TUNING NITRIC OXIDE SYNTHASE: INVESTIGATING THE THIOLATE "PUSH" AND NO RELEASE

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ABSTRACT

All heme thiolate enzymes have conserved hydrogen bonding networks surrounding the axial thiolate ligand. In order to understand the role of this proximal hydrogen bonding network in nitric oxide synthases (NOS), three mutants of the NOS enzyme from *Geobacillus stearothermophilus* were expressed and characterized. The wild type enzyme has a tryptophan residue at position 70 that π-stacks with the porphyrin ring and donates a long hydrogen-bonding interaction to the thiolate ligand of the heme iron. The native Trp was replaced with His, Phe, and Tyr. These three residues were selected to investigate the two effects of the Trp, H-bonding and π-stacking. Several different spectroscopic techniques were used to investigate the stability and properties of these mutant enzymes. The identity of each mutant was confirmed by mass spectrometry. Both UV-visible absorption and circular dichroism spectroscopies were used to assess the stability of the new proteins. It was shown using binding assays, generation of the ferrous-CO species, and redox titrations that the σ-donating abilities of the thiolate are increased after removal of the hydrogen bonding group in the Trp. Finally, electron paramagnetic resonance spectroscopy and Evans method nuclear magnetic resonance spectroscopy were used to characterize the spin state of the iron center in each mutant, reflecting the increased σ-donating capabilities of the thiolate upon removal of the hydrogen bonding group. The reduction potential of wild type and W70H were determined by chemical titration to be -362 and -339 mV vs. NHE, respectively. This is the first report of the reduction potential of any bacterial nitric oxide synthase.

The reactivity of each the wild type enzyme and the three new mutants was tested using stopped-flow mixing coupled with UV-visible absorption spectroscopy and the

Griess Assay. Autoxidation rates measured by stopped-flow suggest that the Tyr and Phe mutants do indeed have significantly more negative reduction potentials, but that the His mutant is particularly slow to oxidize. The Griess Assays showed that all four enzymes produce nitrite in solution, when provided with substrate, cofactor and hydrogen peroxide (as a source of reducing equivalents). In single turnover experiments, however, only three of the four enzymes showed evidence of ferric-NO production. The His mutant showed no intermediate absorbance near 440 nm (which would be indicative of ferric-NO formation), suggesting that it releases NO^- rather than the radical species $NO\cdot$. The role of this hydrogen bond is concluded to be an electronic one, rather than playing any part in positioning the heme. It prevents formation of the inactive P420 species, and tunes the reduction potential to one high enough to be reduced by a reductase but low enough to still deliver an electron to the redox active cofactor, tetrahydrobiopterin, at the end of catalysis.

The rate at which NO is released by each NOS enzyme varies greatly among isoforms and species, over nearly two orders of magnitude. One residue (an isoleucine located above the heme in bacterial enzymes) involved in the gating of NO release has been previously identified by Stuehr. However, this single residue does not account for the entirety of the differences among the forms of NOS. Another residue, a histidine at position 134 in NOS from *Geobacillus stearothermophilus* (gsNOS), was hypothesized to also participate in gating NO release based on an observed correlation between rates of NO release and the bulk of side chains at this position. Each single point mutation, H134S and I223V, and the double mutant were expressed in gsNOS and their reactivity toward the diatomic molecules CO and NO were studied. CO rebinding was investigated using laser flash photolysis and NO release using stopped flow UV-visible spectroscopy. The presence

of both monomer and dimer was observed in solution, and position 134 was shown to be another key residue in gating NO release. Wild type gsNOS contains both the bulkier Ile223 and His134 and has the slowest measured NO release (0.039 s^{-1}) of all NOS enzymes. A new, more accurate kinetics model for turnover is proposed. Each single mutation increased NO release substantially, while the double mutant has a rate constant of 1.0 s^{-1}, nearly as fast as mammalian iNOS at 2.3 s^{-1}, identifying position 134 as another important factor determining rate constants for NO release.

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