Ultrafast dynamics of ligand and substrate interaction in endothelial nitric oxide synthase under Soret excitation

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RECEIVED DATE (to be automatically inserted after your manuscript is accepted if required according to the journal that you are submitting your paper to)

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ABSTRACT. Ultrafast transient absorption spectroscopy of endothelial NOS oxygenase domain (eNOS-oxy) was performed to study dynamics of ligand or substrate interaction under Soret band excitation. Photo-excitation dissociates imidazole ligand in less than 300fs, then followed by vibrational cooling and recombination within 2 ps. Such impulsive bond breaking and late rebinding generate proteinquakes, which relaxes in several tens of picoseconds. The photo excited dynamics of eNOS-oxy with L-arginine substrate mainly occurs at the local site of heme, including ultrafast internal conversion within 400 fs, vibrational cooling, charge transfer, and complete ground-state recovery within 1.4 ps. The eNOS-oxy without additive is partially bound with water molecule, thus its photoexcited dynamics also shows ligand dissociation in less than 800 fs. Then it followed by vibrational cooling coupled with charge transfer in 4.8 ps, and recombination of ligand to distal side of heme in 12 ps.

KEYWORDS. Ultrafast spectroscopy, Nitric Oxide Synthase, Photo-dissociation, Ligand rebinding

Graphical Abstract

1. Introduction

Nitric oxide (NO) is a key signaling molecule for vasodilation and neurotransmission at low concentrations and a defensive cytotoxin at higher concentrations^{1,2}. In mammals, NO is biosynthesized with *L*-citrulline from *L*-arginine by three isoforms (neuronal, inducible, and endothelial) of nitric oxide synthases (NOS) called as nNOS, iNOS, and eNOS, respectively³. The NO produced by eNOS in the endothelial cells signalizes blood pressure control, one produced by nNOS in the brain is for nerve signal transduction⁴, and one produced by iNOS in macrophages triggers immune defense⁵. All of these three isoforms share similar biochemical composition and enzymatic characteristics. They are all homodimeric enzymes including heme, FAD, FMN and tetrahydrobiopterin (BH₄)^{6,7,8,9,10}. Comparison of sequence homology of the isoforms suggested that these isozymes possess a bidomain structure similar to that of cytochrome P450_{BM-3} being catalytically self-sufficient¹¹. In the bidomain structure, the C-terminal half (reductase domain) contains the NADPH, FAD, and FMN recognition sites. The other half, which is called the N-terminal half (oxygenase domain), contains heme¹², BH₄, and *L*-arginine binding site¹³. A Ca²⁺/CaM binding site located in the middle of the two domains¹⁴ is considered to be important in facilitating electron transfer from the reductase domain to the oxygenase domain¹⁵.

To control the density of NO via pharmacological intervention against individual isoforms, systematical and detailed understanding of the NOS reaction mechanism is essentially important. In the NOS reaction mechanism, the oxygenase domain of NOS especially plays the key role as follows. The

oxygenase domain contains the thiolate ligand in proximal side of heme, the distal heme pocket as ligand or substrate binding regions. The catalytic process of NO production occurs in the heme active site of the NOS oxygenase domain.

Because of the biological importance of the NOS, NOS and NOS-like proteins have been widely studied in various fields. Chen et al.¹⁶ has shown the spectral evidence of domain structure and catalytic activity of human eNOS from two separate domains, i.e. oxygenase domain and reductase domain. Gerber *et al.*¹⁷ have demonstrated that the heme ligand (imidazole) or substrate (L-arginine) could induce spin state change showing a clear Soret peak shift of heme in wild-type eNOS. Ligand-protein interaction in NOS has been systematically studied by the resonance Raman scattering spectroscopy¹⁸ showing that the electronic properties and the structure of the proximal iron-cysteine bond of heme in NOS system are important to assess its contribution to the catalytic function. The rapid kinetics of the intramolecular electron transfer between the FMN and heme domains in NOS system was directly determined using laser flash photolysis of CO dissociation and stopped flow system in comparative studies with millisecond time-resolution^{19,20}. Previously, the ultrafast dynamics of the NOS proteins were partially studied with picosecond time-resolution^{21,22}, which is still not enough to fully understand the mechanism of the NOS protein dynamics. Especially, elucidation of the ultrafast heme dynamics in the oxygenase domain is thought to be mostly important because the catalytic process of NO production occurs in the heme active site of the oxygenase domain. Previous studies show that the catalytic center of NOS is affected by heme ligands and substrates to modify the enzymatic activity²³.

In our presented work, the ultrafast dynamics of eNOS oxygenase domain (eNOS-oxy) have been studied by measuring transient absorption (TA) with femtosecond time resolution and estimated effect of ligand or substrate coordination to heme.

2. Experimental section

2.1 Experimental setup of TA spectroscopy

The light source of TA spectroscopy was the Ti:sapphire regenerative amplifier (Legend, Coherent Inc.), which generates NIR pulse with central wavelength of 800 nm, repetition rate of 5 kHz, spectral bandwidth of 30 nm, and pulse duration of 35 fs. The NIR pulse was frequency-doubled by a 0.5-mm-thick β -BaB₂O₄ crystal to generate UV pulse with bandwidth of 20 nm around 400 nm. The remnant NIR pulse was cut by a glass filter, which passes light with wavelength shorter than 750 nm. A beam sampler was inserted to separate the UV pulse into two copies with the power ratio of about 9:1. The UV pulse transmitted through the beam sampler with higher intensity was used as pump pulse. The UV pulse reflected on the beam sampler with lower intensity was served as probe pulse. The pump pulse was modulated by chopper rotating at 2.5 kHz which is synchronized with the 5-kHz repetition rate of the regenerative amplifier. To suppress coherent artifact in the TA signal, the polarizations of pump pulses and probe pulses were set orthogonal to each other. Both pulses were focused on the sample by an off-axis parabolic mirror with focal length of 100 mm. The transmitted probe pulse through the sample was coupled into an optical fiber and guided into a polychromator to be dispersed

with grating (150 g/mm). The dispersed spectrum of the probe pulse was detected by a fast scan-rate CCD with 256 pixel for every laser pulse. The timing of the data collection in the CCD was synchronized with the pulsed probe light source at 5-kHz repetition rate. The TA spectra can be calculated at every 0.4 ms for every two probe pulses (with pump and without pump). To enhance the signal to noise ratio, we have accumulated the TA spectra for 0.5 s (2500 shots) at each time delay and averaged the TA time trace scanning the time delay for four times. The temporal resolution in the pump-probe measurement system was estimated to be 100 fs. The pump and probe pulse energies were 45 nJ and 5 nJ, respectively. To suppress the effect of material chirp, the TA spectroscopy of the liquid sample was performed storing the sample solution in a thin glass cell with 1-mm optical path length (6210-12501, GL Sciences Inc.). All measurements were performed at room temperature of 295 K.

2.2 Preparation of eNOS oxygenase domain

2.2.1 Expression and Purification of eNOS oxygenase domain—The Sf21 cells were cotransfected with Eoxyhis1392 plasmid and linearized BaculoGold baculovirus DNA (BD Biosciences/Pharmingen) to generate recombinant viruses. Due to low heme biosynthetic capability of Sf21 cells, supplemental heme chloride (4 µg/ml) was added into the culture medium 18 h postinfection to enrich heme content of the expressed eNOS oxygenase domain. Cells were harvested at 60 h postinfection, suspended in 5 volumes of buffer A containing 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 µM leupeptin, 1 µM antipain, 1 µM pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 10% glycerol. The cell suspension was

sonicated three times for 20 s each and centrifuged twice at 30,000 x g for 20 min at 4°C. The supernatant was applied to a 5-ml column of Ni-NTA-agarose equilibrated with buffer A. The column was washed with buffer A, then with buffer A plus 5 mM imidazole. The column was eluted with buffer containing 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol plus 100 mM imidazole. The eluate was concentrated by using Centriprep-30 (Amicon) and dialyzed against buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM DTT, and 10% glycerol. The concentration of eNOS oxygenase domain was either determined by Bradford method or estimated spectroscopically using $1 A_{280\text{nm}} = 0.63 \text{ mg/ml}$. The purified enzyme preparations were stored at -80 °C.

2.2.2 Sample Measurement — Imidazole and L-arginine were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purifications. Imidazole and L-arginine were dissolved in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM DTT, and 10% glycerol. The solution was filtered through Nalgene 0.45 µm PES filter from Thermo Scientific (Waltham, MA). The final concentration of the eNOS-oxy solution was determined to be $\approx 10 \text{ µM}$ from the UV/Vis absorption spectrum in the Soret region. Imidazole and L-arginine were added to the solution to form corresponding complexes at final concentrations of 1 mM and 100 µM, respectively.

3. Results and Discussion

3.1 Stationary absorption spectra

Figure 1 shows stationary absorption spectra in Soret band region of eNOS-oxy, eNOS-oxy with addition of imidazole ligand (eNOS-oxy/Im), and eNOS-oxy with addition of L-arginine substrate (eNOS-oxy/L-Arg).

The Soret band associated with the π to π^* electronic transition of the porphyrin macrocycle is sensitive to the oxidation, coordination and spin states of the heme iron in heme protein. Ferric derivative of NOS adopts a six-coordinate low-spin (LS) state configuration, possibly coordinating a solvent water molecule at the distal binding site of the heme. Addition of BH_4 destabilizes the putative heme-bound water molecule through steric interference and structure changes to the protein matrix. Binding of L-arginine and imidazole to the eNOS was characterized by direct heme spectral perturbation²⁵. When both L-arginine and BH_4 are present with eNOS, the Soret transition peak further shifts to 395 nm, indicating a full conversion to the five-coordinated high-spin (HS) state. When imidazole is added to the eNOS, imidazole acts as a genuine heme ligand coordinating to distal side of heme iron in eNOS directly and thus the Soret transition shifts to 430 nm. The red shift represents that heme in eNOS has the six-coordinated LS. When neither of L-arginine and imidazole is added to eNOS, the Soret peak shifts to ~400 nm indicating co-existence of the six-coordinated LS and the five coordinated HS.

Our observation is consistent with the results of absorption spectroscopy of rat brain NOS and eNOS added with imidazole or L-arginine^{24,25}. According to experimental work by Maes et al.²⁶, the Soret peak of Fe(III) and Fe(II) of Nitrophorin 4 (NP4) bound with water molecule appears at 404 nm

and 421 nm, respectively. The Soret peak of Fe(III) and Fe(II) of tetraphenylporphine tetrasulfonic acid (TPPS) locates at 393 nm and 421 nm, respectively²⁷. Thus, the observed broad Soret peak of eNOS-oxy is attributed to the electronic configuration mixing in heme between ferric five-coordination HS and ferrous six-coordination LS.

3.2 Transient absorption time traces and analysis method

Figures 2, 3, and 4 show the femtosecond spectra integrated TA traces of the three samples for eNOS-oxy/Im, eNOS-oxy/*L*-Arg, and eNOS-oxy, respectively. The TA traces were measured pumping at 405 nm and probing also at 405 nm with spectra bandwidth of ~20 nm. The measured TA traces were fitted with triple-exponential decay function and the fitting results are summerized in Table 1.

The femtosecond TA traces show clear difference between the three samples. The TA signal of the eNOS-oxy and eNOS-oxy/Im is negative just after photo-excitation gradually increasing to have positive sign in a few picosecond. Meanwhile, the TA signal of eNOS-oxy/*L*-Arg exhibits only negative signal with ultrafast relaxation. Thus, the three samples have distinct excited-state dynamics and different protein responses. The following sections provide detail explantions about the difference observed in the ultrafast dynamics of these samples.

3.3 Photoexcited dynamics of eNOS-oxy bound with imidazole ligand

As shown in figure 2, the spectra integrated TA trace of eNOS-oxy/Im initially show negative

signal of photo-bleaching within time delay ~ 0.3 ps then followed by positive signal of induced absorption. The ultrafast change is caused by heme-ligand dissociation in eNOS-oxy/Im transforming heme structure from six to five coordination after photo-excitation. Just after the photo-excitation, heme in eNOS-oxy/Im has six coordination bound with imidazole. After the ultrafast heme-ligand dissociation, the sign of TA signal changes to be positive reflecting the induced absorption of the newly formed five-fold coordinated heme. The observed time scale of photo-dissociation of ligand is consistent with that reported by Negre et al.²⁸ at Q-band excitation and that by Wang et al.²⁹ at the Soret band (B-band) excitation. The heme dynamics of cytochrome c pumped at the Soret band and probed in UV and visible region was reported by Zang et al.³⁰, which indicates that ligand dissociates in less than 100 fs. Theoretical work by molecular dynamics (MD)³¹ shows that ligand dissociation occurs in ~ 200 fs. The ultrafast photo-excitation was reported to break the heme-ligand bond in less than 300 fs, which perturbs protein structure significantly forming protein-quake^{32, 33, 34} at the local heme site. The quake spreads outward to fluctuate protein conformation.

The TA trace probed at 405 nm is plotted in Fig. 2, which shows that negative TA signal rapidly decays in 0.36 ps (76.5%) by photo-dissociation of imidazole ligand from heme of eNOS-oxy/Im. Then, the positive TA signal relaxes in 1.2 ps (18.3%) and 144 ps (5.2%). The former time constant corresponds to the vibrational cooling. The latter one reflects heme-imidazole recombination and protein conformation change induced by the impulsive protein quake recovers in 144 ps. Thus, the ultrafast dynamics of eNOS-oxy/Im is summarized as follows. The photo-dissociation of

heme-imidazole is the primary reaction breaking the bond in less than 0.36 ps, and forms the vibrational hot state of the five-fold coordinated heme. Then the cascaded vibrational cooling occurs within 2 ps, accompanying heme-imidazole ligand recombination and relaxation of the local heme site within 37.5 ps (see supporting information and Fig. S2). Finally, the collective structural relaxations take 144 ps to completely dissipate energy to the heme environment and have the conformation to be fully recovered.

3.4 Photoexcited dynamics of eNOS-oxy domain with *L*-arginine substrate

The TA trace of eNOS-oxy/*L*-Arg excited at 405 nm is shown in Fig. 3. At all of the probe wavelengths, we have observed negative TA signal for eNOS-oxy/*L*-Arg being different from that for eNOS-oxy/Im. Fitting result of the TA signal estimated two lifetimes of 0.35 and 1.4 ps. A water molecule is bound on distal side of heme in stationary condition of eNOS-oxy. Meanwhile, in the stationary condition of eNOS-oxy/*L*-Arg, *L*-arginine kicks out the water molecule on distal side of heme to form five coordination. Thus, the observed ultrafast dynamics of the eNOS-oxy/*L*-Arg is not representing heme-ligand dissociation but the relaxation of the excited five-fold coordinated heme of eNOS-oxy. It should be emphasized here that the TA signal probed at 405 nm did not show relaxation later than 20 ps, which implies that slow conformation relaxation observed in >20ps for eNOS-oxy/Im does not occur in eNOS-oxy/*L*-Arg.

The ultrafast decay in 0.35 ps corresponds to the relaxation of population distributed in the higher vibrational levels to the lower levels in the Soret band electronic state *via* vibrational relaxation. The

results of time resolved resonance Raman scattering show that heme charge-transfer band is vibronically coupled to the Soret band and indicated axial iron out-of-plane displacement is primarily responsible for the structure-dynamics correlations observed in myoglobin³⁵. Moreover, the longer time constant of 1.4 ps in TA signal could be related to the porphyrin ring to iron charge transfer. It is similar with photophysical pathway arises from porphyrin ring-to-iron back charge transfer to produce a porphyrin ground state configuration a nonequilibrium iron d-orbital population³⁶.

3.5 Photoexcited dynamics of eNOS-oxy

As shown in Fig. 4, the TA signal of eNOS-oxy shows initial negative signal decaying in ~2.5 ps followed by gradual change to be positive. This trend is similar with eNOS-oxy/Im. The decay lifetime of the negative signal is similar to that of eNOS-oxy/_L-Arg. Water molecule weakly is bound in distal side of heme in eNOS-oxy results in imperfect six coordination with low spin configuration. Thus, the dynamics of eNOS-oxy can be consisted of that of eNOS-oxy/_L-Arg (five coordination) and that of eNOS-oxy/Im (six coordination). The negative TA signal of eNOS-oxy reflects photo-dissociation of water molecule, metal to porphyrin charge transfer, and vibrational cooling, all of which have been estimated to occur earlier than 2.5 ps in previous sections. Then, positive TA signal is thought to be due to water molecules rebinding to the distal side of heme occurring in a few tens of picoseconds.

The TA signal of eNOS-oxy was fitted to three exponential functions with the time constants as 0.79, 4.76, and 12.2 ps. It implies that the photo-excitation dissociate water molecule on distal side of eNOS-oxy with time constant of 0.79 ps. Then, the vibrational cooling couple with charge transfer have

occurred in 4.76 ps, and the dissociated water molecule rebinds to distal side of heme in 12.2 ps.

The photophysics of oxidized cytochrome (Cyt) c complex has been studied with femtosecond Raman and TA spectroscopy indicating that vibration relaxation of heme does occur in ~ 7 ps²⁸. The results of Ursula *et al.*³⁷ suggest that photoexcitation of the ferric heme domain of direct oxygen sensor dissociates ligand of H₂O and rebinds it in ~ 20 ps^{38,39}. Femtosecond infrared (IR) spectroscopy of deoxy-hemoglobin and deoxy-myoglobin (Mb) has shown that D₂O molecule works as heat acceptor perspective with thermal relaxation in double exponential function with a fast component of ~ 8 ps and a slow component of ~ 20 ps. Femtosecond near-IR transient absorbance study of the band III of photoexcited Mb where the longest relaxation time constant of about 6 ps was obtained and interpreted as vibrational cooling of the electronically relaxed heme⁴⁰. Previously study has shown that vibrational cooling proceeds in ferrous Cyt c with relaxation time constants ~ 3 ps, while the lifetime of ~ 6 ps was attributed to ligand rebinding²⁹. The ultrafast dynamics of eNOS-oxy obtained in the present work agrees with above mentioned previous works.

Referring the result obtained for eNOS-oxy/Im and eNOS-oxy/L-Arg, we have clarified the ultrafast dynamics of eNOS-oxy as following. The photo-dissociation of heme-H₂O is the primary reaction breaking the binding in 0.79 ps. After ligand dissociation, five-fold coordinated heme is formed in vibrational excited state, which is cooled in 4.76 ps accompanying heme-H₂O rebinding processes. The complete relaxation of the local heme site takes 12.2 ps.

4. Conclusion

We have performed TA spectroscopy of eNOS-oxy, eNOS-oxy/Im, and eNOS-oxy/L-Arg pumping and probing at Soret band with femtosecond time-resolution. The experimental result has elucidate local heme dynamics and photo-induced conformation relaxation affected by ligand or substrate as follows.

When the eNOS-oxy/Im is photo-excited, heme-imidazole bond was dissociated in 300 fs to form proteinquake and followed by a series of vibrational cooling including local structure relaxation of porphyrin in heme with 2 ps. The proteinquake at the local heme site faded away recovering heme in few tens ps. Total recovery of conformation change took 144 ps. After photo-excitation of the eNOS-oxy/L-Arg, intramolecular and subsequent intermolecular vibrational redistributions occurred in less than 400 fs. The metal to porphyrin ring charger transfer was found to take 1.4 ps.

The eNOS-oxy in stationary state is mixed state consisted of five coordination and six coordination. The observed photo-induced dynamics of eNOS-oxy could be explained as mixture of the dynamics of eNOS/L-Arg (initially 5-coordination) and the dynamics of eNOS/Im (initially 6-coordination). We have demonstrated that the photo-excitation of eNOS-oxy leads the fast (0.79 ps) photo-dissociation of water molecule ligand to create a non-equilibrium five coordinate species. The vibrational cooling coupled with charge transfer is estimated to occur in 4.76 ps accompanied with heme-H₂O rebinding process. It is followed by heme relaxation with a time constant of 12.2 ps.

In conclusion, we have reported comprehensive investigation of the ultrafast dynamics for ligand and substrate interaction in heme of eNOS-oxy domain, which is expected to give key knowledge for

future work on pharmacological intervention against individual isoforms of NOS contributing to human health.

Acknowledgement. This work was supported by ministry of science technology, Taiwan (MOST 104-2112-M-009-001-MY2) and JSPS KAKENHI Grant Number 16K17784. This work was also supported in part by Japan Science and Technology Agency (JST).

Figure Captions

Fig. 1 Stationary absorption spectra of the three eNOS-oxy samples; eNOS-oxy (black curves), eNOS-oxy/Im (blue curves), and eNOS-oxy/L-Arg (brown curves).

Fig. 2 Transient absorption trace (blue solid curves) of eNOS-oxy/Im excited and probed both at 405 nm. The fitting curve (yellow dash curves) contains three components of negative signal with time constant 0.31 ps (green dash curves), short-life positive signal with lifetime of 1.44 ps (orange dash curves), long-life positive signal decaying in 144 ps (purple dash curves). The inset shows the estimated percentage of signal amplitude for each lifetime component.

Fig. 3 Transient absorption trace (brown solid curves) of eNOS-oxy/L-Arg pumped and probed both at 405 nm. The fitting curve (yellow dash curves) contains two components plotted by green and orange dash curves representing negative signal decaying with time constant of 0.35 ps and 1.41 ps, respectively. The inset shows the percentage of signal amplitude of each lifetime component.

Fig. 4 Transient absorption trace (black solid curves) of eNOS-oxy domain excited and probed both at 405 nm. The fitting curve (yellow dash curves) consists of three components of negative signal with lifetime of 0.79 ps (green dash curves), negative signal decaying within 4.76 ps (orange dash curves), and positive signal with time constant of 12.2 ps (purple dash curves). The inset shows the percentage of signal amplitude of each lifetime component.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1.

Fitting parameters								
Sample	A ₁ (%)	τ_1 (ps)	A ₂ (%)	τ_2 (ps)	A ₃ (%)	τ_3 (ps)	A ₄ (%)	τ_4 (ps)
eNOS-oxy/Im	-76.5	0.31	-	-	18.3	1.16	5.2	144
eNOS-oxy/L-Arg	-47.6	0.36	-52.4	1.4	-	-	-	-
eNOS-oxy	-74.8	0.79	-12.4	4.76	15.3	12.2	-	-

Fitting parameters of ΔA traces of each sample at probing wavelength 405 nm

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