Gating NO Release from Nitric Oxide Synthase

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Supporting Information

Sample preparation. The plasmid for the nitric oxide synthase from *Geobacillus stearothermophilus* was a gift from Brian Crane. The enzyme was expressed as described previously with no significant deviations in procedure. A QuikChange site-directed mutagenesis kit from Stratagene was used to introduce the desired mutations in the amino acid backbone. Primers were designed according to the guidelines outlined by the QuikChange kit manual. Unless otherwise noted, protein solutions were prepared using the following buffer: 50 mM Tris (2-amino-2-hydroxymethyl-propane-1,3-diol), 150 mM NaCl, pH 7.5. Steady-state UV-visible spectra were recorded on an Agilent HP 8452 diode array spectrophotometer.

Stopped Flow UV-visible Spectroscopy. Stopped flow UV-visible spectroscopy has been employed to study the kinetics of NO release. The unique spectroscopic features of heme enzymes permit the differentiation of various species during catalysis. Single turnover experiments, in which anaerobic, fully-reduced, substrate-bound enzyme is mixed rapidly with oxygenated buffer, have allowed the determination of rates of NO release in many NOS enzymes.

Samples were prepared anaerobically and transferred to an anaerobic tonometer with 1.5 equivalents of dithionite to scavenge any residual oxygen. Dithionite was used to scavenge oxygen from the stopped flow spectrophotometer (HiTech Scientific) syringes and excess dithionite was removed by repeated washing with anaerobic buffer. Protein samples (4-6 μM gsNOS, 60 μM H4B and 200 μM N-hydroxy-L-arginine) were rapidly mixed with air saturated buffer at 4 °C. The formation and release of NO was monitored using a diode array detector and the rates fit globally using SpecFit32 (HiTech Scientific).

Data Analysis. Transient absorption traces were fit using Igor-Pro graphing software. Traces monitoring the signal over time at a single wavelength were fit empirically. For stopped-flow mixing experiments, all data were fit to a double exponential decay function (as is consistent with all previously published reports of such mixing experiments with NOS, Eq. 1), with residuals less than 1% of the signal.

$$y = A_1 \times \exp(-t/\tau_1) + A_2 \times \exp(-t/\tau_2) \quad (\mathbf{1})$$

Global analysis of NO release kinetics was then performed using SpecFit32 software. This program fits data to a particular model, thus differing chemical models can be compared.