Tryptophan 409 Controls the Activity of Neuronal Nitric-oxide Synthase by Regulating Nitric Oxide Feedback Inhibition*

(Received for publication, June 25, 1999)

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The free radical nitric oxide (NO)1 has diverse roles in cellular processes including blood flow, neurotransmission, and the immune response (1–3). NO is synthesized by a family of enzymes termed NO synthases (NOSs). All NOSs catalyze a two-step, NADPH-and O2-dependent oxidation of L-arginine (Arg) to NO and citrulline forming N-hydroxyarginine oxidation, or Arg and tetrahydrobiopterin binding. In the absence of Arg, NADPH oxidation measurements showed that electron flux through the heme was actually slower in the Trp-409 mutants than in wild-type nNOS. However, little or no NO complex accumulated during NO synthesis by the mutants, as opposed to the wild type. This difference was potentially related to mutants forming unstable 6-coordinate ferrous-NO complexes under anaerobic conditions even in the presence of Arg and tetrahydrobiopterin. Thus, Trp-409 mutations minimize NO feedback inhibition by preventing buildup of an inactive ferrous-NO complex during the steady state. This overcomes the negative effect of the mutation on electron flux and results in hyperactivity. Conservation of Trp-409 among different NOSs suggests that the ability of this residue to regulate heme reduction and NO complex formation is important for enzyme physiologic function.

The heme of neuronal nitric-oxide synthase participates in oxygen activation but also binds self-generated NO during catalysis resulting in reversible feedback inhibition. We utilized point mutagenesis to investigate if a conserved tryptophan residue (Trp-409), which engages in π-stacking with the heme and hydrogen bonds to its axial cysteine ligand, helps control catalysis and regulation by NO. Surprisingly, mutants W409F and W409Y were hyperactive compared with the wild type regarding NO synthesis without affecting cytochrome c reduction, reductase-independent N-hydroxyarginine oxidation, or Arg and tetrahydrobiopterin binding. In the absence of Arg, NADPH oxidation measurements showed that electron flux through the heme was actually slower in the Trp-409 mutants than in wild-type nNOS. However, little or no NO complex accumulated during NO synthesis by the mutants, as opposed to the wild type. This difference was potentially related to mutants forming unstable 6-coordinate ferrous-NO complexes under anaerobic conditions even in the presence of Arg and tetrahydrobiopterin. Thus, Trp-409 mutations minimize NO feedback inhibition by preventing buildup of an inactive ferrous-NO complex during the steady state. This overcomes the negative effect of the mutation on electron flux and results in hyperactivity. Conservation of Trp-409 among different NOSs suggests that the ability of this residue to regulate heme reduction and NO complex formation is important for enzyme physiologic function.

The free radical nitric oxide (NO)1 has diverse roles in cellular processes including blood flow, neurotransmission, and the immune response (1–3). NO is synthesized by a family of enzymes termed NO synthases (NOSs). All NOSs catalyze a two-step, NADPH-and O2-dependent oxidation of L-arginine (Arg) to NO and citrulline forming N\(^{-}\)hydroxy-L-arginine (NOHA) as an intermediate (4, 5). Three different isoforms are constitutively expressed, whereas iNOS is expressed in response to cytokines or bacterial products. Each NOS is comprised of an N-terminal oxygenase domain that binds iron protoporphyrin IX (heme), 6R-tetrahydrobiopterin (H\(_4\)B), and L-arginine (Arg) and a C-terminal reductase domain that binds FMN, FAD, and NADPH (6). To be active, two NOS oxygenase domains interact to form a homodimer. NOS oxygenase and reductase domains are linked together on the polypeptide by a calmodulin (CaM) binding sequence (7, 8). CaM binding activates NO synthesis by triggering electron transfer from the flavins to the heme (9). Sequential transfer of electrons enables the heme to bind and activate O\(_2\), in both steps of NO synthesis (10, 11).

The NOS heme iron has a proximal cysteine thiolate ligand (12–14), as occurs in the cytochrome P450s. This enables a similar chemistry for oxygen activation and mixed function oxidation of substrate (4, 5). However, the heme environments in NOS and the cytochrome P450s differ in a number of important ways. Crystal structures show that H\(_4\)B binds at the heme edge in NOS perpendicular to its plane (12–14). Two conserved aromatic residues (Phe-584 and Trp-409 in rat nNOS) engage in aromatic stacking with the heme on its distal and proximal sides, respectively (Fig. 1). This aromatic stacking is absent in cytochrome P450s but is present in peroxidases and influences their electronic and catalytic properties (15). NOS crystal structures predict that the indole nitrogen of Trp-409 hydrogen bonds with the cysteine thiolate heme ligand in nNOS. In cytochrome P450s, hydrogen bonding to the coordinating thiolate is minimal, but it does occur in the thiolate-ligated hemeprotein chloroperoxidase (16). Aromatic and hydrogen bond interactions of Trp-409 in nNOS could conceivably influence heme iron electronegativity (17, 18), but its actual role has not been tested.

The NOS heme also binds self-generated NO during NO synthesis (19). The resulting ferrous heme-N0 complex is inactive but resists normal catalysis upon O\(_2\)-dependent decay of the NO complex. This distinguishes nNOS from cytochrome P450s, which usually are irreversibly inactivated if NO binds to their ferrous heme (20). Because decay of the ferrous-N0 complex is relatively slow, a majority of NOS enzyme molecules (70–90%) exist as an NO complex during steady-state NO synthesis (19). Although this suggests that heme-NO binding is an important regulator of NOS catalysis, it is currently unclear what, if any, structural features that are unique to NOS allow it to function in this capacity.

To address this issue, we used site-directed mutagenesis to investigate what role Trp-409 has in regulating nNOS activity. Surprisingly, substitution with Phe or Tyr generated mutants that had greater NO synthesis than wild-type nNOS. Mutant hyperactivity appeared to result from combined changes in
and made anaerobic by repeated cycles of evacuation and equilibration with deoxygenated argon gas. A 40 mM EPPS buffer containing 4 mM Hb, 1 mM Arg, and 200 mM DTT was separately made anaerobic, and 1 ml of this solution was transferred to the cuvette. NO gas was added to the head space and dissolved by mixing. Dithionite solution was then added in some cases. 

H$_2$O$_2$-dependent NOHA Oxidation—H$_2$O$_2$-dependent nNOS oxidation of NOHA to nitrite was assayed in 96-well microplates at 30 °C as described previously (23) with modification. The assay volume was 100 μl and contained 40 mM EPPS, pH 7.6, 250 mM nNOS or mutants, 1 mM NOHA, 1 mM DTT, 25 units/ml superoxide dismutase, 0.5 mM EDTA, and 4 mM Hb. Reactions were initiated by adding 30 mM H$_2$O$_2$ and stopped after 10 min by adding 1300 units of catalase. Nitrite was detected at 550 nm using the Griess reagent (100 μl) and quantitated based on nitrite standards.

Arg Binding—Arg binding affinity was studied at 30 °C by perturbation difference spectroscopy according to methods described previously (24). The buffer contained 40 mM EPPS, 5% glycerol, 1 mM DTT, 20 μM Hb, and 1 or 2 μM enzyme. 10 mM imidazole was added to the cuvette prior to titration with L-Arg (0–200 μM). The $K_a$ of Arg was calculated by double reciprocal analysis of the absorbance difference versus substrate concentration.

RESULTS

The optical spectrum of the W409F and W409Y mutants closely resembled that of wild-type nNOS at pH 7.6 in the absence of Hb and Arg (data not shown). Addition of 20 μM Hb and 1 mM arginine to either mutant caused a spectral shift from low spin to high spin, indicating that these molecules bound. Dithionite reduction in the presence of Arg, Hb, and Co produced a 444-nm absorbance peak for the ferrous-CO complex in both cases, indicating that their heme iron ligation is the same as in wild-type nNOS. Arg binding affinities were determined by spectral perturbation in the presence of 10 mM imidazole and 20 μM Hb (data not shown). Arg completely displaced bound imidazole during the titrations, and the Arg $K_a$ values for W409F and W409Y were 60 and 68 μM, respectively, compared with 55 μM for wild-type nNOS. Thus, Arg and Hb binding were not significantly perturbed by the mutation of Trp-409 to Tyr or Phe.

Table I compares the catalytic turnover numbers of wild-type nNOS and the Trp-409 mutants with regard to NO synthesis from Arg or NOHA, NADPH oxidation, and cytochrome c reduction in the presence or absence of Ca$^{2+}$/CaM. Substitution of Trp-409 with Phe or Tyr altered rates of NO synthesis and NADPH oxidation but did not alter cytochrome c reduction in any case, suggesting that the mutations only affect the oxygenase domain of nNOS. Surprisingly, the W409F and W409Y mutants had 3- and 1.8-fold faster rates of NO synthesis from Arg compared with the wild type, respectively. Corresponding rates of NADPH oxidation were increased, indicating a proportional increase in electron flux through the enzyme. The calculated NADPH stoichiometries were 1.7, 1.6, and 1.5 mol of NADPH oxidized/mol of NO generated from Arg for wild-type nNOS, W409F, and W409Y, respectively. These values are close to the theoretical minimum of 1.5 (4, 6) and therefore indicate tight coupling between NADPH oxidation and NO synthesis by the mutants. When NOHA replaced Arg as the substrate, an even greater hyperactivity was observed for both mutants (Table I).

To understand how the mutations increased rates of NO synthesis by nNOS, we first examined mutant activities in the H$_2$O$_2$-supported NOHA oxidation assay. This measures nitrite formation from NOHA and is useful because the reaction does not require electrons from the reductase domain and does not result in formation of a heme-NO complex (23) as shown. As shown in Fig. 2, initial rates of nitrite formation by the mutant proteins were equivalent to wild-type nNOS. This suggests that the mutations increase NO synthesis in the NADPH-supported reaction by changing the rate of electron flux and/or the dynamics of heme-NO complex formation.

To examine how the mutations affect electron flux through...
nNOS, we compared their NADPH oxidation rates under a number of different conditions (Table II). CaM-bound, wild-type nNOS had a relatively high rate of NADPH oxidation in the absence of H$_4$B and Arg, and this rate was increased approximately two times when H$_4$B bound, consistent with previous reports (18). NADPH oxidation rates for the CaM-bound Trp-409 mutants were slower in the absence of Arg and H$_4$B but increased proportionally as for the wild type in response to H$_4$B (–2×). Addition of the heme reduction inhibitor nitro-L-Arg methyl ester (18) to the H$_4$B-bound proteins decreased their NADPH oxidation rates to a level seen for the nNOS reductase domain alone, indicating that any additional NADPH oxidation above this value was associated with heme reduction. Together, these data indicate that electron flux through the heme is actually slower in the two mutants than in wild-type nNOS in the absence of NO synthesis. The addition of Arg to the CaM-bound, H$_4$B-saturated enzymes initiated NO synthesis in all cases and lowered the rate of NADPH oxidation in wild-type nNOS, as reported previously (9, 18). In contrast, the Arg addition increased NADPH oxidation rates in both H$_4$B-bound mutants. To test if NO was involved in modulating the Arg effects, we utilized agmatine, a substrate analog that binds to nNOS without supporting NO synthesis. Agmatine decreased NADPH oxidation by wild-type nNOS compared with H$_4$B alone but to a lesser extent than seen with Arg. For the mutants agmatine either did not effect the NADPH oxidation rate (W409F) or only increased it slightly (W409Y). These data show that electron flux through the heme is actually slower in the mutants under all conditions except when NO synthesis is taking place. This suggests that mutant hyperactivity and associated increase in electron flux must arise from a difference in NO interaction with the enzyme.

NO down-regulates the rate of NO synthesis and associated electron flux by binding to the nNOS heme (19). We therefore investigated if NO binding to the heme was altered by the Trp-409 mutations during aerobic steady-state catalysis. Fig. 3 shows wavelength scans of nNOS and mutants before and after activating NO synthesis at 15 °C. For wild-type nNOS, a significant percentage accumulated as the 6-coordinate ferrous-NO complex during the steady-state, as judged by the buildup of characteristic absorbance peaks at 436 and 560 nm (A and D), as reported earlier (19, 25). Under the same conditions, the Trp-409 mutants had either a small (W409F) or no (W409Y) detectable absorbance buildup at 436 nm (B, C, and D), indicating their 6-coordinate NO complexes did not accumulate during steady-state NO synthesis.

Given the above, we investigated if either mutant could form stable ferric or ferrous NO complexes with authentic NO under an anaerobic atmosphere in the presence of Arg and H$_4$B, as occurs for wild-type nNOS (19, 25). Fig. 4, A–C, shows that wild-type nNOS and both mutants all formed stable 6-coordinate ferric NO complexes that display a Soret peak at 440 nm (A and D), as reported earlier (19, 25). Under the same conditions, the Trp-409 mutants had either a small (W409F) or no (W409Y) detectable absorbance buildup at 436 nm (B, C, and D), indicating their 6-coordinate NO complexes did not accumulate during steady-state NO synthesis.

**DISCUSSION**

Mutating conserved residues near the active site of an enzyme usually diminish activity. This makes the hyperactivity of the Trp-409 nNOS mutants unusual but not unique among
hemeproteins. In fact, mutation of Trp-51 to Phe in yeast cytochrome c peroxidase results in hyperactivity with regard to cytochrome c oxidation (15). Like Trp-409 in nNOS, Trp-51 engages in \( \pi \)-stacking with the peroxidase heme (26) but on its distal rather than proximal side as in nNOS. Peroxidase hyperactivity appears to result from a faster reduction of a compound I-like heme intermediate by the substrate cytochrome c.

However, the hyperactivity observed for our nNOS mutants occurs through a distinct mechanism. After initiating NO synthesis, nNOS molecules quickly partition between a catalytically active form and an inactive ferrous-NO complex (19). This creates two cycles that each have their own rate-limiting step and together determine the observed rate of NO synthesis during the steady-state (Fig. 5). A given nNOS molecule circulates in the active or inactive cycles depending on whether it binds \( \text{O}_2 \) or NO to the heme. The rate-limiting step in the active cycle, which leads to NO synthesis, could be substrate binding, electron transfer, oxygen binding, a bond-making or -breaking step involved in oxygen activation or product formation, or product release. It is still unclear which of these is rate-limiting for wild-type nNOS, but related work clearly shows that heme reduction becomes rate-limiting if it is slowed relative to wild type (27, 28). If an nNOS molecule binds NO, it is placed in the inactive cycle and the rate-limiting step becomes the \( \text{O}_2 \)-dependent decay of the NO complex (29). Once decay occurs, the enzyme can bind \( \text{O}_2 \) and rejoin the active cycle.

Mutation of Trp-409 to Phe or Tyr appears to effect the rate-limiting step in both cycles of nNOS. In the active cycle, our NADPH oxidation measurements suggest the mutants have a slower electron flow to the heme relative to wild type. Because mutant cytochrome c reduction rates were normal, it is the flavin-to-heme reduction step that is likely slowed. Indeed, the equilibrium reached between ferric and ferrous heme during anaerobic NADPH-dependent reduction is greatly shifted toward ferric in both Trp-409 mutants compared with wild type.\(^3\) This is consistent with structural and resonance Raman data that predicts the indole nitrogen of Trp-409 and forms a hydrogen bond with the cysteine thiolate heme ligand.

\(^3\) S. Adak and D. J. Stuehr, unpublished observation.
in nNOS (see Fig. 1), and loss of this hydrogen bond by mutation to Phe or Tyr should increase the negative charge density on the cysteine thiolate and thus lower the reduction potential of the heme iron (17, 30).

A slower rate of heme reduction would slow NO synthesis by any Trp-409 mutant nNOS molecules present in the active cycle relative to the wild type (27, 28). However, we saw that mutant rates of NO synthesis were actually greater than the wild type. This could occur if the mutations minimized partitioning of enzyme molecules into the inactive cycle (i.e. heme-NO complex) during the steady-state. This appears to be the case, because we observed little or no NO complex accumulation for either Trp-409 mutant in the steady-state. Thus, it appears that the Trp-409 mutations offset their negative effect on heme reduction by allowing a greater proportion of the enzyme to circulate in the active cycle during the steady-state.

Why does this occur? There are several possibilities. (a) An increase in cysteine thiolate electronegativity resulting from the loss of the Trp-409 hydrogen bond should weaken the heme-NO bond (31). (b) Slower heme reduction in the mutants might favor formation of the ferrous-NO species during the steady-state from which NO more rapidly dissociates (32). (c) Reactivity of the heme-NO complex toward O₂ may increase, speeding the return of enzyme molecules to the active cycle. (d) Conversion from a 6- to 5-coordinate ferrous NO-complex may occur (33), although NO generally dissociates slower from 5-coordinate than from 6-coordinate ferrous hemes (34). We are presently working to distinguish between these and other potential mechanisms.

Given the hyperactivity of the Trp-409 mutants, it is astonishing that a Trp appears at this position in all 29 NOSs sequenced to date. This implies that in nature the most “appropriate” form of NOS may not be a more catalytically active version of nNOS. Why is this so? Although a definitive answer is still forthcoming, we suspect that this Trp is conserved either to ensure an appropriate rate of heme reduction or to maintain NO complex formation and its resultant effect on enzyme O₂ response (29, 35, 36).

Acknowledgments—We thank Qian Wang for her expert technical assistance.

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