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Kinetics of CO recombination to the heme in *Geobacillus stearothermophilus* nitric oxide synthase ☆

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ABSTRACT

We report the kinetics of CO rebinding to the heme in His134Ser, Ile223Val and His134Ser/Ile223Ser mutants of *Geobacillus stearothermophilus* nitric oxide synthase (gsNOS). The amplitudes of the two observed kinetics phases, which are insensitive to CO concentration, depend on enzyme concentration. We suggest that two forms of gsNOS are in equilibrium under the conditions employed (6.1–27 μM gsNOS with 20 or 100% CO atmosphere). The kinetics of CO rebinding to the heme do not depend on the identity of the NO-gate residues at positions 134 and 223.

1. Introduction

Michelle Millar made major contributions to the understanding of iron–sulfur bonding in biological molecules [1,2]. She also was interested in blue copper proteins [3], cytochromes P450 [4] and nitric oxide synthases (NOS) [5]. In this tribute to her, we report investigations of photoinitiated carbon monoxide (CO) release and rebinding to the heme in the nitric oxide synthase from *Geobacillus stearothermophilus* (gsNOS).

Nitric oxide (NO) is an important signaling molecule in all eukaryotes [6–9] and may be used by prokaryotes to combat host immune response [10–12]. The enzyme (NOS) produces NO [5,13] in a catalytic cycle in which the first turnover involves two-electron oxidation of arginine to *N*-hydroxy-L-arginine. Then, in a second turnover, a one-electron oxidation converts *N*-hydroxy-L-arginine to L-citrulline and heme-bound NO. How the protein controls the rate of NO release in the various forms of NOS enzymes is not fully understood.

The 200-fold difference in NO-release rates between bacterial and mammalian enzymes [14] is striking, particularly in view of high sequence similarities and conserved folds among NOS isoforms. Crystallographic studies suggest that Val is the first gate for NO release in mammalian enzymes (e.g. Val396 in human endothelial NOS [15])[1]; bacterial homologues have Ile resides at the analogous position (based on α-carbon overlays) [7,16]. Stuehr and coworkers showed that Val346 → Ile346 mutation in mouse inducible NOS slows NO release, while Ile224 → Val224 substitution in *Bacillus subtilus* NOS increases the reaction rate [17]. Of interest here is that smaller residues at a second gate also promote NO release: for example, neuronal NOS releases NO with a rate constant of 5 s^{-1}, with Val567at the first gate and Ser477at the second gate (rat enzyme numbering [18,19]). NOS from *Sorangium cellulosum* NOS has the fastest recorded NO release kinetics (7–10 s^{-1}), where Val1014 and Gly923 are at the two gating positions [20]. We have investigated four site-directed mutants of gsNOS that have different amino acids at the two NO-gate positions, 134 and 223 (Fig. 1): wild type (wt), I223V, H134S, and the double mutant H134S/I223V. In single-turnover experiments we found that the rate of NO release in the gsNOS double mutant (1.0 s^{-1}) approached that of fast NO releasing isoforms at 298 K [14]. We have extended our work on these isoforms to include kinetics of CO rebinding to the gsNOS heme.

2. Materials and methods

2.1. Sample preparation

The plasmid for the nitric oxide synthase from *G. stearothermophilus* was a gift from B.R. Crane (Cornell University). The enzyme was expressed as described previously [21]. Mutations were introduced using the QuikChange site-directed mutagenesis protocol from Stratagene. Primers were designed according to the guidelines outlined by the QuikChange manual. Unless otherwise noted, protein solutions were prepared using 50 mM Tris (2-amino-2-hydroxymethyl-propane-1,3-diol), pH 7.5 and 150 mM NaCl. Steady-state UV–Vis spectra were recorded on an Agilent 8453 diode array spectrophotometer.

☆ In memory of Michelle Millar.

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[1] The numbering of the NO-gate residues in the different NOS isoforms corresponds to the numbering system for each respective enzyme. UniProtKB ID: Q5KZC5, gsNOS; P29476, rat neuronal NOS; P29477, mouse inducible NOS; P29474, human endothelial NOS; A9FD96, *Sorangium cellulosum* NOS; O34453, *Bacillus subtilus* NOS.

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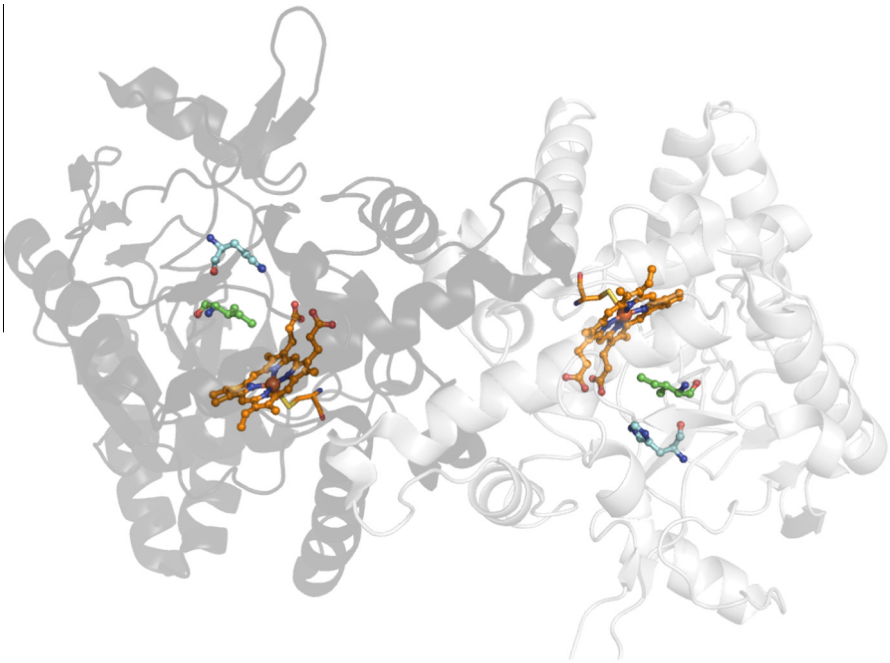

Fig. 1. Structure of wt gsNOS (PDB ID 2FLQ) viewed along the pseudo-C_2 axis. The cysteine-ligated heme is shown in orange: The first NO-gate, Ile134, is in green and the second gate, His223, is in cyan. The two molecules that comprise the dimeric structure are shown in black and gray. (color online)

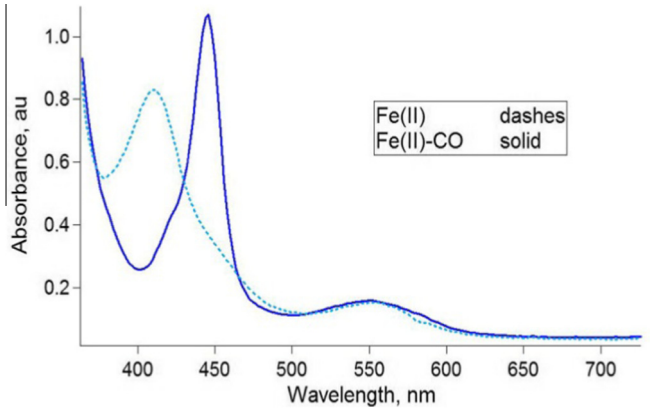

Fig. 2. Absorption spectra of Fe(II) and Fe(II)–CO forms of wt gsNOS.

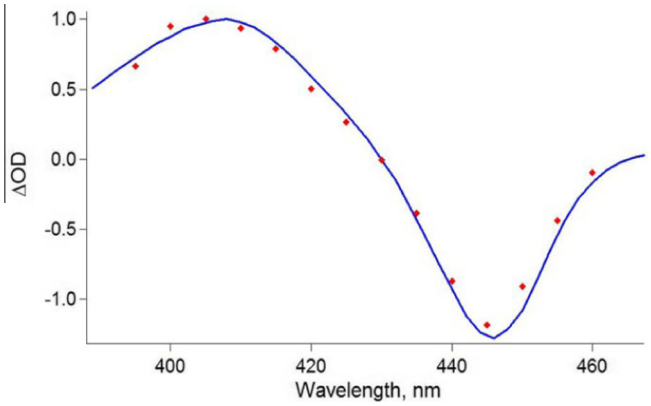

Fig. 3. Scaled steady-state difference spectrum produced by subtracting the spectrum of the wt ferrous–CO complex from the five-coordinate ferrous species (solid blue line) and the transiently generated difference spectrum (red dots, 1 ms after laser pulse, 6.1 μM wt gsNOS). (color online)

For laser experiments, oxygen-free samples were pumped into an anaerobic chamber (with an atmosphere of 100% N_2) and reduced using an excess of sodium dithionite ($Na_2S_2O_4$). A small excess $Na_2S_2O_4$ was left in samples to ensure that NOS remained in the ferrous state throughout the course of the experiment. Samples were then placed in a quartz cuvette (Starna Cells) with a graded seal connecting the cuvette to a vacuum stopcock. The cuvettes were sealed and removed from the anaerobic chamber. The side arm of the cuvette was attached to a Schlenk line, evacuated, then backfilled with carbon monoxide (100% CO or 20% CO + 80% N_2) three times. Once the side arm was under the desired atmosphere, the stopcock was opened to the side arm. The headspace of the cuvette, above the protein solution, was evacuated and back-filled with CO from the Schlenk line and sealed under this new atmosphere. The sample shielded from light and was gently shaken over night at 4 °C to allow for full equilibration of the atmosphere with the solution. Inadequate equilibration time resulted in irreproducibility between samples.

Formation of the ferrous–CO complex was confirmed using its characteristic absorption band at 446 nm [22]. The stability of the sample was monitored by UV–Vis spectroscopy after its generation, and immediately before and after laser irradiation.

2.2. Nanosecond transient absorption spectroscopy

All transient UV–Vis spectroscopic measurements were conducted at the Beckman Institute Laser Resource Center at Caltech. Excitation for time-resolved measurements (560 nm, 8-ns) was provided by an optical parametric oscillator (Spectra-Physics Quanta-Ray MOPO-700) pumped by the third-harmonic of a Q-switched Nd:YAG laser (Spectra-Physics Quanta-Ray PRO-Series). The details of this setup have been described [23]. Kinetics traces were collected at 410 and 440 nm for each protein sample; all measurements were made in duplicate.

2.3. Data analysis

Transient absorption traces were fit using Igor-Pro (WaveMetrics Inc., Lake Oswego, OR, USA) graphing software. Traces