Molecular Basis for Hyperactivity in Tryptophan 409 Mutants of Neuronal NO Synthase*

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A ferrous heme-NO complex builds up in rat neuronal NO synthase during catalysis and lowers its activity. Mutation of a tryptophan located directly below the heme (Trp409) to Phe or Tyr causes hyperactive NO synthase and less heme-NO complex buildup in the steady state (Adak, S., Crooks, C., Wang, Q., Crane, B. R., Tainer, J. A., Getzoff, E. D., and Stuehr, D. J. (1999) J. Biol. Chem. 274, 26907–26911). To understand the mechanism, we used conventional and stopped flow spectroscopy to compare kinetics of heme-NO complex formation, enzyme activity prior to and after complex formation, NO binding affinity, NO complex stability, and its reaction with O2 in mutants and wild type nNOS. During the initial phase of NO synthesis, heme-NO complex formation was 3 and 5 times slower in W409F and W409Y, and their rates of NADPH oxidation were 50 and 30% that of wild type, probably due to slower heme reduction. NO complex formation slowed NADPH oxidation in the wild type by 7-fold but reduced mutant activities less than 2-fold, giving mutants higher final activities. NO binding kinetics were similar among mutants and wild type, although in ferrous W409Y (and to a lesser extent W409F) the 436-nm NO complex converted to a 417-nm NO complex with time. Oxidation of the ferrous heme-NO complex to ferric enzyme was 7 times faster in Trp409 mutants than in wild type. Thus, mutant hyperactivity derives from slower formation and faster decay of the heme-NO complex. Together, these minimize partitioning into the NO-bound form.

Neuronal nitric-oxide synthase (nNOS)† functions in neurotransmission, synaptic plasticity, peristalsis, excitotoxicity, and neurodegenerative diseases (1–3). The enzyme generates nitric oxide (NO) by catalyzing a two-step oxidation of L-Arg that utilizes NADPH and O2 as cosubstrates (4). nNOS is composed of a C-terminal reductase domain that contains binding sites for heme, tetrahydrobiopterin (H4B), and L-Arg (5). A calmodulin (CaM) binding site lies between the two domains. CaM binds in response to elevated Ca2+ and triggers NADPH-dependent heme reduction (6), which is obligatory for NO synthesis.

As in the cytochrome P-450s, the nNOS heme iron is ligated to a cysteine thiolate and can bind a variety of molecules besides O2 (7–13). NO binding is particularly relevant because nNOS accumulates as a ferrous heme-NO complex during normal catalysis (13, 14). This markedly reduces its steady-state activity and increases the apparent Kcat/(O2) (15). Although the ferrous NO complex of nNOS is stable under inert atmosphere when L-Arg and H4B are bound, it decays in the presence of O2 to form ferric NOS, which can again participate in NO synthesis.

Mechanisms that control nNOS partitioning between its active and NO-bound forms are a topic of current interest. In rat nNOS, a conserved tryptophan (Trp409) that is located on the distal side of the heme has been implicated in this process (16). Surprisingly, substitution of Trp409 with Tyr or Phe generated hyperactive mutants that had greater rates of NO synthesis and NADPH consumption than wild type nNOS. From this work, it appeared that mutant hyperactivity was associated with less NO complex formation in the steady state. However, it is unclear why this occurs and how it might be regulated by Trp409.

To address these questions, we compared the Trp409 mutants and wild type nNOS in a number of ways using conventional and stopped flow spectroscopy. We analyzed heme-NO complex formation during catalysis and upon reaction with reagent NO, and measured electron flux through the enzymes prior to and after NO complex formation. Our results explain how Trp409 controls NO complex formation in nNOS and identify a basis for mutant hyperactivity.

EXPERIMENTAL PROCEDURES

Materials—Oxygen and NO gas were purchased from Liquid Carbonic Company and Matheson Inc., respectively. All other reagents and materials were obtained from Sigma or sources as previously reported (16, 17).

Molecular Biology—Trp409 mutations were made in nNOSox as described for full-length nNOS (16) using the same primers and the QuickChange polymerase chain reaction in vitro mutagenesis kit from Stratagene. Mutations were confirmed by DNA sequencing at the Cleveland Clinic core facility.

Protein Expression and Purification—Wild type and Trp409 mutant nNOS proteins (oxygenase domain and full-length) were expressed in E. coli and purified as described previously (7, 17). UV-visible spectra were recorded on a Hitachi U3110 Spectrophotometer in the absence or presence of 20 μM H4B and 1 mM L-Arg. The ferrous CO adduct absorbing at 444 nm was used to quantitate the heme protein content using an extinction coefficient of 74 μM cm⁻¹ (Aext=444 Ånm⁻¹).

Ferrous Nitrosyl Complex Formation during NO Synthesis—For experiments done in the presence of an NADPH regenerating system, an air saturated solution that contained 40 mM EPPS buffer, pH 7.6, 3 μM nNOS protein, 0.5 mM EDTA, 6 μM CaM, 200 μM dithiobisreitol, 20 μM H4B, 30 μM NADPH, 0.5 unit of glucose-6-phosphate dehydrogenase,
and 0.5 mM glucose 6-phosphate was rapidly mixed at 10 °C with an equal volume of 2.4 mM Ca2+ in buffer. Reactions were monitored with a Hi-Tech Ltd. rapid scanning diode array detector (MG6000) designed to collect 96 spectra in a specific time frame.

For experiments done without an NADPH-regenerating system, an air-saturated solution that contained 40 mM EPSS, pH 7.6, 2 μM nNOS protein, 0.4 mM dithiothreitol, 2 mM L-Arg, 20 mM Hb, B, 4 mM CaM, 100 μM NADPH, and 0.5 mM EDTA was rapidly mixed at 10 °C with an equal volume of buffer containing 2.4 mM Ca2+. Absorbance change at 436 nm (ferrous NO formation and decay) and at 340 nm (NADPH oxidation) were monitored separately in a Hi-Tech Ltd. 51MX stopped flow instrument equipped with a variable wavelength detector. The concentration of ferrous NO complex formed during NO synthesis was estimated from absorbance change at 436 nm using an extinction coefficient of 49,800 M⁻¹ cm⁻¹ (14). Signal/noise ratios were improved by averaging 4–6 individual experiments. Each experiment was performed three separate times. The time courses of absorbance change were fit using methods provided by the instrument manufacturer.

Characteristics and Kinetics of NO Binding—Spectra were collected with the rapid scanning diode array detector. Anaerobic solutions containing wild type or mutant nNOS oxygenase domains (nNOSox; 3 μM), 100 mM EPSS buffer, pH 7.6, 1 mM L-Arg, 10 mM Hb, B, 0.2 mM dithiothreitol were prereduced with excess dithionite (100 μM) and then rapidly mixed with NO-saturated buffer at 10 °C. To study NO binding kinetics, full-length nNOS proteins were used. The initial NO concentration was varied by diluting the saturated solution with anaerobic buffer, and absorbance change was recorded with a single wavelength detector.

Reaction of Ferrous NO Complexes with Oxygen—Anaerobic solutions of full-length nNOS enzymes (4 μM) containing saturating L-Arg and Hb were reduced with a minimum amount of dithionite, and then saturated NO solution was added to give an NO concentration of 0.1 mM. This solution was transferred to the stopped flow reservoir and rapidly mixed with air-saturated buffer solutions at 10 °C. Spectra were collected with the rapid scanning diode array detector. In replica experiments absorbance changes at 436 and 393 nm were monitored using a single wavelength detector.

Formation and Reduction of the W409Y Ferric NO Complex—An anaerobic buffer solution containing 3 μM W409Y oxygenase domain (W409Yox), L-Arg, and Hb in a cuvette had NO-saturated solution added to form the ferric NO complex. This was transferred to the stopped flow instrument and rapid mixed at 10 °C with a 0.1 mM dithionite solution. Scans were collected between 0 and 8 s.

RESULTS

Heme-NO Complex Formation during NO Synthesis—We utilized rapid scanning stopped flow spectroscopy to carefully compare heme-NO complex formation in the Trp409 mutants and wild type nNOS. In Fig. 1, air-saturated solutions containing enzyme, L-Arg, cofactors, and an NADPH-regenerating system were rapid mixed at 10 °C with an air-saturated solution containing excess CaCl2 to trigger NO synthesis. A majority of absorbance change attributed to the buildup best fit to a two-exponential equation, whereas in the Trp409 mutants with 3 and 1.7 times faster rates of NADPH oxidation after NO complex formation (Table I), the percentage of absorbance change attributed to each phase (k1 and k2) were the same in wild type and W409F (46 and 54%). The apparent k1 values for W409F and W409Y were 3 and 5 times slower than for wild type, respectively, and the W409F k2 value was 5 times slower.

The rate of NADPH oxidation prior to NO complex buildup was about 2- and 4-fold faster in wild type nNOS compared with W409F and W409Y (Fig. 2; Table I). However, in wild type nNOS the NADPH oxidation rate was decreased 7-fold after heme-NO complex formation, whereas in the Trp409 mutants almost no change was observed (Fig. 2). This left the W409F and W409Y mutants with 3 and 1.7 times faster rates of NADPH oxidation after NO complex formation (Table I), consistent with their being hyperactive in the steady state.

Properties and Kinetics of Heme-NO Complexes—Fig. 3 contains data from rapid scanning experiments where anaerobic ferrous enzymes containing L-Arg and Hb were rapidly mixed at 10 °C with an equal volume of NO saturated buffer. In wild type nNOS, a stable heme-NO complex formed with Soret absorbance at 436 nm and visible band near 560 nm, and loss of absorbance at 395 nm, consistent with previous reports (14, 15). In the W409F and W409Y mutants, there was much less partitioning into a heme-NO complex even at the beginning of the reaction. A majority of mutant enzymes remained ferric as judged by continued strong absorbance at 395 nm in both cases. These levels of NO complex were maintained for at least 150 s of the reaction in all cases. This confirms that the Trp409 mutants generate less heme-NO complex during steady state NO synthesis than wild type nNOS.

In Fig. 2, reactions were similar except that they contained a limiting amount of NADPH and no regenerating system. Absorbance changes at 436 and 340 nm were monitored versus time to follow NO complex formation and NADPH oxidation, respectively, and the results are shown at two different time scales. In all cases, the level of heme-NO complex reached a plateau within the first several seconds of the reaction and then began to decrease after most of the NADPH was oxidized. The peak concentrations of heme-NO complex for wild type, W409F, and W409Y were 0.60, 0.25, and 0.14 μM, respectively, as estimated from the absorbance gain at 436 nm (14). This represents 60, 25, and 14% conversion of wild type, W409F, and W409Y into the heme-NO complex. For wild type and W409F, the buildup best fit to a two-exponential equation, whereas buildup in W409Y best fit to a single exponential equation (Table I). The percentage of absorbance change attributed to each phase (k1 and k2) were the same in wild type and W409F (46 and 54%). The apparent k1 values for W409F and W409Y were 3 and 5 times slower than for wild type, respectively, and the W409F k2 value was 5 times slower.

In Fig. 3, conversion to the 417-nm species with absorbance closer to 420 nm. In W409Y, two NO complexes with Soret absorbance at 417 and 436 nm were present even in the earliest scan after mixing (0.003 s), with the 417-nm complex predominant. More of the W409Y 436-nm NO complex converted to the 417-nm complex with time. As shown in the right panels of Fig. 3, conversion to the 417-nm species was slow in W409F and about 25 times faster in W409Y.
Subsequent air oxidation of the Trp409 NO complexes regenerated ferric enzymes that had normal NO synthesis activities (data not shown), indicating that conversion to the 417-nm species did not irreversibly inactivate the enzymes.

To study the 417-nm complex in more detail, the ferric NO complex of W409Yox (which is stable; Ref. 16) was reacted with dithionite in the stopped flow instrument. As shown in Fig. 4, the ferrous NO complex that initially formed has a broad visible absorbance with peak at 556 nm, essentially identical to the ferrous NO complex of wild type nNOS (see Fig. 3). However, after time this species converted to the 417-nm ferrous NO complex, which has two visible absorbance bands at 534 and 536 nm (Fig. 4). This differs from the ferric NO complex of W409Y (Fig. 4) and from the five-coordinate ferrous NO complex of nNOS, which only forms in the absence of L-Arg and H4B and has a Soret absorbance at 392 nm and broad visible band at 570 nm (18). However, the spectrum of the 417-nm W409Y NO complex is similar to ferrous NO heme proteins that have a weakened transaxial bond (19, 20), consistent with less hydrogen bonding to the cysteine thiolate in W409Y nNOS (16).

We next compared rates of NO binding to ferrous mutants and wild type nNOS. As shown in Fig. 5, graphs of the observed binding rates versus NO concentration were nearly identical in their concentration dependence and magnitude. This also held true for ferric nNOS proteins (data not shown). Thus, NO binding rates appear to be similar between the Trp409 mutants and wild type nNOS.

**TABLE I**

Rate constants for heme-NO complex buildup, NADPH oxidation, and NO complex decay during NO synthesis

| Enzyme | $k_{1}$ | $k_{2}$ | $m_{1}$ | $m_{2}$ | $k_{\text{decay}}$ |
|--------|--------|--------|--------|--------|------------------|
| nNOS   | 10.4   | 2.1    | 0.022  | 0.003  | 2.5 × 10⁻²       |
| W409F  | 3.4    | 0.38   | 0.012  | 0.009  | ND               |
| W409Y  | 2.0    | ND     | 0.006  | 0.005  | ND               |

**FIG. 2.** Kinetics of heme-NO complex formation, decay, and simultaneous NADPH oxidation. A solution containing 2 µM enzyme, L-Arg, cofactors, CaM, NADPH, and EDTA was rapidly mixed with a solution containing CaCl₂ at 10 °C. Absorbance changes at 436 nm (solid line) or 340 nm (dotted line) are shown in a longer (left) and shorter (right) time frame. The results are representative of three independent experiments.

**FIG. 3.** Spectra and stability of ferrous NO complexes. Left, rapid scanning spectra were recorded at 10 °C before and after mixing a buffered solution of dithionite-reduced nNOSox proteins (3 µM) containing H4B and L-Arg with an anaerobic NO-saturated buffer solution. The dashed line is dithionite-reduced enzyme, while the solid and dotted lines were recorded 0.003 and 144 s after mixing. Right, cross-sections of absorbance change at 417 nm. The data are representative of two similar experiments.
contains spectral and kinetic data from experiments in which anaerobic ferrous NO enzyme solutions were rapidly mixed with an air-saturated solution. Overlay of consecutive scans (left panels) shows that a decrease in absorbance occurred between 415 and 445 nm and that an increase occurred between 360 and 410 nm as time progressed. These changes indicate concomitant decay of the heme-NO complex and generation of ferric enzyme, respectively. Cross-sections of absorbance change at 436 and 393 nm were monophasic in all cases and are shown in the right panels. Identical results were obtained for W409Y when NO complex decay was followed at 417 nm (data not shown). Analysis revealed that reaction rates with O2 (and rates of ferric enzyme formation) were about 7 times faster in the mutants compared with wild type nNOS (Table II).

**DISCUSSION**

nNOS quickly reacts with self-generated NO during aerobic catalysis to partition between a catalytically active form and an inactive ferrous NO complex (14, 15). We have incorporated partitioning into a two-cycle model for nNOS catalysis (Fig. 7). The active and inactive cycles each have their own rate-limiting step that together determine the rate of NO synthesis during the steady-state. The rate-limiting step in the active cycle may be heme reduction (21, 22) or product release (23). After NO forms, its binding to ferric heme is fast and nearly quantitative (18, 28), and enough heme-NO complex is reduced prior to NO dissociation to form the ferrous NO complex. NO dissociation from the ferrous NO enzyme is slow, and the rate-limiting step appears to be its O2-dependent decay to generate ferric enzyme that can rejoin the active cycle (15).

Mutating Trp409 to Phe or Tyr caused hyperactivity that was associated with buildup of less NO complex during steady state NO synthesis (16). This suggested that Trp409 can control nNOS partitioning between the active and inactive forms in some way. We examined several steps that make up Fig. 7 to clarify a basis for the Trp409 effect. Our stopped flow data confirm that the mutant enzymes partition less into a NO complex even during the initial phase of NO synthesis. In the initial period, buildup of the ferrous NO complex was also slower in the mutants compared with wild type nNOS (Table II).

**TABLE II**

Rates of ferrous-NO complex reaction with O2 and ferric enzyme formation

| Enzyme | Decrease in ferrous-NO complex | Buildup of ferric enzyme |
|--------|--------------------------------|--------------------------|
| nNOS   | 0.19                           | 0.16                     |
| W409F  | 1.3                            | 1.7                      |
| W409Y  | 1.4                            | 1.2                      |

**FIG. 4.** Visible spectra of W409Y heme-NO complexes. The ferric NO complex of W409Yox was rapidly mixed with dithionite, and spectra were collected over 8 s. Spectra shown are the resting ferric NO complex (solid line) and ferrous NO complex taken at 0.003 s after mixing (dashed line) or 8 s after mixing (dotted line).

**FIG. 5.** Kinetics of NO binding to ferrous enzymes. A buffered solution of dithionite-reduced nNOS (3 μM) containing H4B and L-Arg was rapidly mixed at 10 °C with buffered, anaerobic NO solutions. Absorbance change at 436 nm was monitored, and the observed rate of change (Kobs) is plotted for each initial NO concentration.

**FIG. 6.** Reaction of ferrous NO complexes with O2. The left panels contain rapid scanning spectra recorded during the reaction of preformed ferrous NO complex (4.0 μM) with a air-saturated buffer solution at 10 °C. 96 scans were collected over 144 s. 12 scans are shown in each panel. Isosbestic points formed at 417, 415, and 413 nm in wild type, W409F, and W409Y proteins, respectively, with absorbance increasing to the left and decreasing to the right of this point with time. The right panels show the kinetics of spectral change at 436 and 393 nm.
NO Complex Formation and nNOS Hyperactivity

suggest that there are little or no differences in some of these factors. For example, like wild type nNOS the mutant NO synthesis rates are insensitive to the NO scavenger oxyhemoglobin and display a high degree of coupling with NADPH oxidation (16). Mutants also exhibited no apparent difference in NO binding affinity compared with wild type (Fig. 5). Thus, we are left to consider differences in \( k_{\text{cat}} \), complex stability, and reactivity with O\(_2\).

Tight coupling of NO synthesis and NADPH oxidation in mutants and wild type means that their initial rates of NADPH oxidation (measured before NO complex buildup) are good estimates of \( k_{\text{cat}} \). A comparison of \( m_1 \) values in Table I shows that W409F and W409Y have 2- and 4-fold slower \( k_{\text{cat}} \) than wild type. The initial rates of NO complex formation display the same rank order as the \( k_{\text{cat}} \) estimates (wild type > W409F > W409Y), implying that they are causally linked. However, the fact that NO complex formation rates exhibit greater fold differences than the \( k_{\text{cat}} \) values indicate other factors must contribute. In W409Y, simultaneous formation of the 417-nm NO complex conceivably reduces buildup of its 436-nm NO complex, but this does not apply for W409F. Surprisingly, reaction of the ferrous NO complex with O\(_2\) was almost 7 times faster in the mutants than in wild type nNOS. Together, our results suggest that Trp\(^{409}\) mutants have less NO complex buildup due to the combined effect of a slower \( k_{\text{cat}} \) and a faster reaction of their ferrous NO complexes with O\(_2\).

It is useful to consider how differences in these two parameters affect mutant enzyme function in the steady state. Although the mutants have slower \( k_{\text{cat}} \), by virtue of their partitioning less into a ferrous NO complex their steady-state rates of NO synthesis and NADPH oxidation end up being faster than wild type. Both mutants have the same rate of O\(_2\)-dependent NO complex decay. Thus, a 2-fold difference in \( k_{\text{cat}} \) may explain why steady state NO synthesis by W409Y is half as fast as W409F. The greater propensity of W409Y to form a 417-nm NO complex also helps explain why it builds up less 436-nm NO complex compared with W409F.

In nNOS, a slower rate of heme reduction leads to a slower rate of NO synthesis (21, 22). The lower \( k_{\text{cat}} \) values of the Trp\(^{409}\) mutants probably reflect a slower rate of heme reduction, as can be inferred from their initial rates of NADPH oxidation (Table I). This would also slow reduction of the ferric NO complex that forms as an initial product of catalysis (28). Thus, buildup of the ferrous NO species is minimized during mutant NO synthesis by a slower formation rate and a faster rate of oxidation (Fig. 7). A slower rate of heme reduction probably derives from a mutational effect on heme, because reductase function in the Trp\(^{409}\) mutants is normal (16). Crystal structures indicate that Trp\(^{409}\) stacks with the heme porphyrin and makes a hydrogen bond between its indole nitrogen and the heme cysteine thiolate ligand (24, 25). This hydrogen bond is expected to modulate heme iron electronegativity. Mutating Trp\(^{409}\) to Phe and Tyr removes the hydrogen bond and probably lowers the heme potential, which is known to impede heme reduction in nNOS (26). Whether this also explains why mutant ferrous NO complexes react so much faster with O\(_2\) is unclear. Our spectral work suggests that Trp\(^{409}\) mutants do form altered ferrous NO complexes compared with wild type, and we are currently investigating if a causal relationship exists between heme electronegativity and ferrous NO complex reactivity.

Our data impact on a fundamental aspect of nNOS, namely the binding of NO during normal aerobic catalysis (14, 27). The ferrous heme-NO complex is catalytically inactive and its formation causes nNOS to operate at a fraction of its true activity. We suggested earlier that NO complex formation controls the rate of NO synthesis during the steady state by setting up a condition in which the O\(_2\)-dependent breakdown of the ferrous NO complex becomes the rate-limiting step (15). Our current results support this model and help explain how Trp\(^{409}\) mutants alter nNOS partitioning between active and inactive forms by affecting rates of heme reduction, NO synthesis, and NO complex decay. Oxidation of the ferrous NO complex by O\(_2\) is a key feature that promotes regeneration of ferric enzyme. Because NO complex formation dramatically affects nNOS partitioning (\( K_m(O_2) \)) (15), it will be important to see if oxygen response is altered by the Trp\(^{409}\) mutations.

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