Gating NO Release from Nitric Oxide Synthase

Charlotte A. Whited‡, Jeffrey J. Warren‡, Katherine D. Lavoie‡, Emily E. Weinert#, Theodor Agapie‡,*, Jay R. Winkler‡,*, and Harry B. Gray‡,*
‡Beckman Institute and Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125
#Departments of Molecular and Cell Biology and Chemistry, University of California, Berkeley, Berkeley, CA 94720-3220

Abstract

We have investigated the kinetics of NO escape from *Geobacillus stearothermophilus* nitric oxide synthase (gsNOS). Previous work has indicated that NO release was gated at position 223 in mammalian enzymes; our kinetics experiments include mutants at that position along with measurements on the wild type enzyme. Employing stopped flow UV-vis methods, reactions were triggered by mixing reduced enzyme/N-hydroxy-L-arginine complex with aerated buffer solution. NO release kinetics were obtained for wt NOS and three mutants (H134S, I223V, H134S/I223V). We have confirmed that wt gsNOS has the lowest NO release rate of known NOS enzymes, whether bacterial or mammalian. We also have found that steric clashes at positions 223 and 134 hinder NO escape, as judged by enhanced rates in the single mutants. The empirical rate of NO release from the gsNOS double mutant (H134/I223V) is nearly as rapid as that of the fastest mammalian enzymes, demonstrating that both positions 223 and 134 function as gates for escape of the product diatomic molecule.

The nitric oxide synthases (NOS) found in all eukaryotes, as well as in a selection of prokaryotes, are responsible for biological production of nitric oxide (NO). Various mammalian isoforms of NOS are involved in processes such as neurotransmission, vasodilation, and immune response. The role of NO in bacteria is still under debate, although it has been proposed to be involved in fighting host immune responses. Different functions likely require different rates of NO production in cells. These rates can be controlled by regulation of protein expression or within the enzyme itself. Our investigations have been aimed at elucidating the manner in which the enzyme regulates NO release.

Nitric oxide synthases contain a thiolate-ligated heme active site, very similar to that found in cytochromes P450. Unlike P450, which catalytically oxygenates a vast array of substrates, NOS catalyzes only one reaction, the oxidation of arginine to NO and citrulline. Conversion of L-arginine to products requires two turnovers: the first involves two-electron oxidation of substrate producing an enzyme-bound intermediate (N-hydroxy-L-arginine); the second is a three-electron oxidation forming a ferric-NO species that releases NO. It is not fully understood how the protein controls NO escape from different NOS enzymes.

Although there is high sequence similarity and a conserved overall fold in the NOS family, empirical rate constants for NO release vary by more than 2 orders of magnitude (Table 1).
One residue in particular, V223 in mammalian enzymes, is known to influence these rates.\(^8\) Stuehr and coworkers showed that substituting an isoleucine for the valine at this “first gate” in inducible NOS slows NO release, while mutating isoleucine to valine in the *Bacillus subtilus* enzyme (bsNOS) increases the rate (Ile223 in Figure 1).\(^9\) Nearly all bacterial enzymes have an Ile at the first gate (I223) and slower NO release than in mammalian forms.\(^10\)

A “second gate” candidate is H134 in *Geobacillus stearothermophilus* NOS (gsNOS), an enzyme with slow NO release (~0.04 s\(^{-1}\));\(^11\) the rate is slightly higher in the *Deinococcus radiodurans* enzyme, which has an alanine at this site.\(^12\) NO release from mammalian neuronal NOS,\(^13\) with V223 near the heme and serine at position 134, is relatively fast (5 s\(^{-1}\));\(^14\) and the corresponding reaction in the *Sorangium cellulosum* heme domain (scNOS, V223 with glycine at the putative second gate) is even faster (≥8 and ≥30 s\(^{-1}\) for tetrahydrobiopterin and tetrahydrofolate cofactors, respectively).\(^13\) The gsNOS and scNOS enzymes have the slowest and fastest release rates, respectively, for NOS enzymes. Herein we describe studies that support the proposal that both positions 134 and 223 are involved in gating NO release in *G. stearothermophilus* NOS.

Single turnover experiments where anaerobic, fully reduced, substrate-bound enzyme is mixed rapidly with oxygenated buffer, allow the determination of NO release kinetics in many NOS enzymes.\(^9a,13,15,16\) We have employed this methodology to investigate the roles of residues at positions 223 and 134 in gating NO release from gsNOS. In our work we focus on wild type, as well as single (H134S, I223V) and double (H134S/I223V) mutant enzymes.

Enzyme reactions were monitored using stopped-flow UV-vis spectroscopy, with spectra (370–710 nm) recorded at regular intervals from ms to s. The transient spectra indicate rapid formation of a ferrous-oxy species that ultimately converts to the resting ferric state of the enzyme (representative kinetics shown in Figure 2). In fitting the kinetics the first trace was discarded, owing to the presence of excess reductant; the remaining traces were subjected to global kinetics analysis using SpecFit\(^{TM}\) software.\(^17\) All four proteins exhibit biexponential kinetics. Empirical rate constants for the second phase (\(k_2\)), corresponding to production of the ferric enzyme, are presented in Table 1.

The resting ferric state reforms slowest in wt gsNOS; enzymes with single mutations are four- to seven-fold faster; and ferric formation in the double mutant is twenty-five times faster than that of the wt enzyme. NO release kinetics for several other NOS enzymes from difference sources have been fit to a three-state kinetics model (Scheme 1).\(^9\) The spectra of the ferrous-oxy (A) and ferric (C) forms can be determined independently, leaving two rate constants and the spectrum of the Fe(III)-NO intermediate (B) to be extracted from the data.\(^13,15\) For the faster mutants, I223V and H124S/I223V, global analysis using the three-state model produces an Fe(III)-NO spectrum that is consistent with those reported previously (Figure 3). In the context of Scheme 1, then, the rate constants reported in Table 1 for I223V and H124S/I223V gsNOS correspond to the NO release step (B→C).

The intermediate spectrum extracted from the kinetics data of wt or H134S gsNOS, however, exhibits multiple peaks in the Soret region (Figure 4), indicating the presence of more than one intermediate prior to formation of ferric NOS. Sudhamsu and Crane found evidence for similar intermediates in manual-mixing experiments with wt gsNOS under single turnover conditions.\(^11\) One of the maxima in the intermediate spectrum appears at 440 nm, consistent with partial formation of a ferric-NO species. These multi-peak Soret spectra also are reminiscent of the intermediate spectrum reported\(^9a\) for a V436I mutant of iNOSox. The presence of three Soret maxima in the wt and H134S gsNOS intermediate
spectra suggests that three rapidly equilibrating species likely are involved and that Scheme 1 does not adequately describe the ferric NOS formation mechanism in the slow NO-release limit. In the wt gsNOS and H134S enzymes, the empirical rate constants for ferric formation need not correspond simply to the NO release step.

The NO release rate of wt gsNOS is slow enough that other intermediate species are able to build up, but what are they? As noted above, one of the species must be ferric-NO. The other intermediates have apparent Soret maxima near 420 and 400 nm (Figure 4). Sudhamsu and Crane suggest that the peak at 400 nm in the intermediate spectrum arises from the ferric enzyme and that a feature at 423 nm can be attributed to the Fe(II)-(O$_2$) species.\textsuperscript{11} The greater time resolution afforded by our stopped-flow measurements provides additional insight. If the intermediate species absorbing at 400 nm is the ferric enzyme, then it is likely formed in a pathway parallel to that of the Fe(III)-NO species, possibly by superoxide dissociation from Fe(II)-(O$_2$). And, if the peak at 423 nm arises from Fe(II)-(O$_2$), then homogeneity of the sample is open to question. Alternatively, owing to the slow release of NO, the 400-nm Soret peak could arise from a ferric heme with NO trapped nearby; this species would likely have a Soret maximum similar to that of ferric NOS alone. In 2008 Steur observed an intermediate with $\lambda_{\text{max}}=422$ nm for a slow NO releasing iNOSoxy where the proximal Trp is replaced with His, and proposed that this intermediate was an iron-oxy species.\textsuperscript{18} This proposal requires the unlikely prospect of rapid (relative to NO dissociation) equilibrium between iron-oxy and Fe(III)-NO complexes,\textsuperscript{19} so a more likely explanation is that the sample is heterogeneous. The species at ~420 nm is probably not the recently reported ferrous-NO complex, which has a maximum a 433 nm.\textsuperscript{20} Finally, we cannot preclude interactions with the citrulline product. In any case, the observation of these different intermediates indicates that NO formation and release in nitric oxide synthases requires more steps than depicted in Scheme 1.

In sum, our kinetics studies of wt and mutant gsNOS are consistent with the presence of two gating sites (residues 124 and 223) for the release of NO from the enzyme. The data suggest further that these two positions play similar roles in regulating NO release kinetics. Indeed, analysis of the crystal structure of gsNOS reveals that the bulky side chains at these positions, His and Ile, respectively, extend well into the proposed NO release channel (Figure 1). Mutations at these two positions (H134S/I223V) produce a NOS that releases NO 25 times faster than wt, and almost as rapidly as fast NO releasing mammalian enzymes.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**REFERENCES**


Figure 1.
Active site of gsNOS (PDB code 2FLQ) including the proposed substrate and diatomic access channel. The heme, arginine substrate (Arg), and two gating side chains (His134 and Ile223) are depicted along with the surrounding protein surface.
Figure 2.
Absorption spectra obtained after mixing 4.4 µM reduced gsNOS (60 µM tetrahydrobiopterin and 200 µM N-hydroxy-L-arginine) with air-saturated buffer at 4 °C. Green arrows indicate formation of the spectrum of the intermediate; blue arrows indicate conversion to the spectrum of the ferric resting state. Red trace: ferrous NOS; green trace: ferrous-oxy NOS; blue trace: ferric NOS (resting).
Figure 3.
Spectra of intermediates generated from global fits for H134S/I223V gsNOS. Conditions: 50 mM Tris, 150 mM NaCl, pH 7.5, 4.4 µM NOS, 60 µM tetrahydrobiopterin, 200 µM N-hydroxy-L-arginine, ~130 µM oxygen: ferric-NO, green; ferrous-ox, yellow; ferric resting state, purple.
Figure 4.
Spectra of intermediates generated from global fits for wild type gsNOS using a biexponential kinetics model. Conditions: 50 mM Tris, 150 mM NaCl, pH 7.5, 4.4 µM NOS, 60 µM tetrahydrobiopterin, 200 µM N-hydroxy-L-arginine, ~130 µM oxygen.
Scheme 1.
Three-state model for the conversion of Fe\textsuperscript{II}-NOS in the presence of O\textsubscript{2}, cofactor, and substrate to the resting Fe\textsuperscript{III} state.
### Table 1

NO release rate constants for four gsNOS and three other NOS enzymes.\(^a\)

<table>
<thead>
<tr>
<th>Mutations</th>
<th>(k_2) (s(^{-1}))</th>
<th>Gate 1(^b)</th>
<th>Gate 2(^b)</th>
<th>Temp. (°C)</th>
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<td>Ser</td>
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<td>this work</td>
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<tr>
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<td>9a</td>
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<tr>
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<td>&gt;8 (&gt;30)(^d)</td>
<td>Val</td>
<td>Gly</td>
<td>10</td>
<td>13</td>
</tr>
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</table>

\(^a\) Rate constants were determined by fitting data to a double exponential function.

\(^b\) Gate 1 and Gate 2 correspond to the residues at positions 223 and 134, respectively.

\(^c\) \(k_2\) typical for mammalian enzymes.

\(^d\) \(k_2\) typical for bacterial enzymes.

\(^d\) Rate depends on the cofactor.