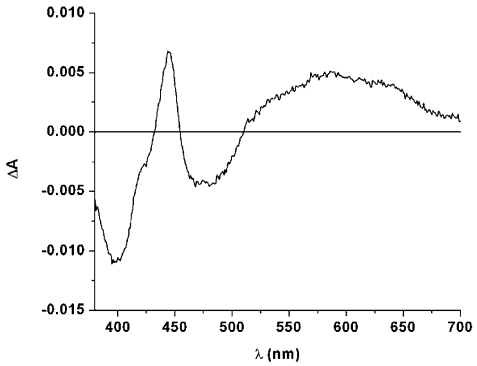

Figure 3. Difference spectra of iNOS oxyFMN in the presence of 20 μM dRF obtained after approximately 1 min of steady light illumination. Anaerobic solutions contained 6 μM iNOS oxyFMN in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-arginine, 1 mM Ca^{2+}, and 10% glycerol).

The oxidized iNOS oxyFMN construct in the presence of dRF and CO was then exposed to steady light illumination for various periods of time to generate sufficient quantities of the [Fe(II)−CO] [FMNH•] form of the protein. The redox potentials of the FMN/FMNH• and FMNH•/$FMNH_2$ couples have been determined to be +70 and −180 mV, respectively.[23] The apparent midpoint potential of the Fe(III)/Fe(II)−CO couple is about −120 mV under these conditions.[25] Therefore, electron transfer from Fe(II)−CO to FMNH• is disfavored, thus allowing the formation of the [Fe(II)−CO][FMNH•] species in high yield. Figure 3 shows the steady-state difference spectrum of the protein in the presence of dRF under such illumination. This closely resembles the flash-induced spectrum and has the characteristic peak of Fe(II)−CO at 446 nm and the broad band of FMNH• around 580 nm.

Electron Transfer Between Heme and FMN Domains in Reduced iNOS oxyFMN. The partially reduced [Fe(II)−CO]-[FMNH•] form was then flashed by 450-nm laser excitation to dissociate CO from the Fe(II)−CO complex with the formation of a transient Fe(II) species. Figure 4 shows 450-nm laser flash-induced difference spectra between 380 and 480 nm for the

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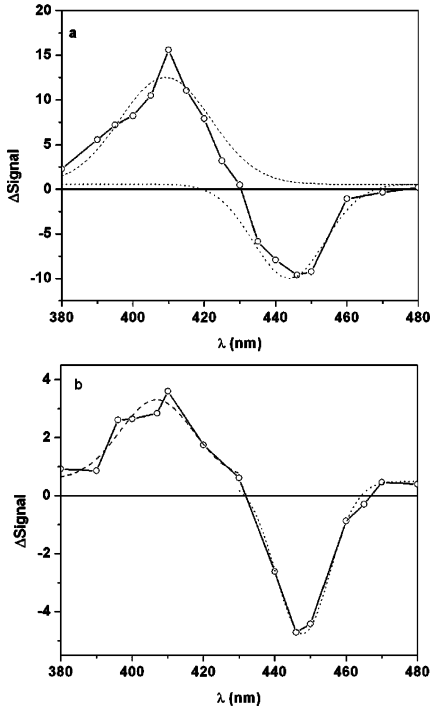

Figure 4. Flash-induced difference spectra (380−480 nm) of (a) reduced iNOS oxyFMN obtained 114 ms after the 450-nm laser flash and (b) reduced iNOS oxygenase construct obtained 198 ms after the 450-nm laser flash. The dotted and dashed lines indicate best Gaussian fits. Note that the two proteins have the same difference spectra in this region with a peak at 407 nm, which is due to the formation of free Fe(II), and a valley at 446 nm, which is characteristic of the Fe(II)−CO complex.

reduced iNOS oxyFMN (Figure 4a) and for the iNOS oxygenase construct iNOSoxy (Figure 4b), which lacks the FMN domain. The similarities in the absorption changes at 446 and 407 nm between these two constructs indicate the prompt generation of CO-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 of CO recombination is consistent with 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) (data not shown), confirming that this is due to the recombination of CO to Fe(II).

CO photolysis of the [Fe(II)−CO][FMNH•] form of iNOS oxyFMN results in a decrease of the redox potential of the Fe(III)/Fe(II) couple by approximately 60 mV (to −180 mV),[25] and thereby electron transfer from Fe(II) to FMNH• may proceed. Figures S1 and S2 show flash-induced difference spectra between 560 and 600 nm for the reduced iNOS oxyFMN and oxygenase constructs, respectively (see Supporting Information). These two constructs show significant differences in the transient spectra between 580 and 600 nm. Specifically, after CO dissociation, the absorption of iNOS oxyFMN decays with a rate constant of 850 ± 50 s^{-1} (Figure 5), whereas the kinetic transient obtained with the iNOS oxygenase construct does not possess this decay feature (Figure 5, inset). In a longer time scale (Figure S3, Supporting Information) at 580−600 nm, both the iNOS oxyFMN and oxygenase constructs recover slowly toward the baseline with rate constants of 6.1 ± 1.7 and 8.9 ± 1 s^{-1}, respectively, close to that of the corresponding CO recombination rates obtained from traces around 446 nm (4.3 ± 0.8 and 11.1 ± 0.9 s^{-1} for iNOS oxyFMN and iNOSoxy,

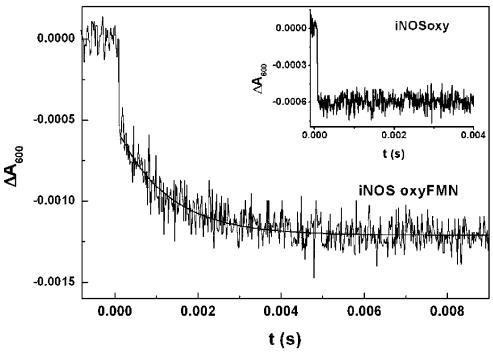

Figure 5. Transient trace at 600 nm at shorter time scale (<10 ms) obtained for [Fe(II)−CO][FMNH•] form of iNOS oxyFMN flashed by 450-nm laser. Anaerobic solution contains 14 μM iNOS oxyFMN, ~20 μM dRF, and 5 mM semicarbazide in the pH 7.6 buffer (see text). Solid line indicates the best single exponential fit. (Inset) Trace of [Fe(II)−CO] form of iNOSoxy flashed by 450-nm laser under same conditions.

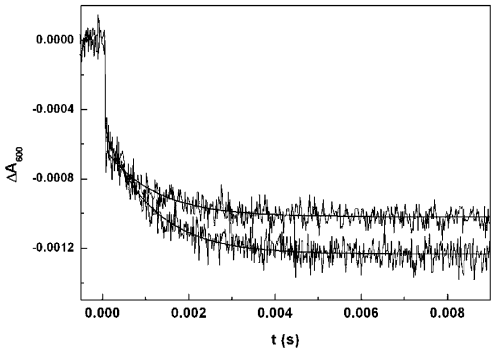

Figure 6. Transient traces at 600 nm for reduced iNOS oxyFMN construct flashed by 450-nm laser, in which the lower trace has an amplitude nearly twice as large as that of the upper trace. This was accomplished by generating different amounts of the [Fe(II)−CO][FMNH•] form during preillumination (see text). Solid lines indicate the best single exponential fits to the traces, giving rate constants of 857 ± 52 and 892 ± 37 s^{-1} for the upper and lower traces, respectively. Experimental conditions were the same as Figure 5.

respectively). Note that FMNH• dominates the absorption in the range of 580−600 nm. More importantly, the rate of the absorption change at 600 nm for iNOS oxyFMN is independent of signal amplitude (Figure 6), i.e., the concentration of reduced protein, indicating an intraprotein process. On the basis of these data, we have assigned the rapid decay at 600 nm in the iNOS oxyFMN (Figure 5) to the following IET process between the heme and FMN domains in the protein (cf. Figure 1):

$$[\mathrm{Fe(II)}][\mathrm{FMNH^{\bullet}}] + \mathrm{H^{+}} \leftrightarrow [\mathrm{Fe(III)}][\mathrm{FMNH_2}] \qquad (1)$$

Because Fe(III)/Fe(II) and FMNH•/$FMNH_2$ are nearly isopotential,[23] the electron transfer between the heme and FMN domains is reversible, and the observed IET rate constant is the sum of the forward electron transfer (k_f) and reverse electron transfer (k_r) rate constants. Thus, both k_f and k_r in iNOS oxyFMN at pH 7.6 are approximately equal to 425 ± 25 s^{-1}.

CO photolysis of the [Fe(II)−CO][FMNH•] form of a rat neuronal NOS oxyFMN in the presence of added CaM gives a similar rapid decay at 600 nm with an observed rate constant of 262 ± 40 s^{-1}, which is also independent of protein concentration.[26] In the iNOS system, added CaM is not required because this protein binds CaM more strongly.[1] This result

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reinforces the assignment of the absorbance change of iNOS oxyFMN at 600 nm to IET between heme and FMN domains. The difference in the rate constants between iNOS and nNOS isoforms may be due to different IET-productive conformations.

Discussion

Previous studies on heme reduction in NOS isoforms gave rate constants of 3−4 and 0.9−1.5 s^{-1} for nNOS and iNOS holoenzymes,[20−22] respectively. The present study demonstrates that these observed slow rates of heme reduction are probably the rates of CO binding to Fe(II). Because the experiments reported here do not involve any step other than the IET between the FMN and heme domains, they provide the first direct determination of the kinetics of this process.

Observation of a rapid phase of electron transfer between FMN and heme domains would be much more difficult in the holoenzyme, because most of the protein is in the "input" state, facilitating the transfer of electrons from FAD to FMN. 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 the reported crystal structures of flavoenzymes of NOS[8] 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. Considerable domain motion to bring the FMN close to the heme in an electron-donating position ("output" state) is required for efficient IET between the heme and FMN domains.

The kinetics reported here are "output" state kinetics and, hence, should not be expected to correspond directly to the kinetics of electron transfer in the holoenzyme, in which FMN has to undergo dissociation from the input state complex and reassociation with the oxygenase domain before rapid electron transfer can occur. We expect the output state of oxyFMN constructs to be a good model for the output state of the holoenzyme, but holoenzyme output state kinetics could be slightly faster or considerably slower than those reported here. Limitations placed on electron transfer by the structures of the FMN, FAD, and oxygenase domains in the holoenzyme probably would prevent much faster rates than observed in the present study. It is possible that the presence of the two domain "dehydrogenase" unit would affect the orientation of the FMN and oxygenase domains in the holoenzyme output state enough to produce a slower rate, but the holoenzyme rate must be faster than the lower limits established by NO synthesis and/or stopped-flow kinetics results. Thus, the precise rate of IET in the holoenzyme will likely fall between that of the two-domain construct (an "output" state model) and the lower value determined from CO binding kinetics.

Recent kinetics studies on holoenzyme indicate that H_4B is involved in the activation of O_2 through electron transfer between the pterin and Fe(II)−O_2 intermediate.[3] In this work, we observed the IET from Fe(II) and $FMNH^{\bullet}$ in anaerobic atmosphere (eq 1), and it is unlikely that the bound pterin is involved in the IET.

The contrast between the oxyFMN and oxygenase time courses is strong additional evidence that the electron-transfer reaction is between heme and FMN, not between heme and H_4B. The spectral features of the smaller pterin ring system are in the ultraviolet, so the observed spectral changes would be assigned to FMN in any case. It is unlikely that tetrahydrobiopterin would participate in FMN−heme electron transfer for several reasons. It is a high potential donor, unable to reduce FMN or heme directly except when a powerful oxidant is generated during catalysis. In addition, it is located in the interior of the oxygenase dimer and cannot be closely approached by FMN, whereas a heme edge is exposed at the surface.[5,6] Adding excess H_4B to oxidized constructs reduced neither the heme nor the FMN.

It is important to maintain H_4B in the sample, however, because the H_4B is closely associated with the heme electronic state and the heme pocket conformation. H_4B, bound in two sites along the dimer interface, is an important factor in dimerization and dimer conformation. Because FMN reduces heme in the oxygenase domain of the opposite monomer, dimerization is essential for native heme/FMN interaction.[20]

Concluding Remarks

In summary, comparative measurements of spectral changes produced upon CO photolysis of reduced iNOS oxyFMN and oxygenase constructs allows the first direct determination of the rates of the IET between the FMN and the heme domains in iNOS. Further laser flash photolysis studies on mutants of residues related to the possible docking between the FMN domain and the heme domain are underway and will provide further insights into the coupling of IET and NO synthesis in this important enzyme.

Experimental Section

Protein Expression. iNOS oxyFMN was expressed and purified as described elsewhere.[23]

Laser Flash Photolysis. A dRF solution in pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-arginine, 1 mM Ca^{2+}, and 10% glycerol) was well degassed by a mixture of Ar and CO (with ratio of ∼3:1), and small aliquots of a concentrated iNOS oxyFMN solution (∼0.3 mM) were injected into the anaerobic cuvette. The solution was further purged with the Ar/CO mixture to remove any 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 $dRFH^{\bullet}$.

The iNOS oxyFMN solution was then illuminated for an appropriate period of time (∼30 s) to obtain a mixture of the [Fe(II)−CO][$FMNH^{\bullet}$] and [Fe(II)−CO] forms of the protein. 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 $FMNH^{\bullet}$ to produce fully reduced FMN. This latter process was followed by the loss of absorbance of $FMNH^{\bullet}$ at 580−600 nm.

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), and confirmed by SVD analysis on a set of traces over a range of wavelengths using Olis GlobalWorks (version 3.0).

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Supporting Information Available: Figures S1-S3, difference spectra of iNOS oxyFMN, and oxygenase constructs by laser flash photolysis, traces obtained at 600 nm at longer time scales upon flashing reduced iNOS proteins with 450-nm laser.

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