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Role of a Conserved Tyrosine Residue in the FMN–Heme Interdomain Electron Transfer in Inducible Nitric Oxide Synthase

Li Chen[†], Huayu Zheng^{†,‡}, Wenbing Li[†], Wei Li[†], Yubin Miao^{||}, and Changjian Feng^{†,‡,*}

[†]College of Pharmacy, University of New Mexico, Albuquerque, NM 87131, United States

[‡]Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, NM 87131, United States

^{||} University of Colorado Denver, Radiology, Denver, CO 80045, United States

Abstract

The interdomain electron transfer (IET) between the flavin mononucleotide (FMN) and heme domains is essential in the biosynthesis of nitric oxide (NO) by the NO synthase (NOS) enzymes. A conserved tyrosine residue in the FMN domain (Y631 in human inducible NOS) was proposed to be a key part of the electron transfer pathway in the FMN/heme docked complex model. In the present study, the FMN–heme IET kinetics in the Y631F mutant and wild type of a bi-domain oxygenase/FMN construct of human inducible NOS were determined by laser flash photolysis. The rate constant of the Y631F mutant is significantly decreased by ~ 75% (compared to the wild type), showing that the tyrosine residue indeed facilitates the FMN–heme IET through the protein medium. The IET rate constant of the wild type protein decreases from 345 to 242 s⁻¹ on going from H₂O to 95 % D₂O, giving a solvent kinetic isotope effect of 1.4. In contrast, no deuterium isotope effect was observed for the Tyr-to-Phe mutant. Moreover, an appreciable change in the wild type iNOS IET rate constant value was observed upon changing pH. These results indicate that the FMN–heme IET is proton coupled, in which the conserved tyrosine residue plays an important role.

Graphical abstract

Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule that plays a crucial role in cardiovascular, nervous, and immune systems.^{1–2} Although there are nonenzymatic sources of NO,³ most of the biological effects of NO are mediated by nitric oxide synthase enzymes (NOSs). Owing to the importance of NO as a molecular messenger, its production by NOS is under stringent control. Deviant NO production by NOS is a major contributor to the pathology of often fatal diseases that currently lack effective treatments, including cancer and stroke.^{4–5} To date, clinical NOS modulators still remain elusive. Yet, there is still much unknown about the mechanism of tight regulation of NO production by NOS.^{6–7}

Mammalian NOS enzyme is a homodimeric flavo-hemoprotein that catalyzes conversion of the L-arginine (L-Arg) substrate to NO and L-citrulline with NADPH and O₂ as co-substrates.^{8–9} Each NOS subunit comprises of an N-terminal oxygenase domain (containing a catalytic heme active site) and a C-terminal electron-supplying reductase domain (that binds the flavins FAD and FMN), along with a calmodulin (CaM) binding linker between the two domains.^{9–10} The substrate, L-Arg, and a cofactor, (6*R*)-5,6,7,8-tetrahydrobiopterin (H4B), both bind near the heme center in the oxygenase domain.

The intraprotein interdomain electron transfer (IET) processes are key steps in the NO synthesis.^{8, 11} In particular, the CaM-controlled inter-subunit FMN–heme IET step¹² is functionally essential by supplying electrons to the heme¹² and H4B¹³ groups, allowing O₂ activation required for NO synthesis.¹⁰ We have developed a laser flash photolysis approach^{14–15} to directly measure the rates of the IET between catalytically significant redox couples of the FMN and heme centers in eq 1 (FMN_{hq} represents FMN hydroquinone).¹¹

CaM control of NO synthesis requires a large conformational change in which the FMN domain shuttles between the NOS's electron-accepting input state and electron-donating

output state.^{15–17} We initially addressed the conformational and structural requirement for NOS electron transfer.¹⁸ In the NOS output state, the FMN and heme domains form a docking complex, thus enabling efficient IET between the FMN and heme centers. Several docking models of the NOS output state were proposed.^{19–22} Our recent molecular dynamics work proposed some IET-competent conformations for human iNOS.²³ Interestingly, in the docked FMN/heme complex models^{20, 23} a conserved human iNOS Tyr631 residue is well positioned to take part in the electron transfer through protein medium (Figure 1).

In the present study, we have investigated the role of Tyr631 in the FMN–heme IET by comparing its IET rate and deuterium isotope effect to those of the wild type (wt) protein. We utilized a bi-domain oxygenase/FMN (oxyFMN) construct, which only consists of the heme-containing oxygenase and FMN domains, along with the CaM-binding region.²⁴ This construct is a minimal electron transfer complex,²⁴ and biochemical and kinetic studies have established that it is a valid model of the NOS output state for NO production.^{14, 25} Herein we have demonstrated that the conserved tyrosine residue indeed facilitates the IET. The solvent isotope and pH dependence data further indicate that the FMN–heme IET is proton coupled, in which the tyrosine residue plays an important role. This work suggests a new proton-coupled facet of the IET mechanism in NOS.

Materials and Methods

Expression and purification of human iNOS oxyFMN proteins

The Y631F mutant plasmid was constructed by site-directed mutagenesis on a pCWori⁺ vector containing cDNA of the wt human iNOS oxyFMN construct.²⁶ The forward primer (with the mutation site underlined) is CCTCGGCTCCAGCATGTTCCCTCGGTTCTGCGCCTTTG. The expression and purification of the oxyFMN proteins were carried out as reported earlier.²⁶ CaM binds tightly to iNOS and co-exists in the purified iNOS proteins.

Laser flash photolysis

CO photolysis experiments were conducted on an Edinburgh LP920 laser flash photolysis spectrometer, in combination with a Q-switched Continuum Surelite I-10 Nd:YAG laser and a Continuum Surelite OPO. A 446 nm laser pulse out of the OPO module was focused onto the sample cell to trigger the IET processes; the laser output power was around 10 mW. A 50 W halogen lamp was used as the light source for measuring the kinetics at ms – s time scales. A LVF-HL filter (Ocean Optics, FL) with band pass peaked at 580 nm or 465 nm was placed before the protein sample to protect it from photo-bleaching and further photo-reduction by the white monitor beam.¹¹

The CO photolysis experiments were performed at 21 °C as previously described.¹⁵ Briefly, a CO/Ar (v/v ~ 1:3) pre-degassed iNOS solution in the presence of 5-deazariboflavin was illuminated for a certain period of time (~ 2 minutes) to obtain a partially reduced form of [Fe(II)–CO][FMNH*]. The sample was subsequently flashed with 446 nm laser excitation (pulse width 4–6 ns) to trigger the FMN–heme IET, which can be followed by the loss of

absorbance of FMNH* at 580 nm and Fe(II) at 465 nm, respectively.²⁶ All the experiments were conducted at least twice. The transient absorbance changes were averaged and analyzed using OriginPro 2015 (OriginLab).

Proton inventory experiments

D₂O, glycerol-d₈, NaOD and DCl were obtained from Cambridge Isotopes Laboratory. Isotopic mixture of buffers at pH 7.6 were obtained by mixing appropriate volumes of separately prepared solutions in H₂O and D₂O, with the latter prepared taking into account the necessary correction of the pH meter reading to obtain the pD (pD = meter reading + 0.4).²⁷ Proton inventory experiments, in which the mole fraction of D₂O was varied from 0 to 95 %, were conducted in the buffers.

Results and Discussions

The FMN–heme IET kinetics in Y631F human iNOS oxyFMN

As expected, upon a 446 nm laser excitation, the absorbance at 580 nm of the partially reduced Y631F human iNOS oxyFMN decays below the pre-flash baseline (Figure 2), which is due to the FMN–heme IET (eq 1 above), resulting in FMNH* depletion,²⁶ with a rate constant of $90.6 \pm 1.6 \text{ s}^{-1}$. This is followed by a much slower recovery toward baseline (apparent rate constant = $2.0 \pm 0.1 \text{ s}^{-1}$), which is due to CO re-binding to Fe(II).²⁶ Note the spectral “transition” (*i.e.*, a reversal in direction of absorption changes over time), which is a signature of successful observation of the FMN–heme IET process.

The IET rate constant of the Y631F mutant is notably decreased by ~ 75% (compared to $342.9 \pm 11.1 \text{ s}^{-1}$ of the wt human iNOS oxyFMN^{28–29}); see Figure 3 for comparison of the IET phases of the two proteins. The mutational effect is even larger than that of E546→N mutation at a primary FMN–heme interdomain docking site.³⁰ This demonstrates that Tyr631 is important in the FMN–heme IET, as predicted from the docking model structure (Figure 1).

The FMN– heme IET kinetics in wild type human iNOS oxyFMN as a function of pH

The IET kinetics of the wild type human iNOS oxyFMN construct were determined over the pH range from 6.7 to 8.7, and the rate constants k_{et} are listed in Table 1. Importantly, the obtained IET rate constant over the pH range is independent of the signal amplitude (data not shown), *i.e.*, reduced protein concentration, confirming an intra-protein process in the pH range we studied. It is also of note that the observed IET rate at pH 7.6 is in good agreement with that obtained on another laser flash photolysis apparatus for a different preparation of the human iNOS oxyFMN protein.²⁶

An appreciable pH dependence of the IET rate constant was observed, and the iNOS protein displays an optimal IET around pH 7.4, decreasing to ~ 70 % of this activity at pH 8.7. Full length NOS proteins displayed a similar pH profile of enzymatic activities.^{31–35} These results suggest that the FMN–heme IET process is very likely a major contributor to the pH dependence of the NOS enzymatic activities. This is reasonable since the IET step is rate-limiting in the NOS catalysis.¹⁰ It is also of note that the magnitude of change in the IET is

much smaller than that of enzymatic activities (e.g. nNOS decreases to 27% of its optimal activity at pH 8.6³³). This is consistent with the global kinetics model in which the overall NOS activity depends on interplay of the IET rate, and other two kinetics parameters.¹⁰

Importantly, surface-enhanced Raman scattering spectra of the FMN domain of neuronal NOS showed that the FMN_hq is neutral (*i.e.*, the FMNH₂ form).³⁶ In combination with the pH dependence profile (Table 1), we conclude that eq 2 below (assigning FMN_hq as FMNH₂), not eq 3 (assuming FMN_hq is FMNH⁻), should be used as the chemical equation for the FMN–heme IET in NOS.

Since the pH dependence data suggest that proton is involved in the FMN–heme IET process, we next compared the solvent kinetic isotope effects of the wt and Y631F mutant proteins to further investigate how the Y631 residue modulates the IET in human iNOS.

Deuterium effect on the FMN–heme IET kinetics in wt human iNOS oxyFMN

The IET kinetics of the wt oxyFMN protein were determined in the buffer containing 0 – 95 % D₂O. The rate constant k_n obtained at a certain molar fraction of D₂O (n), along with the ratio of k_n to rate constant k_0 in pure H₂O buffer (k_n/k_0), are listed in Table 2. Notable decrease in the IET rate constant value was observed with increasing the D₂O contents: the rate constant decreases from 345 to 242 s⁻¹ on going from H₂O to 95 % D₂O.

The proton inventory data were analyzed by plotting the ratio k_n/k_0 versus n (Figure 4). The data were fitted linearly using eq 4:

$$k_n/k_0 = (1 - n + n/a) \quad \text{eq 4}$$

where a = SKIE (solvent kinetic isotope effect).^{37–38} The linear fitting gives a SKIE of 1.38 ± 0.04 . Similar isotope effects on protein electron transfer kinetics have been observed.³⁹ However, it is not possible to draw definite conclusions about the number of proton-transfer steps for a kinetic deuterium isotope effect of 1.4. Theoretical graphs for >1 protons involved in the rate-limiting step in the transition state would most likely be so close to the linear fit (*i.e.*, 1 proton) that they would fit with the data as well as the line.

Nonetheless, the deuterium effect on the wt iNOS IET kinetics shows that the FMN–heme IET is proton coupled (eq 2 above). The FMN semiquinone receives an electron from heme Fe(II) and is reduced to FMNH⁻, which also picks up a proton (presumably from the nearby protein environment; see below), giving a neutral form of FMN_hq (FMNH₂). Proton transfer

participates in the IET process shown in eq 2, and the observed IET kinetics should thus be affected by solvent isotope (as observed here).

The FMN–heme IET kinetics of Y631F human iNOS oxyFMN in deuterated solvent

From what is known about proton transfer in other proteins,^{38, 40} reactions with relatively small SKIE (~ 2) originate from proton transfer limited by hydrogen bond breaking or hydrogen bond reorientation. A plausible mechanism underlying the deuterium effect is that a NOS residue critical in the IET receives a proton, and its protonation is required for the role of this residue in facilitating the IET. We did not find charged residues in potential electron transfer pathways in the docking model²⁰ (Figure 1). Interestingly, the conserved Tyr631 residue is within hydrogen-bonding distance of N1 atom of the FMN cofactor (Figure 1). Assuming that Tyr631 mediates the proton transfer, removal of the hydroxyl group in the Y631F mutant should alter the deuterium effect. Indeed, the IET rate constant of Y631F mutant in the 95 % D₂O buffer is $96.4 \pm 4.0 \text{ s}^{-1}$ (Figure 5), which is similar to its rate in the H₂O buffer ($90.6 \pm 1.6 \text{ s}^{-1}$), *i.e.*, no deuterium isotope effect was observed for this mutant. In contrast, the IET rate constant of the wt protein is decreased by 30% when going from 0 to 95 % D₂O (Table 2). Taken together, the Tyr631 residue is very likely to cause the observed kinetic isotope effect in the wt iNOS protein. Figure 6 gives a tentative schematic view of a proton coupled electron transfer (PCET) mechanism, in which Tyr631 in human iNOS acts as an intermediary proton shuttle between the reduced FMN cofactor and water solvent.

We recognize that the position of Tyr631 in the crystal structure of human iNOS FMN domain¹⁹ is not ideal for Tyr631–OH $\cdots$ FMN hydrogen-bonding geometry since its phenyl ring is nearly parallel to the FMN isoalloxazine ring. Note that the crystal structure¹⁹ contains oxidized FMN cofactor. However, it has been shown that the reduction of the FMN cofactor in flavoproteins is often accompanied by a local conformation change.⁴¹ For example, in *D. vulgaris* flavodoxin, protein conformation and solvation dynamics in the vicinity of the FMN isoalloxazine ring are closely connected to the flavin redox states, which in turn can be correlated to the electron transfer properties.⁴² The current molecular dynamics methods do not yet allow simulation of protein dynamics at the time scale of IET (milliseconds), and the docked complex model²⁰ may not fully represent the potential structural rearrangements during the IET. Small redox-linked local dynamics may likely take place in NOS⁴³ such that a hydrogen bond could be formed between Tyr631–OH and the reduced FMN cofactor.

PCET plays a key role in functions of many redox enzymes⁴⁴ including cytochrome oxidase⁴⁵ and sulfite oxidase.⁴⁶ These enzymes use PCET to avoid formation of high-energy intermediates and to transfer electrons and protons over long distances. To the best of our knowledge, the present work is the first solvent isotope study of a discrete key step of NOS catalysis (*i.e.*, the NOS FMN–heme IET), which allows us to elucidate a new proton-coupled facet of the IET mechanism. PCET has been observed for other flavoproteins, and concomitant process and discrete chemical steps mechanisms have been proposed for xanthine oxidase and trimethylamine dehydrogenase, respectively.⁴⁷ The different behavior may be due to the extent of stabilization by the protein environment of the protonation state

of the reduced flavin.⁴⁷ It will be interesting to study the extent to which the NOS proteins can accommodate one or another ionization state of the hydroquinone. The present work should also inspire future studies to address the mechanism of PCET in NOS. For example, it remains an open question whether other residues participate in the proton transfer, and the transfer of a proton and an electron in NOS (Figure 6) may proceed synchronously or asynchronously.

Emerging evidence demonstrates that the large scale shuttling motion of the FMN domain between the FAD and heme domains,¹⁶ as well as the short-range sampling motions to productively dock onto the heme domain,^{29–30} enable efficient FMN–heme IET in the output state.^{48–49} These conformational changes are in part guided by complementary charged residues on the domain-domain FMN-heme interface.^{21, 30} In addition to the docking surface residues, our present study stresses the importance of protein medium of the docked FMN/heme complex in efficient FMN–heme IET. Interestingly, a conserved tryptophan nearly equidistant between the heme and FMN cofactors (human iNOS W372 in Figure 1) has been shown to facilitate the long-range electron transfer from FMN to heme in iNOS.²² Importantly, the interdomain docking site (E546, E603)⁵⁰ is not on the IET pathway (Figure 1), and it thus appears that the interdomain binding and the IET through protein medium are relatively independent. The selected mutation only affects either the interdomain docking (in E546N³⁰) or the intrinsic IET rate in the docked complex (in Y631F; this work), but not both. This has allowed us to distinguish the two effects.

In conclusion, the IET rate constant of Y631F human iNOS oxyFMN is decreased by ~ 75% (compared to the wt), showing that the tyrosine residue facilitates the IET in the docked FMN/heme complex. No deuterium isotope effect was observed for the IET kinetics of this mutant, while the wt protein possesses a solvent kinetic effect of 1.4. We also observed an appreciable change in the IET rate constant value upon changing pH. These results indicate that the FMN–heme IET is proton coupled, in which the conserved tyrosine residue plays an important role. The mechanism underlying this PCET process in NOS enzymes merits further investigations.

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Abbreviations

NO	nitric oxide
NOS	nitric oxide synthase
iNOS	inducible NOS
CaM	calmodulin
FMN	flavin mononucleotide

oxyFMN	bi-domain NOS construct in which only the heme-containing oxygenase and FMN domains along with the CaM binding region are present
FMNH[•]	FMN semiquinone
FMN_hq	FMN hydroquinone
H4B	(6R)-5,6,7,8-tetrahydrobiopterin
IET	interdomain electron transfer
SKIE	solvent kinetic isotope effect
PCET	proton coupled electron transfer

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Figure 1. Potential electron transfer pathway in a docked FMN/heme complex of human iNOS.²⁰ The conserved Tyr631 is proposed to facilitate the FMN–heme IET through the protein medium: IET presumably takes place through FMN → Tyr631 → Trp372 → heme. An equivalent Trp366 in murine iNOS has been shown to participate in the IET.²² The FMN and heme domains are shown in blue and gray line ribbons, respectively. The docking surface residues on the FMN domain (E546 and E603)⁵⁰ are colored in green, while the other residues and ligands are colored in element. The FMN and heme cofactors are shown in ball and stick.

Figure 2.

Transient traces at 580 nm obtained for the [Fe(II)-CO][FMNH^{*}] form of Y631F human iNOS oxyFMN mutant flashed by 446 nm laser. The graph is a combined plot of two traces at 0 – 0.08 s and 0 – 1.8 s using a logarithmic timescale. Solid lines correspond to the best single-exponential fit to the data: upon a laser excitation, the absorption at 580 nm decays below the pre-flash baseline (red solid line, 0 – 0.08 s), which is due to the FMN–heme IET resulting in FMNH^{*} depletion, followed by a much slower recovery toward the baseline (green solid line, 0.08 – 1.8 s), which is due to the CO rebinding to Fe(II). The 0 – 0.08 s trace is an average of seven traces, while the 0 – 1.8 s trace is of a single trace. The sample temperature was set at 21 °C. Anaerobic solutions contained 10 μM Y631F iNOS oxyFMN, ~ 20 μM 5-deazariboflavin and 5 mM fresh semicarbazide in a 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 3.

Transient trace (0 – 0.018 s) at 580 nm obtained for the [Fe(II)-CO][FMNH[•]] form of Y631F human iNOS oxyFMN protein flashed by 446 nm laser, in comparison with the trace of the wild type protein. Note that the wild type trace has become leveled within this time scale, while the Y631F mutant trace has not finished yet. The experimental conditions are the same as those in Figure 2.

Figure 4. Proton inventory plot of wild type human iNOS oxyFMN. The ratio of the IET rate constant at the indicated mole fraction of D₂O (n) to that in 100 % H₂O (k_n/k_0) is plotted versus n . $R = 0.92$ for the linear fit of the data using eq 4.

Figure 5. Transient trace at 465 nm obtained for the [Fe(II)-CO][FMNH*] form of Y631F human iNOS oxyFMN protein in 95 % D₂O buffered solution flashed by 446 nm laser. Solid line corresponds to the best single-exponential fit to the data. The sample temperature was set at 21 °C. The experimental conditions are the same as those in Figure 2.

Figure 6.

A possible PCET mechanism in which human iNOS Tyr631 acts as an intermediary proton shuttle between anionic FMN hydroquinone (FMN^{•-}) and water solvent. The radical and negative charge symbols are colored in blue.

Table 1

The FMN–heme IET rate constants k_{et} of wild type human iNOS oxyFMN construct at pH 6.7–8.7 ^a

pH	k_{et} (s ⁻¹)
6.7	338.5 ± 2.8
6.9	371.2 ± 3.1
7.2	462.5 ± 5.0
7.4	499.0 ± 4.5
7.6	390.8 ± 1.5
8.7	347.2 ± 3.2

^aThe IET rates were determined by laser flash photolysis at 21 °C; buffer: 40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 1 mM Ca²⁺, and 10 % glycerol. The rate constant values are average of results from at least two experiments.

Table 2

The FMN–heme IET rate constant k_{et} of wild type human iNOS oxyFMN protein in H₂O/D₂O buffered solutions (pH 7.6) at 21 °C, along with the ratio of the FMN–heme IET rate constant k_n obtained at a certain molar fraction of D₂O (n) to rate constant k_0 in pure H₂O buffer (k_n/k_0). The values are average of rate constants obtained from at least two experiments.

Mole fraction of D ₂ O	k_{et} (s ⁻¹)	k_n/k_0
0	345.3 ± 2.2	1
0.27	323.1 ± 4.3	0.94 ± 0.02
0.55	304.3 ± 5.2	0.88 ± 0.02
0.77	285.6 ± 2.0	0.82 ± 0.01
0.95	242.0 ± 2.4	0.70 ± 0.01