A Tryptophan that Modulates Tetrahydrobiopterin-Dependent Electron Transfer in Nitric Oxide Synthase Regulates Enzyme Catalysis by Additional Mechanisms†

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ABSTRACT: Nitric oxide synthases (NOSs) are flavo-heme enzymes that require (6R)-tetrahydrobiopterin (H4B) for activity. Our single-catalytic turnover study with the inducible NOS oxygenase domain showed that a conserved Trp that interacts with H4B (Trp457 in mouse inducible NOS) regulates the kinetics of electron transfer between H4B and an enzyme heme—dioxy intermediate, and this in turn alters the kinetics and extent of Arg hydroxylation [Wang, Z.-Q., et al. (2001) Biochemistry 40, 12819–12825]. To investigate the impact of these effects on NADPH-driven NO synthesis by NOS, we generated and characterized the W457A mutant of inducible NOS and the corresponding W678A and W678F mutants of neuronal NOS. Mutant defects in protein solubility and dimerization were overcome by purifying them in the presence of sufficient Arg and H4B, enabling us to study their physical and catalytic profiles. Optical spectra of the ferric, ferrous, heme—dioxy, ferrous—NO, ferric—NO, and ferrous—CO forms of each mutant were similar to that of the wild type. However, the mutants had higher apparent Kₘ values for H4B and in one mutant for Arg (W457A). They all had lower NO synthesis activities, uncoupled NADPH consumption, and a slower and less prominent buildup of enzyme heme—NO complex during steady-state catalysis. Further analyses showed the mutants had normal or near-normal heme midpoint potential and heme—NO complex reactivity with O₂, but had somewhat slower ferric heme reduction rates and markedly slower reactivities of their heme—dioxy intermediate. We conclude that the conserved Trp (1) has similar roles in two different NOS isozymes and (2) regulates delivery of both electrons required for O₂ activation (i.e., kinetics of ferric heme reduction by the NOS flavoprotein domain and reduction of the heme—dioxy intermediate by H4B). However, its regulation of H4B electron transfer is most important because this ensures efficient coupling of NADPH oxidation and NO synthesis by NOS.

Nitric oxide synthases (NOSs) are flavo-heme enzymes that catalyze a stepwise oxidation of l-arginine (Arg) to form nitric oxide and l-citrulline (1–3). In the first reaction, Arg is hydroxylated to form N⁶-hydroxy-l-arginine (NOHA), and in the second reaction, the NOHA intermediate is oxidized to form NO and citrulline (4). Both steps consume one molecule of O₂ and utilize NADPH-derived reducing equivalents. Three mammalian NOS isozymes have been characterized: neuronal NOS (nNOS, type I), mouse macrophage inducible NOS (iNOS, type II), and endothelial NOS (eNOS, type III) (5–7). Each NOS is only active as a homodimer (8, 9). Their subunits are comprised of an N-terminal oxygenase domain, a C-terminal flavoprotein domain, and a central calmodulin binding motif (9–11). The oxygenase domain binds Fe—protoporphyrin IX (heme), the substrate Arg, and the essential cofactor (6R)-tetrahydrobiopterin (H4B) (12, 13), while the flavoprotein domain binds FAD, FMN, and NADPH (14–16). During catalysis, the flavoprotein domain provides electrons to the heme in the NOS oxygenase domain, which enables the heme to bind O₂ and initiate oxygen activation in both reactions of NO synthesis (17–19).

H4B appears to have novel structural, redox, and electronic roles in NOS (12, 18, 20–23). For example, combined stopped-flow, rapid-quench, and rapid-freeze kinetic studies have demonstrated that H4B transfers an electron to a heme—dioxy intermediate (Fe⁶O₂) that is formed when O₂ binds to the ferrous heme in both reactions of NO synthesis (17, 18, 24). This is an important function for H4B because the electron it provides enables the creation of the heme-based oxidants that react with Arg or NOHA (Scheme 1). Crystal structures of iNOS, nNOS, and eNOS oxygenase domain dimers (i.e., iNOSOxy) (12, 13, 25) showed that H4B binds near the heme such that its 2-amino-4-hydroxypyrimidine
that slowing H₄B electron transfer might cause NO synthesis is primarily due to combined kinetic defects in electron transfer from H₄B and from the flavoprotein domain.

Figure 1: Residues whose side chains interact with the ring of H₄B. Green residues are provided by the same iNOS Ox subunit in which the H₄B is bound, and yellow residues are provided by the partner subunit of the dimer. Bound heme and Arg are indicated. This structure is based on the crystal structure of the mouse iNOS Ox dimer [PDB entry 1NOD (12)].

EXPERIMENTAL PROCEDURES

Materials. All reagents and materials were obtained from Sigma, Aldrich, Alexis, or sources described previously (28, 30, 31).

Mutagenesis. Site-directed mutagenesis were performed on the rat nNOSox domain (amino acids 1–720), full-length rat nNOS (amino acids 1–1570), and mouse Δ65NOS (amino acids 65–1269) using the mutagenesis kit from Stratagene. Mutation bases (bold and underlined) and a silent restriction site (italic) were introduced into the primers: W678A, 5′ ACT GGG TGG CGA TTG TGC CTC CCA TGT CGG GAT CCA CCC CTG TCT TCC 3′; W678AR, 5′ GGA AGA CAG GGG TGA TGG ATC CCG ACA TGG GAG GCA CAA TCG CCC AGT 3′; W678F, 5′ ACT GGG TGT TCA TTG TGC CTC CCA TGT CGG GAT CCA TCC CTG TCT TCC 3′; W678FR, 5′ GGA AGA CAG GGG TGA TGG ATC CCG ACA TGG GAG GCA CAA TGA ACA CCC AGT 3′; W457A, 5′ GAA TGA GGA CGC CCG TGG AGG CTG CCC GGC AGA ATG GAT CCG GCT GGT CCC AGC 3′; W457FR, 5′ GGT TGC CTC CCA TGT CGG TCC AG 3′; and W457AR, 5′ ACA TGG GAG GCA CAA TC TGT CG 3′.

DNA isolation, restriction enzyme digestion, and transformation were carried out using standard protocols (32).

Protein Expression and Purification. Wild-type NOS enzymes and mutants contained a six-histidine tag and were overexpressed in Escherichia coli BL21 using the pCWori vector and purified as reported previously (20, 28, 33). NOS concentrations were determined from the 444 nm absorbance of the ferrous–CO complex, using an extinction coefficient of 76 mM⁻¹ cm⁻¹ (34).

Imidazole and Arg Binding Affinities. The binding affinity of Arg was measured by perturbation difference spectroscopy based on methods reported previously (28, 35). In general, enzymes were incubated with H₄B overnight at 4 °C, and then known amounts of imidazole were gradually added to the diluted protein solution in cuvettes at 15 °C. Spectra were taken when equilibrium was reached after each addition. Plots of the peak to trough absorbance difference versus the concentration of Arg was measured by perturbation difference spectroscopy (36).

Measurement of Apparent Kₘ Values for Arg or H₄B. Apparent Kₘ and Vₘₐₓ values were determined by fitting plots of the NO synthesis activity versus Arg or H₄B concentration. Each protein sample was incubated with Arg and H₄B for 30 min at room temperature before the assay. In the case of Kₘ measurements for Arg, mutant enzymes that were purified in the presence of Arg and H₄B were rapidly passed through a PD-10 desalting column to remove Arg from the enzyme and then immediately incubated with 200 μM H₄B and the indicated concentrations of Arg in the assay cuvette prior to initiation of the NO synthesis assay.

Dimerization Assay. Protein stock solutions were incubated overnight at 4 °C in the presence of 1 mM H₄B and 10 mM Arg. The dimer:monomer content of the proteins was estimated by chromatography on an Amersham Pharmacia Biotech Superdex-200 HR size-exclusion column.
equilibrated with 40 mM EPPS buffer (pH 7.6), containing 10% glycerol, 250 mM NaCl, and 3 mM DTT. The dimer: monomer content was derived from measuring the integrated area of the corresponding elution peaks determined at 280 nm. Molecular weights of the protein peaks were estimated relative to protein molecular weight standards (35, 36).

**NO Synthesis and NADPH Oxidation Rates.** Steady-state rates of NO synthesis were determined by the spectrophotometric oxyhemoglobin assay using a difference extinction coefficient of 38 mM\(^{-1}\) cm\(^{-1}\) for the oxyhemoglobin to methemoglobin transition at 401 nm (37). Sample solutions contained 200 \(\mu M\) H\(_2\)B, 10 mM Arg, 0.6 mM DTT, 0.1 mg/mL BSA, 10 units/mL SOD, 346 units/mL catalase, 10 \(\mu M\) oxyhemoglobin, 4 \(\mu M\) FMN and FAD, and 0.1 \(\mu M\) NOS. For nNOS, 0.60 mM EDTA, 0.83 mM CaCl\(_2\), and 12.5 \(\mu g\)/mL CaM were also added in the solution. Reactions were initiated by adding NADPH (final concentration of 100 \(\mu M\)), and the absorbance change at 401 nm was recorded at room temperature or 10 °C. For the NADPH oxidation rate measurements, we omitted oxyhemoglobin and used an extinction coefficient of 6.22 mM\(^{-1}\) cm\(^{-1}\) at 340 nm. In some cases, Arg was also omitted as explained in the text.

**Formation of the Heme—Nitrosyl Complex during NO Synthesis.** Aerated solutions containing 400 \(\mu M\) H\(_2\)B, 5 mM Arg, 1.2 mM DTT, 43 units/mL SOD, 3.5 units/mL catalase, and \(\sim 0.5–2 \mu M\) iNOS enzymes were rapidly mixed with an aerated solution of 130 \(\mu M\) NADPH at 10 °C in a stopped-flow spectrophotometer (Hi-Tech Ltd.). Spectral changes were recorded using a diode array instrument (Hi-Tech Ltd.). The absorbance change at 436 nm was used to follow formation of the heme—NO complex, and the absorbance change at 340 nm was used to follow NADPH oxidation in the reactions (37, 38). The concentration of the ferrous heme—NO complex formed during the reactions was estimated from the absorbance change at 436 nm using an extinction coefficient of 48 200 M\(^{-1}\) cm\(^{-1}\) (31). At least 10 individual mixing experiments were averaged for each experiment to improve the signal-to-noise ratio. For experiments using nNOS and its mutants, 0.25 mM EDTA, 20 \(\mu M\) CaM, and 150 \(\mu M\) NADPH were also present in the solutions that contained 3.5–5 \(\mu M\) iNOS prior to mixing. The nNOS reactions were initiated by rapidly mixing the enzyme solution with an aerated buffer solution containing 2.83 mM CaCl\(_2\).

**Rates of Heme and Flavin Reduction.** The kinetics of ferric heme reduction and flavin reduction were determined as described previously (39). Reactions were carried out at 10 °C in a stopped-flow spectrophotometer and were initiated by rapidly mixing an anaerobic buffered solution containing NOS enzyme, Arg, and H\(_2\)B with an anaerobic solution containing excess NADPH and a saturating concentration of CO. For nNOS, the reaction mixtures also contained CaM, Ca\(^{2+}\), and EDTA at the concentrations indicated above. Heme reduction was followed by the absorbance increase at 444 nm due to formation of the ferrous—CO complex, while flavin reduction was followed from the absorbance decrease at 485 nm under the same conditions.

**Kinetics of Ferrous Heme—NO Complex Formation and Oxidation.** For the formation measurements, wild-type nNOSoxy and mutant proteins were diluted in 40 mM EPPS buffer (pH 7.6) that contained 40 mM Arg and 400 \(\mu M\) H\(_2\)B, were made anaerobic, and were then reduced with dithionite in an anaerobic optical cell (18, 40). The ferrous protein solutions were transferred to the stopped-flow instrument using a gastight syringe and were then rapidly mixed at 10 °C with an anaerobic buffered solution containing 10 \(\mu M\) NO. Sequential spectral scans were collected and fit to an A \(\rightarrow\) B reaction model using a Specfit global analysis program (Hi-Tech Ltd.). Eight to 10 scans were analyzed and then averaged for each experimental condition.

For the oxidation measurements, the procedure for forming ferrous nNOSoxy enzymes was identical to that described above. The ferrous proteins were then titrated by adding small amounts of an anaerobic NO-saturated buffer to form the ferrous NO complex, which was then transferred to the stopped-flow instrument and rapidly mixed at 10 °C with air-saturated buffer (41). Sequential spectral scans were collected and fit to an A \(\rightarrow\) B reaction model using the Specfit global analysis program. Eight to 10 scans were analyzed and then averaged for each experimental condition.

**Arg Hydroxylation Single-Turnover Reactions.** Single-turnover reactions to study Arg hydroxylation by the nNOSoxy enzymes were carried out in the stopped-flow spectrophotometer at 10 °C as reported previously (30), in this case using nNOSoxy enzymes in the presence of 40 mM Arg and 400 \(\mu M\) H\(_2\)B.

**Kinetics of H\(_2\)B Radical Formation During Arg Hydroxylation.** Single-turnover Arg hydroxylation reactions were carried out as described previously (30). Briefly, an anaerobic buffer solution containing substrate, H\(_2\)B, and \(\sim 400 \mu M\) ferrous W678F nNOSoxy protein was rapidly mixed at 10 °C with equal amount of O\(_2\)-saturated buffer in a Hi-Tech rapid-quench instrument that was modified for rapid-freezing sample collection. The mixture was aged for various times, and then ejected into a rapid freezing solution housed in a bath apparatus from Update Instruments, Inc. (Madison, WI). The samples were then kept in liquid nitrogen until measurement. The EPR spectra of the frozen samples were recorded in a Bruker ER300 spectrometer equipped with an ER 035 NMR gauss meter and a Hewlett-Packard 5352B microwave power controller. Temperature control was achieved using Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and ITC4 temperature controller. All spectra were recorded at 150 K using a microwave power of 2 mW, a frequency of 9.5 GHz, a modulation amplitude of 10 G, and a modulation frequency of 100 kHz.

**Midpoint Potential Measurement of W457A iNOSoxy.** The heme midpoint potential measurement was performed as previously described with some modifications (42). Spectroelectrochemical titrations were carried out at room temperature in the presence of 40 \(\mu M\) neutral red, 20 \(\mu M\) phenosafranin, and 100 \(\mu M\) methyl viologen. The W457A iNOSoxy (10 \(\mu M\)) was dissolved in a 40 mM phosphate buffer (pH 7.0) that included 200 \(\mu M\) H\(_2\)B and 4 mM Arg. Protein solutions and the working electrode (Au) were made anaerobic by alternating cycles of evacuation and flushing with nitrogen. The anodic reference electrode [AgCl(s)/Ag(s)/Cl(aq)] and auxiliary electrode [Ag(s)/KCl(aq)] were promptly put into the cell under N\(_2\) pressure. Reductive titrations were performed by gradually adding small amounts of current to the system using a Radiometer PGP201 potentiostat/galvanostat. Spectra were then recorded when the potential in the system stabilized after each addition. The midpoint potential was determined using the absorbance
chages at 400 and 645 nm, which indicate the ferric to ferrous heme transition. At these wavelengths, changes due to the dye mediators are negligible. The midpoint potential was calculated according to the Nernst equation and is reported relative to the standard hydrogen electrode.

RESULTS

Spectral Properties, Stability, and Dimer Content of Mutants. The W678A and W678F nNOS proteins and W457A iNOS displayed Soret absorbance peaks at 418 nm when purified in the absence of H_{4}B and Arg, indicating that they contained low-spin heme iron as found in wild-type nNOS and W457A iNOS when they are purified under similar conditions (28, 35, 43). When our mutants were purified in the presence of H_{4}B and Arg, they displayed Soret peak maxima at 398–400 nm, indicating that Arg and H_{4}B could bind and stabilize the heme iron in a five-coordinate high-spin state as in wild-type NOS (Figure 2). Addition of dithionite to the mutants generated ferrous–CO complexes that had maximal Soret absorbance at 444 nm (Figure 2), similar to that of the wild type. These spectral properties demonstrate that the mutations do not significantly perturb the electronic properties of the heme or the spectral response to Arg and H_{4}B binding in nNOS or iNOS. This finding agrees well with crystal structures of iNOSoxy mutants W457A and W457F that show there are very limited structural changes compared to wild-type iNOSoxy (27).

The Trp678 nNOS mutants purified without Arg and H_{4}B were less stable in solution than the wild type and often precipitated during incubation at 15 °C. Adding NaCl increased their solution stabilities somewhat. These proteins also had difficulty binding Arg or H_{4}B, because incubating the mutants with either Arg or H_{4}B at 4 °C overnight did not cause a high-spin shift in the heme iron spin state that is characteristic of their binding (data not shown). However, we did observe the spin-state shift if the enzymes were incubated with H_{4}B and Arg, consistent with cooperative binding of these two molecules by NOS (3, 35). However, even under this condition, their binding was slow, as judged by the spin-state shift, taking 1 h to be completed at 15 °C and ~25 min to be completed at room temperature when the mutants were incubated with 100 μM H_{4}B and 10 mM Arg (data not shown).

The dimer:monomer ratio of each Trp678 mutant was estimated by gel filtration chromatography (28, 35). W678A nNOSoxy and W678F nNOSoxy that were purified in the absence of H_{4}B and Arg were approximately 45 and 55% dimeric, respectively, and preincubating either protein with Arg and H_{4}B alone or together increased their percentage of dimer by only 10%. In contrast, full-length nNOS Trp678 mutants that were purified with H_{4}B and Arg were approximately 95% dimeric (data not shown). Because of these properties, we henceforth utilized full-length and NOSoxy proteins that were purified in the presence of Arg and H_{4}B for our studies, unless noted otherwise.

Binding Affinity and Apparent K_{m} Values for H_{4}B and Arg. We attempted to determine K_{m} values for Arg by a method that relies on spectral change associated with Arg displacing imidazole that is bound to the NOS ferric heme (28, 44, 45). The K_{m} values for imidazole binding to wild-type nNOS and the W678A and W678F mutants were 200 (46), 102, and 93 μM, respectively, indicating similar affinities for imidazole. Unfortunately, during the subsequent Arg titrations, we observed only a partial displacement of bound imidazole in the mutants (data not shown), and consequently could not derive the Arg K_{m} values by this method. A similar situation was previously observed for the W457A and W457F iNOSoxy mutants (28). This suggests that the Trp mutations reduce the capacity of Arg to displace heme-bound imidazole in both nNOS and iNOS enzymes.

We then ran NO synthesis assays for each mutant to determine their apparent K_{m} values for H_{4}B and Arg. Because the mutant enzymes were purified in the presence of Arg and H_{4}B, they were buffer exchanged and then preincubated with the indicated concentrations of H_{4}B and Arg before the assay was carried out. Figure 3 shows how the NO synthesis activity of W457A iNOS increased as a function of preincubation time with 200 μM H_{4}B and 10 mM Arg at room temperature. Maximal activity required a 25 min preincubation period, so we routinely preincubated our mutant enzyme samples for 30 min at each concentration of H_{4}B or Arg prior to assaying for the NO synthesis activity. In Figure 4, the right panels are graphs of NO synthesis activity versus H_{4}B concentration for each of the Trp678 nNOS and Trp457 iNOS mutants, which were used to derive apparent K_{m} values for H_{4}B (Table 1). The left panels of Figure 4 contain graphs of NO synthesis versus Arg concentration for the same mutants and were used to derive apparent K_{m} values for Arg (Table 1). The apparent K_{m} values of W457A iNOS for Arg...
or H_2B are both ~60–100 times greater than in wild-type iNOS. In contrast, the apparent K_m values for Arg in the W678F and W678A nNOS mutants are similar to that of wild-type nNOS, while their apparent K_m values for H_2B are 125 and 570 times greater than the wild-type value, respectively.

**NO Synthesis and NADPH Oxidation.** We next measured the rates of NO synthesis and NADPH oxidation at 25 and 10 °C. The measures were taken in the presence of H_2B and in the presence or absence of Arg. Enzymes were preincubated with H_2B and with or without Arg prior to the assay as required. Results are summarized in Table 2. Mutant NO synthesis activities determined at 25 or 10 °C were lower than in the wild-type enzymes. However, their concurrent rates of NADPH oxidation were either reduced to a lesser degree (W678A) or not reduced at all (W678F nNOS). Both the Ala and Phe mutants had decreased rates of NADPH consumption in the absence of Arg compared to the wild type.

NO synthesis from Arg displays a minimum stoichiometry of 1.5 NADPH molecules oxidized per NO molecule formed (10, 47). This minimum value was observed for wild-type iNOS at 25 °C, while W457A iNOS had a value of 7. Similarly, we obtained values of 2, 8, and 16 for wild-type nNOS and its W678F and W678A mutants, respectively (Table 2). This indicates that a majority of the NADPH oxidation by the iNOS and nNOS Trp mutants is uncoupled from their NO synthesis.

**Kinetics of Flavin and Heme Reduction.** We measured rates of NADPH-dependent flavin and heme reduction at 10 °C in a stopped-flow spectrophotometer. Enzymes were mixed with excess NADPH under anaerobic conditions, and for heme reduction, the buffer included CO at a saturating concentration (48, 49). The flavin reduction rates in the mutants were very similar to those of the wild-type enzymes (Table 3), consistent with the Trp mutations being located in the oxygenase domain and not affecting the flavoprotein domain. Figure 5 contains representative absorbance traces at 444 nm that indicate the ferric heme reduction rates in wild-type nNOS, iNOS, and the three mutants. The initial absorbance decrease that is present in some of the traces is due to flavin reduction that always takes place before electrons can transfer to the ferric heme (31, 37). The heme reduction rates calculated from the data are listed in Table 3. Heme reduction was monophasic in nNOS, iNOS, and W678F nNOS. However, the rate in W678F nNOS was ~3 orders of magnitude slower than in wild-type nNOS. In contrast, heme reduction was biphasic in the W457A iNOS and W678A nNOS mutants, with the fast phase being a minor component of the total absorbance change in both cases (from 19 to 33%). The fast phase rate in W678A nNOS was 8 times slower than in the wild type, whereas in W457A iNOS, the fast phase was equivalent to that of the wild type. The slow phase in either Ala mutant was ~3 orders of magnitude slower than in the wild-type enzymes. To test if the slow rates of heme reduction were caused by a hindrance to CO binding, we measured rates of CO binding to the prereduced ferrous mutants. These rates were much faster than the aforementioned heme reduction rates (for example, the observed rate of CO binding by ferrous W457A iNOS was 25.2 s^{-1}), and thus indicated no hindrance toward CO binding in the mutants.

**Heme Midpoint Potential.** We determined the heme midpoint potential in W457A iNOSoxy to determine if the Trp mutation influences this thermodynamic parameter. A spectroelectrochemical reductive titration was performed for the ferric enzyme in the presence of Arg, H_2B, and mediator dyes (42, 50). Representative data are shown in Figure 6. On this basis, the heme midpoint potential was estimated to be -271 ± 3 mV in W457A iNOSoxy, which is ~10–20 mV more negative than the heme midpoint values for wild-type iNOSoxy when measured under similar conditions [-263 mV (50)] (-253 mV).^2^

**Steady-State Enzyme Distribution.** Light absorbance spectra of the nNOS enzymes were recorded before and during NO synthesis to see if the Trp678 mutations might alter the amount of heme—NO complex that builds up during catalysis. The three panels in Figure 7 contain spectra recorded for each enzyme prior to NADPH addition (dotted line), after NADPH addition to reduce the enzyme flavins (dashed line), and after addition of Ca^{2+} to trigger CaM binding, ferric heme reduction, and NO synthesis (solid line). NADPH addition caused the flavins to become reduced as indicated by an absorbance decrease between 400 and 550 nm for each enzyme. During NO synthesis, there was a smaller buildup of six-coordinate ferrous heme—NO species in the W678A and W678F nNOS mutants compared to wild-type nNOS, as judged by their having smaller 437 nm peaks and a greater degree of ferric heme absorbance at 650 nm in the steady state. The amount of enzyme present as a six-coordinate heme—NO complex during NO synthesis was estimated using an extinction coefficient of 48 200 M^{-1} cm^{-1} at 437 nm (31) and found to be 60% for wild-type nNOS, 14% for W678A, and 25% for W678F. The mutant spectra also displayed a hump near 400 nm in the steady state, which could indicate buildup of other species such as a five-coordinate heme—NO complex, the heme—dioxy (Fe^III O_2) intermediate, or low-spin ferric heme.

**Kinetics of Formation of the Heme—NO Complex and NADPH Oxidation during Catalysis.** We next examined the
kinetics of formation of the heme—NO complex and NADPH oxidation just after initiating NO synthesis in wild-type nNOS (31) and in the Trp678 mutants. The NADPH-reduced, anaerobic, CaM-free enzymes were rapidly mixed in the stopped-flow spectrophotometer with an aerated buffer containing Ca2+ to trigger CaM binding and initiate NO synthesis at 10 °C (31, 51). Formation of the heme—NO complex was monitored by the absorbance gain at 436 nm, and concurrent NADPH oxidation was monitored by the absorbance loss at 340 nm. In Figure 8, the left panels depict light absorbance spectra that were collected just after mixing to initiate the reaction, during NO synthesis, and after NO synthesis had ceased (due to NADPH depletion) for wild-type nNOS and the two Trp678 mutants. The corresponding absorbance changes at 437 and 340 nm that took place during the reactions are shown in the middle and right panels of Figure 8. The spectral traces in the left panels confirm that
there was less buildup of a six-coordinate heme–NO complex during NO synthesis in the Trp678 mutants than in wild-type nNOS. The absorbance increases at 437 nm were best fit to a two-exponential equation for all three enzymes, as reported previously for wild-type nNOS (31, 51). However, the fast and slow phases were 4–10 times slower in the Trp678 mutants than in wild-type nNOS (Table 4). In wild-type nNOS, the heme–NO complex buildup was associated with a 10-fold deflection in the rate of NADPH oxidation (rates listed in Table 4), similar to what was reported previously (31, 51). After ~2 s, the wild-type enzyme reaction reached a steady-state phase where the concentration of the heme–NO complex and the NADPH oxidation rate remained relatively constant until the NADPH became depleted (middle panels, Figure 8). After this point, the absorbance at 437 nm decreased to a level that was 0.02 absorbance unit above the original value, which takes into account an absorbance increase that occurs at this wavelength due to reoxidation of the nNOS flavins (see spectral traces in the left panel). In contrast, for the Trp678 mutants, the

Table 3: Observed Rates of NADPH-Dependent Flavin and Heme Reduction

<table>
<thead>
<tr>
<th>NOS</th>
<th>Flavin reduction (s⁻¹)</th>
<th>Heme reduction (s⁻¹)</th>
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<tr>
<td></td>
<td>k₁ (%)</td>
<td>k₂ (%)</td>
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<tr>
<td>WT nNOS</td>
<td>14.5 ± 1.9 (60%)</td>
<td>3.3 ± 0.4 (40%)</td>
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<tr>
<td>W678F</td>
<td>13.8 ± 5.5 (40%)</td>
<td>3.3 ± 0.7 (60%)</td>
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<td>W678A</td>
<td>ND</td>
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<tr>
<td>WT iNOS</td>
<td>5.2 ± 0.7 (60%)</td>
<td>0.76 ± 0.19 (40%)</td>
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<tr>
<td>W457A</td>
<td>7.24 ± 1.0 (63%)</td>
<td>1.2 ± 0.5 (37%)</td>
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Reactions were initiated in a stopped-flow instrument at 10 °C. Data are the mean and standard deviation and are representative of two separate experiments for each enzyme. WT is wild type and ND not determined.

Figure 5: Kinetics of ferric heme reduction. Anaerobic ferric enzyme solutions containing 400 μM Hb, 10 mM Arg, 1.2 mM DTT, 2 μM CaM (for nNOS proteins), 1.2 mM CaCl₂ (for nNOS proteins), and ~0.8–2 μM NOS were rapidly mixed at 10 °C with an anaerobic CO-saturated buffer solution containing 100 μM NADPH. Heme reduction was followed by formation of the ferrous–CO complex at 444 nm. Dotted lines are the fitting curves. Inset plots in panels C and E show the absorbance changes over shorter time frames.
absorbance gain at 437 nm indicating buildup of the heme–NO complex was more gradual and much smaller and did not reach a clear steady state prior to NADPH depletion. Again, the 437 nm absorbance signals did not return to their initial values after the NADPH was consumed in either mutant reaction (middle panels, Figure 8) because of their being offset by an absorbance gain due to the reoxidation of the flavins (see spectral traces in the left panels). There was an initial rapid NADPH oxidation in the W678F and W678A mutant reactions that consumed 1.5 and 1.3 NADPH molecules per mole of enzyme, respectively, and took place prior to significant buildup of the heme–NO complex. These initial rates of NADPH consumption were either equivalent to (W678F) or slightly slower than (W678A) the initial rate seen in the wild-type nNOS reaction (see NADPH oxidation $k_1$ values in Table 4). The subsequent buildup of the heme–NO complex in either Trp678 mutant was not associated with a clear deflection of their NADPH consumption rates, consistent with only a small proportion of each mutant enzyme accumulating as a heme–NO complex. The NADPH oxidation rates during NO synthesis were then calculated from the slopes of the absorbance decrease at 340 nm within the 2–8 s time window (Figure 8). This showed that the mutant NADPH oxidation rates were either equivalent to (W678A) or greater than (W678F) the rate of NADPH oxidation by wild-type nNOS (see NADPH oxidation $k_2$ values in Table 4). This relationship corresponds with what we observed for the mutant NADPH oxidation rates when they were measured during NO synthesis in a conventional spectrophotometer at 10 °C (Table 2). The stopped-flow data suggest that the Trp678 mutants catalyze uncoupled NADPH oxidation even in the initial very early phase of their NO synthesis.

Heme–NO Binding Kinetics and Oxidation Rate of the Ferrous Heme–NO Complex. Because NO binding kinetics and stability of the heme–NO complex are altered in some NOS mutants (39, 40), we examined these properties in W678A and W678F nNOSoxy. The formation rates and stabilities of their ferrous heme–NO complexes were determined in stopped-flow reactions that mixed dithionite-reduced nNOSoxy mutants in the presence of H4 B and Arg with a 10 μM NO solution under anaerobic conditions. The light absorbance data shown in Figure 9 (left panels) indicate that both mutants predominantly formed stable six-coordinate ferrous heme–NO complexes whose spectral features were similar to those of the wild-type ferrous heme–NO complex, with the exception that the Soret peak of either mutant was broader on its left (lower wavelength) side. The right panels in Figure 9 depict the kinetics of ferrous enzyme disappearance and heme–NO complex formation during the NO synthesis.
binding reaction. The transitions were monophasic, and the observed rates of formation of the ferrous heme-NO complex were 62 ± 11, 59 ± 9, and 27 ± 4 for W678F, W678A, and wild-type nNOSoxy, respectively, under our experimental conditions, indicating that the Trp678 mutations do not greatly alter the kinetics of binding of NO to the heme.

The oxidation rate of the NOS ferrous heme-NO complex is a key parameter that helps determine NO synthesis activity. This parameter \( k_{\text{ox}} \) exhibits a 10-fold range among the three mammalian NOSs (39, 41). We determined \( k_{\text{ox}} \) values for the wild-type and mutant nNOSoxy proteins by mixing their ferrous heme-NO complexes with air-saturated buffer in stopped-flow reactions and following their subsequent conversion to the ferric enzyme (39, 41). In Figure 10, the left panels depict the light absorbance spectra of the beginning ferrous heme-NO species and ending ferric heme species in each reaction, while the right panels depict the time courses of absorbance change at 436 nm (or 340 nm). Fits to the 437 nm absorbance traces are shown as dotted lines in panels G–I. Data are representative of two or three separate experiments for each enzyme.

Table 4: Observed Rate Constants for Formation of the Heme-NO Complex and NADPH Consumption after Initiation of NO Synthesis

<table>
<thead>
<tr>
<th>NOS</th>
<th>heme-NO complex formation rate (s⁻¹)</th>
<th>NADPH consumption rate (s⁻¹)</th>
<th>% NO complex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_1 (%) )</td>
<td>( k_2 (%) )</td>
<td>( k_1 )</td>
</tr>
<tr>
<td>WT nNOS</td>
<td>3.3 ± 0.4 (18%)</td>
<td>0.83 ± 0.01 (82%)</td>
<td>0.021</td>
</tr>
<tr>
<td>W678F</td>
<td>0.64 ± 0.01 (79%)</td>
<td>0.16 ± 0.01 (21%)</td>
<td>0.021</td>
</tr>
<tr>
<td>W678A</td>
<td>0.77 ± 0.07 (39%)</td>
<td>0.08 ± 0.03 (61%)</td>
<td>0.017</td>
</tr>
</tbody>
</table>

NO synthesis was initiated in a stopped-flow spectrophotometer at 10 °C. The percentages in parentheses give the proportion of absorbance change for each kinetic phase. The \( k_1 \) and \( k_2 \) for NADPH consumption are the approximate linear rates observed between 0 and 0.4 s and between 2 and 8 s after mixing, respectively. Data are representative of two or three similar experiments. WT is wild type.
Arg, and the subsequent heme transitions were followed by UV-visible scanning (18, 30, 53). Figure 11 depicts representative data collected during reactions catalyzed by wild-type, W678A, and W678F nNOSoxy. The left panels show the light absorbance spectra of the three enzyme species that were detected during each reaction, while the right panels show how the concentration of each species changed with time during the reaction. In all three cases, we could identify a beginning ferrous species, a heme-dioxy (Fe$^{II}$ O$_2$) intermediate, and an ending ferric species. Their calculated Soret absorbance maxima are listed in Table 5. Formation and disappearance of the Fe$^{II}$ O$_2$ intermediate were best described by monophasic transitions, consistent with previous reports (18, 53). The kinetics of Fe$^{II}$ O$_2$ formation in the mutants were similar to or somewhat less than the rate in wild-type nNOSoxy (Table 5). However, the transition of the Fe$^{II}$ O$_2$ intermediate to ferric enzyme was 2.8 and 5.6 times slower in the W678F and W678A nNOSoxy mutants, respectively, than in the wild type (Table 5). On the basis of our work with the analogous W457A and W457F iNOSoxy mutants (30), the kinetic differences we observed here regarding Fe$^{II}$ O$_2$ disappearance rates in the Trp678 nNOSoxy mutant reactions imply that their rates of Fe$^{II}$ O$_2$ reduction by H$_4$B are proportionally slower (18, 30).

We tested this possibility by measuring the rate of H$_4$B radical formation during the Arg single-turnover reaction catalyzed by W678F nNOSoxy at 10°C (data not shown). The rate of H$_4$B radical formation was 5 s$^{-1}$, similar to the rate of Fe$^{II}$ O$_2$ disappearance in the same reaction (5.2 s$^{-1}$, Table 5). This supports the contention that Fe$^{II}$ O$_2$ reduction by H$_4$B is slower in the Trp678 nNOS mutants.

**DISCUSSION**

Here we investigated how mutations of a conserved Trp known to influence H$_4$B electron transfer (24, 30) would impact NADPH-driven NO synthesis by nNOS and iNOS. As expected, mutation of this Trp affected many enzyme properties, consistent with its indole engaging in an extensive π-stacking interaction with H$_4$B (27) and with H$_4$B modulating the structural, electronic, binding, thermodynamic, and kinetic properties of NOS (54, 55). Fortunately, the negative effects caused by the mutations on NOS protein solubility, dimer stability, and Arg or H$_4$B binding affinity were largely overcome by purifying, preincubating, and assaying the
In general, the lower NO synthesis activities of the Trp mutants could arise for two different reasons. (A) Mutant enzyme molecules generate NO more slowly than the wild type, or (B) a smaller subset of the mutant enzyme molecules generate NO at the normal rate. Our data suggest that both circumstances are at play for the Trp mutants, causing them to have a slower and less efficient NO synthesis. Consider the spectral data we collected during NO synthesis by the Trp678 nNOS mutants, which showed that the heme–NO complex builds up more slowly and to a smaller extent than in wild-type nNOS. The speed and extent of the buildup of the heme–NO complex depends on the intrinsic rate of NO biosynthesis (which is limited by $k_r$, the rate of ferric heme reduction by the NOS flavoprotein domain), the kinetics of heme–NO association and dissociation, the stability of the six-coordinate heme–NO complex, the rate of reaction between the ferrous heme–NO complex and O$_2$ (specified by kinetic parameter $k_{ox}$), and, finally, the proportion of total NOS molecules that generate NO in unit time ($41$). Our related measures can dismiss some of these potential contributors. For example, the Trp678 NOSoxy mutants formed stable six-coordinate ferrous and ferric heme–NO complexes, had relatively normal NO binding kinetics, and had $k_{ox}$ values similar to those of wild type nNOS. Thus, their diminished heme–NO complex formation during steady-state NO synthesis was potentially due to their having a slower rate of NO biosynthesis and/or fewer enzyme molecules making NO in unit time.

The rates of ferric heme reduction in the Trp678 nNOS mutants were up to 1000 times slower than in wild-type nNOS when they were measured at 10 °C under a CO atmosphere. Unfortunately, these rates are much too slow to account for the rates of buildup of the heme–NO complex and NO synthesis that we observed for these mutants in our aerobic experiments. Such “O$_2$ enhancement” of the ferric heme reduction rate has been observed before in eNOS (48) and in certain nNOS mutants (48), and its basis remains to be explained. However, in this study, the O$_2$ effect seen in
the Trp mutants could involve a change in the thermodynamic driving force for their ferric heme reduction, given that the heme midpoint potential was somewhat decreased in the W457A mutant compared to that of the wild type. In any case, we can obtain reasonable estimates for the rates of ferric heme reduction under aerobic conditions from the NADPH oxidation rates that were measured in the absence of Arg. These measures indicated that electron flux through the heme of the W678A and W678F nNOS mutants was diminished to 66 and 78% of that of wild-type nNOS, respectively. This suggests that the mutants do have somewhat slower rates of ferric heme reduction, consistent with their having decreased NO synthesis activity and smaller buildup of the heme-NO complex.

This leaves us to consider what proportion of the mutant enzyme molecules engage in NO synthesis in unit time. The degree to which product formation is coupled to ferric heme reduction in NOS enzymes can be determined in single-turnover Arg hydroxylation reactions catalyzed by ferrous NOSoxy domains. Our previous studies with W457A and W457F iNOSoxy mutants established that they have slower rates of H4B electron transfer to the FeII O2 intermediate, and this kinetic defect is associated with a proportionally slower disappearance of their FeII O2 intermediate and with a proportionally slower and less efficient Arg hydroxylation. Specifically, the rate of disappearance of the FeII O2 intermediate (which is determined by the rate of H4B radical formation) was 12.5 s⁻¹ in the wild-type iNOSoxy reaction, but fell by 50 and 75% in the W457F and W457A iNOSoxy reactions, respectively. These slower rates of H4B electron transfer were associated with...
60 and 73% less Arg hydroxylation per heme in the nNOSoxy single-turnover reactions. In this study, we observed slower rates of Fe(II)O2 disappearance in the Arg hydroxylation reactions catalyzed by the W678F and W678A nNOSoxy mutants, and slower H2B radical formation in the W678F nNOSoxy reaction, quite similar to what was observed in the iNOSoxy counterparts. This strongly implies that the Trp678 mutants also exhibit slower Fe(II)O2 reduction by H2B, and therefore catalyze a less coupled Arg hydroxylation.

If two mutational effects are in play in our W678F and W678A mutants (i.e., a slower ferric heme reduction by the flavoprotein domain and a slower Fe(II)O2 reduction by H2B), can we discern their individual impacts on catalysis? To address this, we ran computer simulations of a local kinetic model for NOS catalysis, to examine how slowing the rate of ferric heme reduction, on its own, will impact buildup of the heme—NO complex and NO synthesis activity in nNOS. As previously explained in detail (41, 51, 52), the global kinetic model relies on the finding that under conditions of sufficient substrate concentrations the steady-state enzyme distribution and NO synthesis activity of a given NOS are largely determined by three kinetic parameters: the rate of ferric heme reduction (kI), the rate of NO dissociation from the ferric heme—NO product complex (kd), and the oxidation rate of the ferrous heme—NO complex (ka). The simulations run here3 revealed that the kI values of the W678F and W678A nNOS mutants would have had to drop to 35 and 23% of the value of wild-type nNOS, respectively, to fully account for their smaller degree of heme—NO complex buildup seen during their NO synthesis. However, such decreases in kI are not supported by the NADPH oxidation rates that we measured for the mutants in the absence of Arg, which ranged from 61 to 78% of that of wild-type nNOS. In addition, experimental work in which the kI of nNOS was decreased to different degrees through the use of CaM mutants (51, 59) provides independent evidence that kI would have to fall below 40% of the wild-type value to approach the diminished levels of NO synthesis activity and heme—NO complex buildup that we observed in our W678F and W678A nNOS mutants. Thus, the slower kI values in the Trp678 mutants appear to account for only approximately one-third of the overall effect regarding their diminished heme—NO complex buildup and NO synthesis rates. This implies that an uncoupled NO biosynthesis, caused by slower H2B electron transfer to the Fe(II)O2 intermediate, accounts for the remaining two-thirds of the overall effect. Our current analysis therefore supports the original proposal developed from experiments with NOsoxy enzymes (17, 30) that slowing H2B electron transfer would decrease the efficiency of NADPH-driven NO synthesis by full-length NOS enzymes.

It is interesting to consider how the H2B electron transfer step and the ferric heme reduction step might be influenced by the Trp mutations. The mechanisms by which Trp457 influences the rate of H2B electron transfer in iNOSoxy, and how this in turn impacts the efficiency of its product formation in single-turnover reactions, have been previously discussed in detail (27, 30, 53, 60). Briefly, the structural and electronic changes brought on by the Phe and Ala substitutions appear to prevent NOS from maximally stabilizing its H2B radical. This in turn slows the rate of H2B radical formation and consequently the rate of Fe(II)O2 reduction by H2B. A slower reduction of the Fe(II)O2 intermediate increases the probability that it will decay to superoxide and ferric enzyme before it can be reduced by H2B (Scheme 1). Such a decay process for the Fe(II)O2 intermediate uncouples ferric heme reduction from Arg hydroxylation (61), and this helps to explain why the iNOS and nNOS Trp mutants studied here have such poor coupling regarding their NADPH-driven NO synthesis. Perhaps more surprising was that the Trp678 mutations have an effect on the rate of ferric heme reduction in nNOS. Ferric heme reduction is catalyzed exclusively by the FMN hydroquinone of the NOS flavoprotein domain, and this electron transfer step is subject to its own unique regulation (16, 48, 51, 59, 62, 63). The FMN module must dock against the NOS oxygenase domain to transfer an electron to the heme; however, this process is thought to involve surface residues that are far from Trp678 (12, 16, 29), and the crystal structures of the W457A and W457F iNOSoxy mutants do not exhibit any obvious structural change in that surface region of iNOSoxy (27). Thus, we speculate that the Trp mutations might impact ferric heme reduction through an effect on the heme itself, either by lowering the heme midpoint potential, as seen here, or by altering some other aspect of the heme, such as the degree of heme ruffling that occurs in response to H2B binding (64). In any case, during nNOS catalysis, the Trp678 mutations appear to slow ferric heme reduction by a factor of less than 2, so the physical basis for the effect might be relatively subtle.

Relation to Other Studies. Our W678F and W678A nNOS mutants have properties that are both similar to and different from those reported previously for nNOS mutants W678L and W678H (29). For example, our mutants form homodimers to a greater degree when provided with Arg and H2B and have relatively normal Km values for Arg, whereas the W678L mutant in particular exhibits very poor dimerization and Arg binding. Both pairs of mutants have a poorer affinity for H2B relative to wild-type nNOS, and all have diminished levels of NO synthesis, uncoupled NADPH oxidation, and a kinetic defect in their ferric heme reduction when measured under anaerobic conditions. Sagami et al. (29) concluded that Trp678 is important to the retention of the appropriated active site conformation for H2B and Arg binding and for electron transfer from NADPH to the heme. Our findings concur with theirs, but in addition emphasize that the ability of Trp678 to regulate H2B electron transfer to the Fe(II)O2 intermediate is likely to be its most important effect with regard to its impact on the catalytic functioning of NOS.

REFERENCES


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3 In the simulations, we varied kI, utilized our measured kout values, and assumed that the Trp mutants had identical kd values as in wild-type nNOS (65).
Tetrahydrobiopterin Function in NOS

identically highly anticoagulative pteridine binding sites, Biochemistry 35, 16735–16745.


