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

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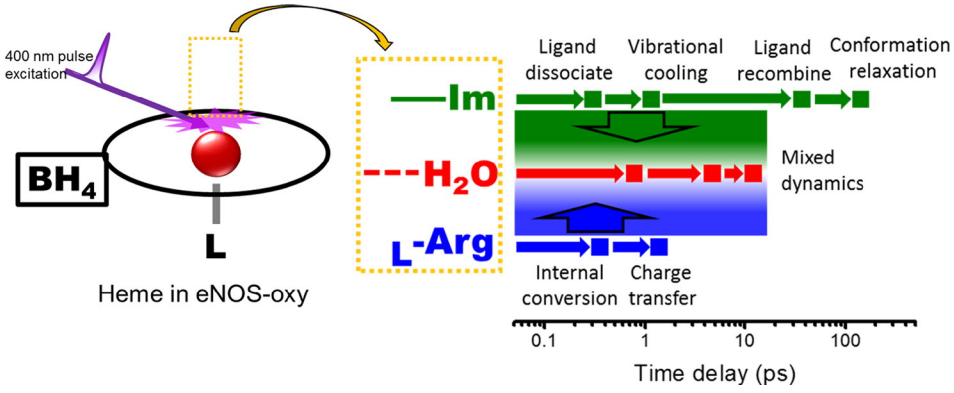
HIGHLIGHTS

- We show dynamics of ligand or substrate interaction of eNOS-oxy.
- Ligand rebinding and conformational relaxation in few-to-tens ps range was found in eNOS-oxy bound with ligand of imidazole.
- Internal conversion via vibrational cooling and charge transfer were found in eNOS-oxy interacted with substrate of L-Arg.
- The dynamics of eNOS-oxy show mixture of that bound with imidazole and that interacted with L-Arg.

GRAPHICAL ABSTRACT



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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 <300 fs, 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 <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.

1. Introduction

Nitric oxide (NO) is a key signaling molecule for vasodilation and neurotransmission at low concentrations and a defensive cytotoxin at

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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, respectively [3]. The NO produced by eNOS in the endothelial cells signalizes blood pressure control, one produced by nNOS in the brain is for nerve signal transduction [4], and one produced by iNOS in macrophages triggers immune defense [5]. All of these three isoforms share similar biochemical composition and enzymatic characteristics. They are all homodimeric enzymes including heme, FAD, FMN and tetrahydrobiopterin (BH_4) [6–10]. 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-sufficient [11]. 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 heme [12], BH_4, and L-arginine binding site [13]. A Ca^{2+}/CaM binding site located in the middle of the two domains [14] is considered to be important in facilitating electron transfer from the reductase domain to the oxygenase domain [15].

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 spectroscopy [18] 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 activity [23].

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-BaB_2O_4 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 $\times g$ 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 $A_{280\ nm} = 0.63$ 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 ≈10 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.