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Comparing the temperature dependence of FMN to heme electron transfer in full length and truncated inducible nitric oxide synthase proteins

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ABSTRACT

The FMN-heme interdomain (intraprotein) electron transfer (IET) kinetics in full length and oxygenase/FMN (oxyFMN) construct of human iNOS were determined by laser flash photolysis over the temperature range from 283 to 304 K. An appreciable increase in the rate constant value was observed with an increase in the temperature. Our previous viscosity study indicated that the IET process is conformationally gated, and Eyring equation was thus used to analyze the temperature dependence data. The obtained magnitude of activation entropy for the IET in the oxyFMN construct is only one-fifth of that for the holoenzyme. This indicates that the FMN domain in the holoenzyme needs to sample more conformations before the IET takes place, and that the FMN domain in the oxyFMN construct is better poised for efficient IET.

1. Introduction

Nitric oxide (NO) 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]. Mammalian NO synthase (NOS) catalyzes the conversion of L-arginine (Arg) to NO with NADPH and O_2 as co-substrates [3,4]. There is still much unknown about how NO production by NOS is tightly regulated [4–6]. It is of biomedical importance to study mechanisms of NOS regulation because unregulated NO production by NOS has been implicated in an increasing number of human pathologies, including cancer and ischemic injury caused by stroke [2,7].

There are three NOS isoforms in mammals: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Mammalian NOS is a homodimeric flavo-hemoprotein. Each NOS subunit comprises of an N-terminal oxygenase domain (containing a catalytic heme active site) and a C-terminal reductase domain (containing FAD and FMN cofactors), with a calmodulin (CaM) binding region between the two domains [4,5]. The substrate, L-Arg, and a cofactor, (6R)-5,6,7,8-tetrahydrobiopterin (H_4B), both bind near the heme center in the oxygenase domain. The iNOS isoform binds CaM tightly while nNOS and eNOS bind CaM reversibly in response to intracellular Ca^{2+} concentration [3,4]. The interdomain (intraprotein) electron transfer (IET) processes are key steps in NO synthesis [3,4,8,9]. Specifically, the CaM-controlled intersubunit FMN-heme IET is essential in coupling electron transfer in the reductase domain with NO synthesis in the heme domain [10]. A laser flash photolysis approach, recently developed in laboratories of Feng and Tollin [11], has been used for direct determination of kinetics of the IET between catalytically significant redox couples of FMN and heme in bi-domain oxygenase/FMN (oxyFMN) constructs [11–13] and full-length NOS enzymes [13,14].

It is generally accepted that CaM-binding has little effect on the thermodynamics of redox processes in NOS [15–17], implying dynamic CaM regulation of IET via redox-linked conformational changes. A FMN-domain tethered shuttle model (Fig. 1) was recently proposed [18] and supported by kinetics [12,14,19,20] and thermodynamic [21] studies. This model involves the swinging of the FMN domain from its original electron-accepting (input) state to a new electron-donating (output) state. The putative output state (bottom panel of Fig. 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. Truncated two-domain NOS

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible NOS; nNOS, neuronal NOS; eNOS, endothelial NOS; CaM, calmodulin; oxyFMN, bi-domain NOS construct in which only the heme-containing oxygenase and FMN domains along with the CaM binding region are present; $FMNH^{\bullet}$, FMN semiquinone; FMN_{hq}, FMN hydroquinone; FAD, flavin adenine dinucleotide; IET, intraprotein electron transfer; ET, electron transfer; k_{et}, rate constant for electron transfer; dRF, 5-deazariboflavin; H4B, (6R)-5,6,7,8-tetrahydrobiopterin

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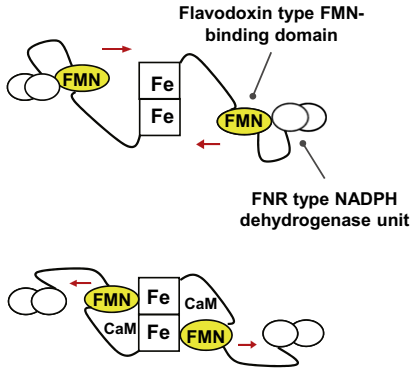

Fig. 1. Tethered shuttle model. Flavodoxin-type FMN-binding domain (yellow) shuttles between the flavodoxin-NADPH reductase (FNR)-type "dehydrogenase unit" and the heme-containing oxygenase domain. Top: input state; bottom: output state. Free FMN domain conformations also exist in between the two docked states.

oxyFMN construct is a valid model of the output state [11,12], which only consists of the heme-containing oxygenase and FMN domains, along with the CaM-binding region [22]. This construct is a minimal electron transfer complex designed to favor the interactions between the FMN and heme domains [22].

An appreciable decrease in the FMN-heme IET rate constant value of a human iNOS oxyFMN construct was observed with an increase in the solution viscosity [23]. The kinetics and NOS flavin fluorescence results indicate that the FMN-heme IET in iNOS is gated by a large conformational change, and that the docked FMN/heme state is populated transiently [23]. In the present work, we have investigated the temperature dependence of kinetics of the FMN-heme IET in truncated oxyFMN and full length human iNOS proteins. To our knowledge, this is the first temperature-dependence study of the NOS FMN-heme IET kinetics, which allows us to directly compare the temperature-dependent behavior of the IET process in the two protein systems.

2. Materials and methods

2.1. Expression and purification of human iNOS oxyFMN and holoenzyme

The full length and oxyFMN human iNOS vectors and CaM expression vector were generous gift from Dr. Guy Guillemette (University of Waterloo, Canada). The iNOS plasmid was co-transfected with CaM expression vector (p209) into *Escherichia coli* BL21(DE3) cells by electroporation (MicroPulser, Bio-Rad). The iNOS protein needs to be co-expressed with CaM *in vitro* because of its tendency to aggregate when residues of the highly hydrophobic CaM-binding region are exposed to an aqueous environment. Expression and purification of the human iNOS proteins was performed as previously described [13]. CaM binds tightly to iNOS and co-exists in the purified iNOS proteins.

2.2. Laser flash photolysis

CO photolysis experiments were conducted using 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 reactions. 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 selected wavelength (580 or 465 nm) was placed before the partially reduced protein sample to protect it from photo-bleaching and further photo-reduction by the white monitor beam [9]. The sample temperature was controlled by using a TLC 50 cuvette holder coupled with a TC 125 temperature-controller (Quantum Northwest). Dry nitrogen gas was purged over the cuvette surface to avoid moisture buildup at lower temperature.

The CO photolysis experiments were performed as previously described [11,12,14]. Briefly, a CO/Ar (v/v ~1:3) pre-degassed iNOS solution was illuminated for a certain period of time to obtain a partially reduced form of [Fe(||)-CO][FMNH·]. The sample was subsequently flashed with 446 nm laser excitation to trigger the FMN-heme IET, which can be followed by the loss of absorbance of FMNH· at 580 nm, and the loss of absorbance of Fe(II) at 465 nm [13]. All the experiments were repeated at least twice. The transient absorbance changes were averaged and analyzed using OriginPro 8.5 (OriginLab).

3. Results and discussions

3.1. The FMN-heme IET kinetics in human iNOS oxyFMN as a function of temperature

As expected, upon a 446 nm laser excitation, the absorption at 580 nm of the partially reduced human iNOS oxyFMN at 15 °C decays rapidly below the pre-flash baseline (Fig. 2), which is due to the FMN-heme IET (Eq. (1), where FMN_{hq} stands for FMN hydroquinone), resulting in FMNH· depletion [13], with a rate constant of 252.8 ± 2.2 s^{-1}.

$$[Fe^{II}][FMNH^{\cdot}] \rightleftharpoons [Fe^{III}][FMN_{hq}] \quad (1)$$

This is followed by a much slower recovery toward baseline (apparent rate constant = 2.5 ± 0.1 s^{-1}; Figure S1 in the Supporting Information), which is due to CO re-binding to Fe(II) [13]. Note the spectral "transition" (i.e., a reversal in direction of absorption changes over time) in the 580 nm traces.

The IET kinetics of the oxyFMN construct was determined over the temperature range from 283 to 304 K, and the rate constants k_{et} are listed in Table S1 in the Supporting Information. Note that an appreciable increase in the IET rate constant value was observed with an increase in the temperature. Importantly, the obtained rate constant of the rapid decay (Fig. 2) over the temperature range is

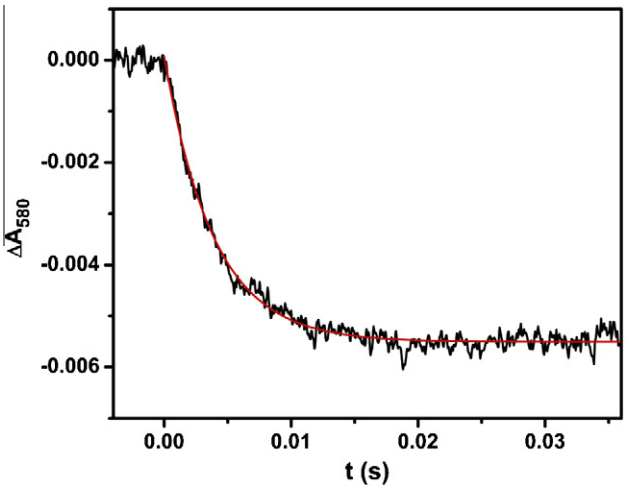

Fig. 2. Transient trace at 580 nm at 0–0.04 s obtained for [Fe(II)–CO][FMNH·] form of the human iNOS oxyFMN construct flashed by 446 nm laser excitation. The sample temperature was set at 15 °C. Red trace stands for the best fit using a single-exponential decay model. Anaerobic solutions contained 10 μM iNOS oxyFMN, ~20 μM dRF 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 H_4B, 1 mM Ca^{2+} and 10% glycerol).