Neuronal Nitric-oxide Synthase Mutant (Ser-1412 → Asp) Demonstrates Surprising Connections between Heme Reduction, NO Complex Formation, and Catalysis*

Received for publication, July 31, 2000, and in revised form, October 6, 2000 Published, JBC Papers in Press, October 18, 2000, DOI 10.1074/jbc.M006857200

Subrata Adak‡‡, Jérôme Santolini‡, Svetlana Tikunova‖, Qian Wang‡, J. David Johnson¶, and Dennis J. Stuehr‡‡‡

From the ‡Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195 and the ¶Department of Medical Biochemistry, Ohio State University, Columbus, Ohio 43210

Rat neuronal NO synthase (nNOS) contains an Akt-dependent phosphorylation motif in its reductase domain. We mutated a target residue in that site (Ser-1412 to Asp) to mimic phosphorylation and then characterized the mutant using conventional and stopped-flow spectrophotometries. Compared with wild-type, S1412D nNOS catalyzed faster cytochrome c and ferricyanide reduction but displayed slower steady-state NO synthesis with greater uncoupling of NADPH oxidation. Paradoxically, the mutant had faster heme reduction, faster heme-NO complex formation, and greater heme-NO complex accumulation at steady state. To understand how these behaviors related to flavin and heme reduction rates, we utilized three soybean calmodulins (CaMs) that supported a range of slower flavin and heme reduction rates in mutant and wild-type nNOS. Reductase activity and two catalytic parameters (speed and amount of heme-NO complex formation) related directly to the speed of flavin and heme reduction. In contrast, steady-state NO synthesis increased, reached a plateau, and then fell at the highest rate of heme reduction that was obtained with S1412D nNOS + CaM. Substituting with soybean CaM slowed heme reduction and increased steady-state NO synthesis by the mutant. We conclude the following. 1) The S1412D mutation speeds electron transfer out of the reductase domain. 2) Faster heme reduction speeds intrinsic NO synthesis but diminishes NO release in the steady state. 3) Heme reduction displays an optimum regarding NO release during steady state. The unique behavior of S1412D nNOS reveals the importance of heme reduction rate in controlling steady-state activity and suggests that nNOS already has a near-optimal rate of heme reduction.

Nitric oxide (NO) is synthesized by one inducible and two constitutively expressed NO synthases (NOSs). The constitutive NOSs (endothelial (eNOS) and neuronal synthases (nNOSs)) are Ca2⁺-responsive and have multiple functions in physiology and pathology (1, 2). All NOSs are homodimers whose subunits contain an N-terminal oxygenase domain that binds heme, (6R)-tetrahydrobiopterin (H₂B), and L-arginine (Arg), and a C-terminal reductase domain that binds FMN, FAD, and NADPH (3, 4). A CaM binding motif is located between the oxygenase and reductase domain, and its occupancy triggers electron transfer between the FMN and heme (5). This enables NOSs to catalyze the NADPH- and O₂-dependent oxidation of Arg to generate NO and citrulline, with N-hydroxyarginine (NOHA) being formed as an enzyme-bound intermediate (3, 4).

The NOSs share many structural features but appear to differ in how their catalysis is regulated. For example, in rat nNOS we have established that heme-NO complex formation governs its rate of NO synthesis during the steady state by setting up a condition where the O₂-dependent breakdown of the ferrous NO complex becomes a rate-limiting step (6, 7). This greatly shifts the apparent Kₘ,O₂ value of the enzyme and thus has physiologic impact (8). In nNOS, ferrous heme-NO complex formation is rapid (6, 9) and results from reduction of a ferric heme-NO species that is an immediate product of catalysis (10). In contrast, eNOS does not build up detectable ferrous heme-NO complex during steady-state catalysis (11), and its slow NO synthesis is associated with slow heme reduction (9, 11).

To better understand the relationship between heme reduction rate, NO complex formation, and NO release, we developed a kinetic model for nNOS catalysis. The companion paper by Santolini et al. (12) describes our kinetic model in detail and demonstrates that it can accurately simulate initial and steady-state features of nNOS catalysis including heme-NO complex formation, a concomitant deflection in NADPH oxidation and NO synthesis, and an increase in enzyme apparent Kₘ,O₂ values. Further simulations predicted that a complex relationship should exist between rates of heme reduction and NO synthesis during the steady state (12). Specifically, there should be an optimal rate of heme reduction beyond which NO synthesis is actually diminished, and nNOS appears to be set near its optimum heme reduction rate. Although this concept is of fundamental importance, only limited information is available on how heme reduction rate affects NO synthesis by nNOS. These include studies with CaM-troponin C chimeras (13), CaM point mutants that contain dysfunctional Ca²⁺-bind-

tetrahydro-L-biopterin; nNOS, neuronal NO synthase; ScAM, soybean calmodulin; NOHA, N-hydroxyarginine; Arg, l-arginine.
ing sites (14), and NOS chimeras whose reductase domains are swapped (15). In the only study where heme reduction rates were measured (13), slower rates were obtained relative to CaM, and NO synthesis was proportional to the heme reduction rate (i.e. was slower). Unfortunately, no information was provided in these studies regarding NOS heme-NO complex formation or how the enzymes might respond if heme reduction became faster than that obtained with CaM.

Given the above, we were interested in reports on serine/threonine kinase Akt-dependent phosphorylation of Ser-1179 in bovine eNOS (16) and the analogous Ser in human eNOS (17). This residue is located in the NADPH-FAD module near the C terminus of the reductase domain. Cotransfection of the Akt-dependent kinase with eNOS led to phosphorylation at Ser-1179, increased NO production, and allowed the enzyme to be activated at lower Ca2+ concentrations. Mutation of Ser-1179 to Asp, which mimics phosphorylation by permanently introducing a negative charge, also resulted in a 2–3-fold increase in NO synthesis and faster cytochrome c reduction (16–18). These results suggest that the mutation may increase electron transfer in eNOS and can explain how eNOS is activated in endothelium in response to shear stress. An Akt phosphorylation motif (RXXRX(S/T)) is also present at the same site in rat nNOS and contains the corresponding residue Ser-1412.

XX

However, cotransfection of the Akt-dependent kinase with nNOS did not result in increased NO release (16), so it is puzzling why rat nNOS responded differently than eNOS.

nNOS did not result in increased NO release (16), so it is puzzling why rat nNOS responded differently than eNOS. However, cotransfection of the Akt-dependent kinase with nNOS did not result in increased NO release (16), so it is puzzling why rat nNOS responded differently than eNOS.

In rat nNOS and contains the corresponding residue Ser-1412.

(S/T)) is also present at the same site in rat nNOS and contains the corresponding residue Ser-1412.

These results suggest that the mutation may increase electron transfer in eNOS and can explain how eNOS is activated in endothelium in response to shear stress. An Akt phosphorylation motif (RXXRX(S/T)) is also present at the same site in rat nNOS and contains the corresponding residue Ser-1412.

Importantly and had the same molecular mass as wild-type nNOS (18). These results suggest that the mutant displayed the same absorbance to nitrite was assayed in 96-well microplates at 25 °C as described previously (19) with modification. The assay volume was 100 µL, which contained 40 mM EPPS, pH 7.6, 250 mM NaCl, an intact 1 mM NOHA, 1 mM dithiothreitol, 25 units/ml superoxide dismutase, 0.5 mM EDTA, and 4 mM H2B. Reactions were initiated by adding 30 mM H2O2 and stopped after 10 min by adding 1300 units of catalase. Nitrite was detected at 550 nm after adding the Griess reagent (100 µl) and quantitated based on nitrite standards.

**Heme and Flavin Reduction**—The kinetics of flavin and heme reduction were analyzed at 10 °C as described previously (13, 19) using a diode array detector and stopped-flow apparatus from Hi-Tech Ltd. (model SF-61) equipped for anaerobic work. Heme reduction was followed by formation of the ferrous-CO complex, and the kinetics were determined by absorbance change at 444 nm. Reactions were initiated by rapid mixing an anaerobic, buffered, CO-saturated solution containing 50 µM NADPH with an anaerobic CO-saturated solution containing wild-type or mutant nNOS (2 µM), 40 mM EPPS buffer, pH 7.6, 10 µM H2B, 0.3 mM dithiothreitol, 5 mM Arg, 4 mM CaM, or 20 mM soybean CaM proteins, and 1 mM Ca2+. Flavin reduction was monitored under the same conditions at 485 nm. Signal-to-noise ratios were improved by averaging at least 10 individual mixing experiments. The time course of absorbance change was fit to single or multiple exponential equations using a nonlinear least square method provided by the instrument manufacturer.

**Kinetics of Ferrous Heme-NO Buildup and Concurrent NADPH Oxidation**—Experiments were done at 10 °C in a Hi-Tech 51MX stopped-flow instrument equipped with a variable wavelength detector. To initiate NO synthesis, an air-saturated solution that contained 40 mM EPPS, pH 7.6, 2 mM NOS or mutant, 0.4 mM dithiothreitol, 2 mM Arg, 20 mM H2B, 4 mM CaM, or 20 mM soybean CaM proteins, 40 µM NADPH, and 0.5 mM EDTA was rapid mixed with a buffered solution containing 2.4 mM Ca2+. Absorbance at 436 nm was monitored to follow ferrous heme-NO formation and at 340 nm to follow NADPH oxidation (7). The concentration of ferrous heme-NO complex formed during NO synthesis was estimated from the absorbance change at 436 nm using an extinction coefficient of 49,800 m−1 cm−1 (6), whereas the amount of NADPH oxidation was determined using an extinction coefficient of 6,220 m−1 cm−1 at 340 nm. Signal-to-noise ratios were improved by averaging six consecutive scans. Each experiment was performed three separate times.

**Expression and Purification of Wild-type and nNOS Mutant**—Wild-type rat nNOS and the S1412D mutant both contained a His6 tag attached to their N termini. They were overexpressed in E. coli strain BL21 (DE3) using a modified pCWori vector and purified as described previously (20, 21).

**EXPERIMENTAL PROCEDURES**

**Materials**—All regents and materials were obtained from Sigma or sources previously reported (19). Soybean CaM proteins Scam-1, Scam-4, and M14V Scam-1 were cloned, expressed, and purified as described previously (20, 21).

**Molecular Biology**—Restriction digestions, cloning, bacterial growth, and transformation and isolation of DNA fragments were performed using standard procedures. Site-directed mutagenesis was done using the Quick Change polymerase chain reaction in vitro Mutagenesis Kit from Stratagen. Incorporated mutations were confirmed by DNA sequencing at the Cleveland Clinic Core Facility. DNA containing the desired mutation was transformed into Escherichia coli for protein expression.

Oligonucleotides used to incorporate the S1412D mutation in nNOS were synthesized by Integrated DNA Technologies. A silent mutation was used to quantitate heme protein content using an extinction coefficient of 74 mM−1 cm−1 (4,445–4,500).

**NO Synthesis, NADPH Oxidation, Ferricyanide Reduction, and Cytochrome c Reduction**—Steady-state activities of wild-type and mutant nNOS were determined separately at 25 °C using spectrophotometric assays that were described previously in detail (19). To assay catalytic activities versus Ca2+ concentration, the standard assay mixtures were modified to contain 20 mM EDTA, and different amounts of CaCl2 were added.

H2O-dependent NOHA Oxidation—H2O-dependent NOS oxidation of NOHA to nitrite was assayed in 96-well microplates at 25 °C as described previously (19) with modification. The assay volume contained 40 mM EPPS, pH 7.6, 250 mM NaCl, 1 mM NOHA, 1 mM dithiothreitol, 25 units/ml superoxide dismutase, 0.5 mM EDTA, and 4 mM H2B. Reactions were initiated by adding 30 mM H2O2 and stopped after 10 min by adding 1300 units of catalase. Nitrite was detected at 550 nm after adding the Griess reagent (100 µl) and quantitated based on nitrite standards.

**RESULTS**

The S1412D nNOS used in our study was purified to homogeneity and had the same molecular mass as wild-type nNOS (Fig. 1). This rules out potential artifacts due to contaminating reductase domain. The mutant displayed the same absorbance spectrum in the presence of Arg and H2B as wild type, and its modified to contain 20 mM EDTA, and different amounts of CaCl2 were added.

H2O2-dependent NOHA Oxidation—H2O-dependent NOS oxidation of NOHA to nitrite was assayed in 96-well microplates at 25 °C as described previously (19) with modification. The assay volume contained 40 mM EPPS, pH 7.6, 250 mM NaCl, 1 mM NOHA, 1 mM dithiothreitol, 25 units/ml superoxide dismutase, 0.5 mM EDTA, and 4 mM H2B. Reactions were initiated by adding 30 mM H2O2 and stopped after 10 min by adding 1300 units of catalase. Nitrite was detected at 550 nm after adding the Griess reagent (100 µl) and quantitated based on nitrite standards.
ferrous CO-bound spectrum contained a Soret peak at 445 nm (data not shown). These properties indicate that mutation of Ser-1412 did not perturb enzyme structure, substrate, and prosthetic group binding or heme electronic environment.

**NOS Catalytic Activities**—We compared catalysis by wild-type and S1412D nNOS by measuring their rates of H$_2$O$_2$-dependent NOHA oxidation, cytochrome c or ferricyanide reduction, and NO production. The wild-type and mutant enzymes catalyzed H$_2$O$_2$-dependent oxidation of NOHA at similar rates (8.4 and 9.0 min$^{-1}$, respectively), consistent with their oxygenase domains being identical and this reaction not requiring electrons from the reductase domain. Both cytochrome c and ferricyanide reduction was faster in the mutant compared with wild-type in the presence or absence of Ca$^{2+}$/CaM (Table I). The mutant did not synthesize NO without CaM and with CaM had a rate of NO synthesis that was 25% diminished compared with wild-type enzyme (Table I). We conclude that the mutant displays slower NO synthesis, but this cannot be explained by defects in oxygenase domain catalysis or electron transfer through the reductase domain.

Electron flux between the reductase and oxygenase domains can be a critical determinant of NO synthesis (9, 11, 13–15). We therefore compared steady-state rates of NADPH oxidation by CaM-bound S1412D and wild-type nNOS under a number of different conditions (Table II). S1412D nNOS had faster rates of NADPH oxidation than wild-type in all cases. This is consistent with the mutant catalyzing faster electron transfer from its reductase domain to external acceptors such as ferricyanide or cytochrome c. However, it cannot account for the observed decrease in NO synthesis. In fact, the concomitant decrease in NO synthesis and increase in NADPH oxidation led to a more uncoupled activity during the steady state, with the calculated NADPH/NO ratio rising to 3.5 for the mutant instead of 2 for wild-type nNOS (Tables I and II).

**Ca$^{2+}$-dependent Activation of Wild-type and Mutant nNOS**—Two different groups (16, 17) reported that phosphorylation at Ser-1412 did not perturb enzyme structure, substrate, and prosthetic group binding or heme electronic environment.

Two different groups (16, 17) reported that phosphorylation at the analogous residue in eNOS (Ser-1179) or substitution with Asp increased enzyme affinity toward Ca$^{2+}$ during CaM activation of NO synthesis. As shown in Fig. 2, at lower Ca$^{2+}$ concentrations the nNOS mutant showed higher steady-state NO synthesis rates compared with wild-type, but this disappeared sharply as Ca$^{2+}$ concentrations increased. The greatest difference in activity was seen at 13 μM Ca$^{2+}$ (Fig. 2, upper inset), which was a suboptimal concentration for both enzymes. In contrast, when the experiment was repeated using cytochrome c reduction to measure activity, mutant activity remained consistently higher than wild-type across the same range of Ca$^{2+}$ concentrations. We conclude that the mutation causes enhanced response to Ca$^{2+}$ in nNOS, but this only occurs at suboptimal Ca$^{2+}$ concentrations and only when activity is measured by steady-state NO synthesis.

**Heme and Flavin Reduction**—We next examined the kinetics of flavin and heme reduction at 10 °C by stopped-flow spectrophotometry. Observed rate constants for heme and flavin reduction are listed along with other data in Tables III and IV. Traces in Fig. 3 panel A illustrate heme reduction in mutant and wild-type nNOS in response to CaM. Heme reduction was monophasic and achieved the same extent of reduction in both enzymes but was 1.4 times faster in the mutant. Thus, a slower steady-state NO synthesis activity in the mutant is actually associated with a faster rate of heme reduction. Panels B and C compare rates of flavin reduction in the presence or absence of CaM. Flavin reduction was biphasic in all cases and was faster in the mutant only in the absence of CaM. This indicates that faster cytochrome c reduction in the mutant is only associated with faster flavin reduction in the CaM-free state.

**Relationships between Flavin and Heme Reduction and Enzyme Behavior**—To understand better the mutant phenotype, we examined how different rates of flavin and heme reduction would impact mutant behaviors relative to wild type. For this purpose we utilized two native soybean calmodulins (ScaM1 and ScaM4) and a point mutant of ScaM1 (M144V), which were shown by Johnson and colleagues (20, 21) to bind to nNOS with good affinity but support different rates of cytochrome c reduction and NO synthesis in the steady state. Our hope was that these “CaM analogs” would behave like CaM-troponin C chimeras in supporting different rates of flavin and heme reduction in mutant and wild-type nNOS (13). As shown in Tables III and IV, this was the case because the ScaM proteins and CaM each supported different rates of flavin and heme reduction in the two enzymes.

Table III shows how different rates of flavin reduction affect cytochrome c reductase activity in mutant and wild-type nNOS. Flavin reduction was biphasic in all cases, with each phase representing one-third to two-thirds of the total absorbance change depending on the CaM used in the experiment. Although the two nNOS started with different basal rates of flavin reduction, they achieved identical rates in response to each CaM and thus showed the same rank order of response (CaM > ScaM4 > M144V ScaM1 > ScaM1) and range of enhancement (100% with CaM to 50% with ScaM1). In both

---

**TABLE I**

Comparative analysis of NO synthesis, cytochrome c reduction, and ferricyanide reduction in wild type and S1412D nNOS

|          | NO synthesis | Cytochrome c reduction | Ferricyanide reduction |
|----------|--------------|------------------------|------------------------|
|          | +CaM         | –CaM                   | +CaM                   | –CaM                   |
| Wild type| 55 ± 5       | 0                      | 5523 ± 500             | 452 ± 40               |
|          |              |                        |                        | 5000 ± 500             | 937 ± 190               |
| S1412D   | 43 ± 4       | 0                      | 9500 ± 700             | 1075 ± 100             |
|          |              |                        |                        | 6631 ± 664             | 1611 ± 139              |

**FIG. 1.** Purification of S1412D nNOS followed by SDS-polyacrylamide gel electrophoresis. Lane 1, Coomassie-stained molecular mass markers; lane 2, wild-type nNOS standard; lanes 3–6, S1412D mutant after final 2',5'-ADP purification step, after elution from the Ni$^{2+}$-nitrilotriacetic acid resin; after ammonium sulfate precipitation, and after the crude extract, respectively.
Table II

Comparative analysis of NADPH oxidation in wild type and S1412D nNOS

Turnover number is expressed as mole of NADPH oxidized per mole of heme per min. Rates were measured at 25 °C. The values are the mean and S.D. for three measurements each.

| System                  | Wild type nNOS | S1412D   |
|-------------------------|----------------|----------|
| - H4B, -Arg             | 381 ± 30       | 508 ± 26 |
| + H4B, -Arg             | 499 ± 17       | 695 ± 5  |
| - H4B, +Arg             | 106 ± 10       | 142 ± 11 |
| + H4B, +Arg             | 105 ± 4        | 157 ± 15 |

Table III

Correlation between rates of cytochrome c reduction and flavin reduction in wild type and S1412D nNOS

Cytochrome c reduction was measured at 25 °C, and flavin reduction was measured at 10 °C as detailed under "Experimental Procedures." Values represent the mean and S.D. for three measurements each.

| System                  | Cytochrome c reduction | Flavin reduction |
|-------------------------|------------------------|-----------------|
|                         | min⁻¹                  | k₁             | s⁻¹           | k₂             |
| nNOS-CaM                | 452 ± 40               | 2.3 ± 0.2 (18%)| 0.9 ± 0.1 (82%)|
| nNOS + CaM              | 6000 ± 500             | 23 ± 2.1 (54%) | 3.4 ± 0.3 (46%)|
| nNOS + ScaM4            | 4200 ± 300             | 17 ± 1.1 (60%) | 2.2 ± 0.2 (46%)|
| nNOS + ScaM1V144M       | 4100 ± 300             | 16.6 ± 1.5 (54%)| 1.9 ± 0.2 (46%)|
| nNOS + ScaM1            | 3400 ± 300             | 13.7 ± 1.5 (40%)| 1.7 ± 0.1 (60%)|
| S1412D-CaM              | 1075 ± 50              | 5.58 ± 0.4 (37%)| 1.7 ± 0.2 (63%)|
| S1412D + CaM            | 9000 ± 500             | 22 ± 2.2 (51%) | 3.4 ± 0.2 (49%)|
| S1412D + ScaM4          | 7500 ± 500             | 17 ± 1.0 (35%) | 2.3 ± 0.2 (66%)|
| S1412D + ScaM1V144M     | 7200 ± 400             | 16 ± 1.2 (69%) | 2.0 ± 0.1 (66%)|
| S1412D + ScaM1          | 6000 ± 500             | 12.8 ± 1.1 (45%)| 1.6 ± 0.1 (55%)|
Characterization of S1412D nNOS

FIG. 4. Correlation between flavin reduction rate and cytochrome c reductase activity. Rates were obtained from Table III. Open and solid symbols denote wild-type and S1412D nNOS, respectively. The enzymes contained wild-type CaM (○ and A), SCaM-4 (□ and □), SCaM-V144M (△ and △), and SCaM-1 (○ and ●). In the absence of CaM the flavin reduction rates are denoted by ∨ and ▼. The lines represent the calculated best fit of values obtained for the CaM- and SCaM-bound enzymes.

FIG. 3. Kinetics of heme reduction (panel A) and flavin reduction (panels B and C) in wild-type and S1412D nNOS. Stopped-flow traces for wild-type and S1412D nNOS are denoted by solid and dotted lines, respectively. The S1412D traces also contain a calculated line of best fit (dashed) in panels A and C. Heme reduction was detected by CO binding, and the kinetics were determined from the change in absorbance at 444 nm with time. CaM-bound enzymes were rapidly mixed with excess NADPH to trigger flavin and heme reduction at 10 °C under anaerobic conditions as described under “Experimental Procedures.” Flavin reduction was followed at 485 nm under anaerobic conditions at 10 °C after mixing excess NADPH with CaM-bound (panel B) or CaM-free (panel C) nNOS proteins. Data are an average of 7–10 individual scans.

DISCUSSION

The S1412D mutant is quite valuable because it helps demonstrate how rates of flavin and heme reduction relate to the initial and steady-state catalytic behavior of nNOS. In the steady state, the mutant displayed increased NADPH oxidase and cytochrome c reductase activities but somewhat slower NO synthesis. Remarkably, this differed from the analogous S1179D eNOS mutant, which displayed an increase in all three activities (16–18). We first discuss how the S1412D mutation alters electron transfer in and out of the nNOS reductase domain, and then we discuss the effect of the mutation on heme reduction in light of our kinetic simulation model (see accompanying paper by Santolini et al. (12)) that can explain the behavior of the S1412D nNOS and why it differs from the analogous eNOS mutant. Reductase Domain Electron Transfer—Before discussing the effects of the mutation, it is important to review some general properties of reductase domain catalysis. All NOS reductase domains are composed of an NADPH-FAD module and an FMN module (22). As established for nNOS by Mayer and co-workers (23) and Gachhui et al. (24), electron transfer to cytochrome c and ferricyanide occurs via direct electron transfer from the reductase domain and does not require heme reduction or superoxide generation. Cytochrome c binds with high affinity to the FMN module and accepts electrons exclusively from this site, whereas ferricyanide accepts electrons from either FAD or FMN (9, 23–25).

Our results are the first to show that modification of Ser-1412 alters both flavin reduction and reductase activities in any NOS. However, it is important to note that S1412D nNOS displayed faster flavin reduction relative to wild type only in the CaM-free state, and this was associated with faster reduction of cytochrome c and ferricyanide. Upon CaM binding, the mutant lost its advantage regarding speed of flavin reduction but still displayed greater cytochrome c reductase activity than wild type. This relationship held when any of three ScA proteins substituted for CaM. We conclude that electron transfer to cytochrome c is directly related to the speed of flavin reduction (see Table IV). However, in CaM-bound nNOS Ser-1412 primarily influences this activity through a mechanism that does not involve changing the speed of flavin reduction. Although the details are not understood, we suspect that phosphorylation at Ser-1412 alters a physical interaction in the FMN module. One mechanism would have the mutation make the FMN group more accessible to external electron acceptors, and we are currently testing this possibility. Because Ser-1412 is in the NADPH-FAD module and therefore “upstream” from the FMN module, the Asp mutation must alter an interaction between these two modules even in the presence of CaM. Recent data suggest the C terminus of the NADPH-FAD module could be a candidate for interacting with the FMN module (26). In any case, the S1412D mutation seems to speed electron transfer to the nNOS heme and to cytochrome c by the same mechanism, because changes in these two parameters correlate precisely in the mutant and wild-type enzymes (see Tables III and IV).

The S1412D mutation may increase electron transfer from NADPH to FAD in CaM-free nNOS by a different mechanism. We suspect the S1412D mutation partly relieves a repression that is exerted by the FMN module on the NADPH-FAD module. This repression of “upstream” electron transfer was first described by Adak et al. (19), who showed that several FMN module mutants had faster electron transfer between NADPH and FAD. The repression in wild-type nNOS is fully relieved by CaM binding or by FMN removal (19), and our current results show that the ScA proteins can also relieve the repression but to lesser degrees than CaM. It is also clear that phosphorylation of Ser-1412 should only provide partial relief of repression.
Characterization of S1412D nNOS

Catalytic behaviors of wild type and S1412D nNOS as a function of the heme iron reduction rate

Different heme reduction rates were achieved using CaM and three SCaM proteins. NO synthesis was measured at 25 °C, and the other parameters were measured at 10 °C. Measurements and analyses are detailed under “Experimental Procedures.” The values are the mean and S.D. for three measurements. The numbers in parentheses indicate the relative proportion of absorbance change for each phase of ferrous-NO complex formation.

| System          | Heme reduction | Steady-state NO synthesis | Ferrous-NO complex formation | % Fe²⁺ of NO complex |
|-----------------|----------------|---------------------------|------------------------------|----------------------|
|                 | s⁻¹            | min⁻¹                     | k₁, s⁻¹                      |                      |
| nNOS + CaM      | 3.9 ± 0.3      | 57 ± 4                    | 7.3 (35%)                    | 65                   |
| nNOS + SCaM     | 3.4 ± 0.2      | 55 ± 4                    | 4.1 (15%)                    | 61                   |
| nNOS + SCaM1V144M | 2.9 ± 0.2      | 29 ± 2                    | 2.6 (10%)                    | 56                   |
| nNOS + SCaM1    | 2.0 ± 0.2      | 17 ± 1                    | 1.7 (30%)                    | 40                   |
| S1412D + CaM    | 5.4 ± 0.4      | 45 ± 3                    | 9.2 (35%)                    | 72                   |
| S1412D + SCaM    | 4.6 ± 0.3      | 69 ± 5                    | 5.5 (15%)                    | 67                   |
| S1412D + SCaM1V144M | 3.2 ± 0.2      | 35 ± 3                    | 3.0 (20%)                    | 61                   |
| S1412D + SCaM1   | 2.8 ± 0.2      | 28 ± 2                    | 2.3 (33%)                    | 48                   |

Fig. 5. The rate of heme-NO complex formation (panel A), the amount of heme-NO complex attained during steady state (panel B), and the rate of steady-state NO synthesis (panel C) measured as a function of heme reduction rate. Data were obtained from Table IV. Graphs include all measurements for wild-type (open symbols) and S1412D nNOS. Panel A shows both fast and slow phases of heme-NO complex buildup with calculated lines of best fit. The enzymes contained wild-type CaM ( ), SCaM-4 ( ), SCaM-V144M (Δ and ▽), and SCaM-1 ( and ●).

fied, a good candidate is a 40-amino acid insert present in the FMN module of nNOS that inhibits ferricyanide and cytochrome c reduction in the CaM-free state (27–29). Perhaps this element inhibits electron transfer between NADPH and FAD through interaction with Ser-1412.

Catalytic Control in S1412D nNOS—From the perspective of NO synthesis there is only one relevant change in the S1412D mutant, its faster heme reduction. But our analysis of catalytic parameters shows apparent contradictions. Indeed, how can a faster heme reduction give rise to greater heme-NO complex formation yet slower NO synthesis in the steady state? These paradoxical effects are in fact related to heme-NO complex formation during NO synthesis and can be understood within the context of our kinetic model for nNOS (12). An abbreviated kinetic model is illustrated in Fig. 6 and involves a forward pathway that generates the ferric nNOS heme-NO complex and citrulline as products (10). This heme-NO species then partitions between two paths to regenerate ferric enzyme. One involves NO dissociation from the ferric heme (step B), and the other involves reduction of the heme-NO species to form the ferrous heme-NO complex (step A'). Importantly, the reductive path is futile because little or no NO is released from the ferrous heme-NO complex before it reacts with oxygen (step C).

Thus, partitioning between productive and futile pathways reflects relative rates of NO dissociation from the ferric heme versus its reduction to the ferrous heme-NO species. Because NO dissociation and heme reduction occur at similar speeds in nNOS (−5 and 3.6 s⁻¹ at 10 °C, respectively), and oxidation of the ferrous heme-NO complex is comparatively slow (−0.19 s⁻¹), a majority of nNOS is present as the ferrous heme-NO complex during steady-state NO synthesis (6–8).

Computer simulations of our kinetic model use rate constants derived for nNOS at 10 °C that are described in the accompanying article (12). As shown there, simulations accurately reproduce the initial and steady-state behaviors of wild-type nNOS and the behaviors of the hyperactive mutant W409F (7, 30). We therefore utilized the kinetic model to understand the pre-steady-state and steady-state behaviors of S1412D nNOS. Because this mutation is in the reductase domain, it is reasonable to assume that it only affects the speed of heme reduction. In fact, several data obtained with mutant and wild-type nNOS distributed along a single line when plotted as a function of heme reduction rate (see Fig. 5) strongly supported this assumption. The nNOS heme can receive an electron from the reductase domain at two different steps in the kinetic model as follows: during NO biosynthesis (step A in Fig.

Table IV

| System          | Heme reduction | Steady-state NO synthesis | Ferrous-NO complex formation | % Fe²⁺ of NO complex |
|-----------------|----------------|---------------------------|------------------------------|----------------------|
|                 | s⁻¹            | min⁻¹                     | k₁, s⁻¹                      |                      |
| nNOS + CaM      | 3.9 ± 0.3      | 57 ± 4                    | 7.3 (35%)                    | 65                   |
| nNOS + SCaM     | 3.4 ± 0.2      | 55 ± 4                    | 4.1 (15%)                    | 61                   |
| nNOS + SCaM1V144M | 2.9 ± 0.2      | 29 ± 2                    | 2.6 (10%)                    | 56                   |
| nNOS + SCaM1    | 2.0 ± 0.2      | 17 ± 1                    | 1.7 (30%)                    | 40                   |
| S1412D + CaM    | 5.4 ± 0.4      | 45 ± 3                    | 9.2 (35%)                    | 72                   |
| S1412D + SCaM    | 4.6 ± 0.3      | 69 ± 5                    | 5.5 (15%)                    | 67                   |
| S1412D + SCaM1V144M | 3.2 ± 0.2      | 35 ± 3                    | 3.0 (20%)                    | 61                   |
| S1412D + SCaM1   | 2.8 ± 0.2      | 28 ± 2                    | 2.3 (33%)                    | 48                   |

Fig. 6. Kinetic model for nNOS catalysis. Both steps of NO biosynthesis (Arg hydroxylation followed by NOHA oxidation) are collapsed for simplicity. The biosynthetic pathway involves heme reduction (panel A) and O₂ binding and generates a ferric heme-NO complex and citrulline as products. The heme-NO product then partitions between NO release (panel B) versus reduction to the ferrous heme-NO complex (A'), which then reacts with O₂ (panel C) to regenerate ferric enzyme. See text for additional explanation and ref. 12 for a detailed kinetic model.
Characterization of S1412D nNOS

could fit the subsequent mono-exponential portion of each curve (which reflects the slow phase of heme-NO complex formation) to obtain simulated rates for heme-NO complex buildup at each heme reduction rate. As shown in panel A of Fig. 7, the simulated rates actually distribute along the same line as the experimental values for slow phase heme-NO complex buildup in S1412D and wild-type nNOS. Thus, results from the simulation are consistent with (a) a faster intrinsic NO synthesis for the S1412D mutant, (b) ferric heme-NO complex buildup prior to NO release, and (c) equivalent reduction rates for ferric and ferric-NO species within a given enzyme.

The amount of ferrous heme-NO complex attained at steady state also correlated with the rate of heme reduction (see Fig. 5). This parameter likely reflects enzyme partitioning between a productive regenerating cycle (release of NO from the ferric heme) and a futile one (reduction of the ferric-NO complex) (see Fig. 6). Thus, as heme reduction speeds up, the balance between these two paths should shift to favor more ferrous-NO complex formation and less NO release. This is well depicted by our model simulations. In Fig. 7, panel B, the measured and simulated percentages of ferrous heme-NO species present at steady state are plotted as a function of heme reduction rate. Although the simulated percentages are all slightly higher than the experimental values, both groups follow an identical distribution pattern that increases as heme reduction rate increases. Hence, an increase in the heme reduction rate will increase the percentage of enzyme that partitions into the futile cycle.

These two facets of enzyme catalysis (intrinsic rate of NO synthesis and enzyme partitioning toward the ferrous heme-NO complex) are both controlled by heme reduction, but they counterbalance one another to different degrees depending on the rate of heme reduction. Thus, their combined effect on steady-state NO synthesis is complex. This was noted in the accompanying paper by Santolini et al. (12) who simulated how steady-state NO synthesis varies with heme reduction rate. The analysis predicted that NO synthesis should increase, reach a maximum, and then fall as the rate of heme reduction increases. This pattern in fact matches what we observed here for wild-type and S1412D nNOS at different rates of heme reduction (see Fig. 5, panel C). Thus, our kinetic model can explain why we observed an optimum range of heme reduction for NO synthesis, and why the S1412D mutant has slower steady-state activity. Specifically, faster heme reduction in the mutant speeds its intrinsic NO formation (resulting in faster ferric heme-NO complex formation) but also causes a greater proportion of enzyme to partition into the futile cycle. Our data suggest that heme reduction rates between 3.5 and 4.5 s⁻¹ strike an optimal balance between these opposing parameters to enable peak NO synthesis by nNOS. Heme reduction in CaM-bound S1412D nNOS is 5.4 s⁻¹ and therefore is too fast to strike an optimal balance, resulting in slower NO synthesis during the steady state. However, when its heme reduction is slowed to 4.6 s⁻¹ by using ScaM4 in place of CaM, a near optimal balance is regained, and the rate of NO synthesis is increased.

Simulation of our kinetic model using currently available rate constants actually predicted an optimal rate for heme reduction between 2 and 3 s⁻¹ (12), which is slightly slower than the experimental optimum we observed here (about 3.8 s⁻¹, see Fig. 5, panel C). However, it is important to realize that the optimum rate of heme reduction depends on the following two facets of the kinetic mechanism, which themselves are functions of multiple reactions: 1) partitioning between productive and futile cycles, which depends on rates of ferric heme-NO formation, NO dissociation, and reduction of the ferric...
heme-NO complex, and 2) the relative rates of the productive and futile cycles, which depend on NO dissociation from the ferric heme and the reaction of O_2 with the ferrous heme-NO complex. Thus, the difference between simulated and experimental values for optimal rate of heme reduction is probably reasonable given such a complex system. Small inaccuracies in the kinetic constants if present can shift the optimum rate for heme reduction (see Fig. 5C in Ref. 12). Additional measurements and refinement of the simulation should reconcile the relatively small difference between the theoretical and experimental optimum and should also reveal mechanisms for “tuning” the optimum.

Kinetic simulations also predict that the rate of NADPH oxidation will reach a maximum as heme reduction rate increases in nNOS (12), but this parameter does not fall off as fast as NO synthesis. Thus, with the faster heme reduction there is a gradual decrease in catalytic efficiency. This is because faster heme reduction favors cycling through the futile cycle, which consumes NADPH without NO release and therefore leads to increased uncoupling between NADPH consumption and NO production. The simulation predicts S1412D nNOS should oxidize 3.65 NADPH per NO formed, which is quite similar to our experimental value of 3.5. These values are greater than the ratio for wild-type nNOS (the simulation predicts 2.8, the experimental value was 2.0) and signifies that a greater proportion of mutant enzyme cycles through the futile pathway during the steady state. In fact, the simulation predicts that 52% of S1412D nNOS cycles through the futile pathway in unit time, which is understandable given that its rate of heme reduction (5.4 s^{-1}) is about the same as its NO release (5 s^{-1}) and gives a partition coefficient near 1.

Another parameter that is affected by this complex regulation is enzyme apparent $K_m$ for O_2. The apparent $K_m$ for O_2 measured during steady state is actually a combined function of ferrous heme affinity for O_2 and the oxidation rate of the ferrous heme-NO complex, which depends differently on O_2 concentration (8). This gives wild-type nNOS a surprisingly high apparent $K_m$ for O_2 that enables NO release in a graded manner across the entire physiologic concentration range for O_2 (0–200 mM) (8). We ran simulations to examine how the S1412D mutant and wild-type nNOS might differ in their steady-state NO synthesis as a function of O_2 concentration (Fig. 7, panel C).

The rate of ferrous heme-NO oxidation was assumed to vary in direct proportion to the O_2 concentration, based on earlier data obtained with wild-type nNOS (8). The simulation predicts that the apparent $K_m$ value of the mutant will shift to a somewhat higher value (738 μM) compared with wild type (482 μM) (using heme reduction rates of 3.9 and 5.4 s^{-1}, respectively). This makes sense, because the increased rate of heme reduction of the mutant causes a greater buildup of ferrous heme-NO complex during steady state. In air-saturated buffer (~250 μM O_2), the mutant rate of NO synthesis is predicted to be 84% that of wild type, which is close to the 78% value calculated from our experimentally determined rates. As shown in the inset of Fig. 7, panel C, the simulation predicts the mutant will always display slower NO synthesis throughout the physiological concentration range of O_2. This is because oxidation of the ferrous heme-NO species remains rate-limiting for the futile cycle within this range of O_2 concentrations. However, as the O_2 concentration further increases to very high levels, the oxidation rate of the ferrous heme-NO complex is predicted to eventually become faster than the rate of heme reduction. At this point, the rate-limiting step in the futile cycle would switch to heme reduction, and under this circumstance the simulation predicts NO synthesis by the mutant would become faster than wild-type nNOS (Fig. 7, panel C).

**Catalytic Control in eNOS and in Other nNOS Mutants—** Our kinetic model may also explain why the analogous mutation in eNOS (S1179D) increased steady-state NO synthesis along with NADPH oxidation and cytochrome c reduction (16–18). Basically, heme reduction in eNOS is much slower than in nNOS (<0.1 s^{-1} at 10 °C; Ref. 11) and thus is in the range where increasing it should speed NO synthesis according to the kinetic model (12).

Two other reductase domain mutants have a similar catalytic phenotype as we observed here for S1412D nNOS. Both are nNOS deletion mutants, one missing the final 33 C-terminal residues (32) and the other missing a 42-residue insertion that is present in the FMN module (28). NADPH oxidation and cytochrome c reductase activities increased in both mutants relative to wild type, whereas their steady-state NO synthesis was either slower (28) or about the same (32). Rates of flavin and heme reduction were measured in the N-terminal deletion mutant under aerobic conditions and were found to be faster than wild type (32). However, neither group was able to explain the catalytic behavior of these mutants. We suspect that their behaviors reflect an increase in heme reduction rate above the optimum value for nNOS and will ultimately be understood based on our kinetic model.

**Biologic Implications—** There is no obvious advantage to speeding heme reduction in rat nNOS, because it already functions near its optimum rate and is predicted to have faster steady-state NO synthesis than the S1412D mutant throughout the physiologic O_2 concentration range. Indeed, our results suggest that phosphorylation will increase NO synthesis relative to native enzyme only within a narrow Ca^{2+} concentration range that is suboptimal for full CaM binding. Thus, the Ser-1412 phosphorylation site in rat nNOS is of uncertain physiologic consequence. On the other hand, eNOS is set to respond differently to phosphorylation by increasing NO synthesis in response to a faster heme reduction rate. It will be interesting to see if auxiliary proteins that increase eNOS activity such as HSP-90 also act by speeding heme reduction. For nNOS, other mechanisms are available to increase its steady-state NO synthesis. For example, in nNOS mutants W409F and W409Y the ferrous heme-NO complex reacts 7 times faster with O_2 (7), and hyperactivity results despite their heme reduction being slower than wild type (7). Although factors that influence ferrous heme-NO complex oxidation are not identified, our analysis suggests that modulation of this parameter would be a good strategy to up-regulate NO release by nNOS.

**Acknowledgments—** We thank Abby Meade, Ritsu Kondo, and D. J. Black for excellent assistance.

**REFERENCES**

1. Dawson, V. L., and Dawson, T. M. (1998) **Prog. Brain Res.** 118, 215–229
2. Michel, T., and Feron, O. (1997) **J. Clin. Invest.** 100, 2417–2425
3. Marletta, M. A., Hurshman, A. R., and Rusche, K. M. (1998) **Curr. Opin. Chem. Biol.** 2, 656–663
4. Stuehr, D. J. (1999) **Biochim. Biophys. Acta** 1411, 217–230
5. Stuehr, D. J. (1995) **Proc. Natl. Acad. Sci. U. S. A.** 90, 10769–10772
6. Abu-Soud, H. M., Wang, J., Rousseau, D. L., Fukuto, J., Ignaaro, L. J., and Stuehr, D. J. (1995) **J. Biol. Chem.** 270, 22997–23006
7. Adak, S., Wang, Q., and Stuehr, D. J. (2000) **J. Biol. Chem.** 274, 17344–17349
8. Abu-Soud, H. M., Rousseau, D. L., and Stuehr, D. J. (1996) **J. Biol. Chem.** 271, 32515–32518
9. Miller, R. T., Martasek, P., Omura, T., and Masters, B. S. S. (1999) **Biochem. Cell Biol. Commun.** 265, 184–188
10. Boggs, S., Huang, L., and Stuehr, D. J. (2000) **Biochemistry** 39, 2323–2339
11. Abu-Soud, H. M., Ichimori, K., Presta, A., and Stuehr, D. J. (2000) **J. Biol. Chem.** 275, 17349–17357
12. Santolini, J., Adak, S., Curran, C. M. L., and Stuehr, D. J. (2001) **J. Biol. Chem.** 276, 12253–12263
13. Gachhui, R., Abu-Soud, H. M., Ghosh, D. K., Presta, A., Blazing M. A., Mayer, B., George, S. E., and Stuehr, D. J. (1998) **J. Biol. Chem.** 273, 5451–5454

3 S. Adak and D. J. Stuehr, unpublished results.
14. Stevens-Truss, R., Beckingham, K., and Marletta, M. A. (1997) Biochemistry 36, 12337–12345
15. Nishida, C. R., and Ortiz de Montellano, P. R. (1998) J. Biol. Chem. 273, 5566–5571
16. Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., Franke, T. F., Papapetropoulos, A., and Sessa, W. C. (1999) Nature 399, 597–601
17. Dimmelser, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A. M. (1999) Nature 399, 601–605
18. McCabe, T. J., Fulton, D., Roman, L. J., and Sessa, W. C. (2000) J. Biol. Chem. 275, 6123–6128
19. Adak, S., Ghosh, S., Abu-Soud, H. M., and Stuehr, D. J. (1999) J. Biol. Chem. 274, 22313–22320
20. Cho, M. J., Vaghy, P. L., Kondo, R., Lee, S. H., Davis, J. P., Rehl, R., Heo, W. D., and Johnson, J. D. (1998) Biochemistry 37, 15593–15597
21. Kondo, R., Tikunova, S. B., Cho, M. J., and Johnson, J. D. (1999) J. Biol. Chem. 274, 36213–36218
22. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., and Snyder, S. H. (1991) Nature 351, 714–718
23. Klatt, P., Heinzel, B., John, M., Karsner, M., Bohme, E., and Mayer, B. (1992) J. Biol. Chem. 267, 11374–11378
24. Gachhui, R., Presta, A., Bentley, D. F., Abu-Soud, H. M., McArthur, R., Brudvig, G., Ghosh, D. K., and Stuehr, D. J. (1996) J. Biol. Chem. 271, 20594–20602
25. Richards, M. K., Clague, M. J., and Marletta, M. A. (1996) Biochemistry 35, 7772–7780
26. Roman, L. J., Miller, R. T., de la Garza, M. A., Kim, J. J. P., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 21914–21919
27. Salerno, J. C., Harris, D. E., Irizarry, K., Patel, B., Morales, A. J., Smith, S. M. E., Martasek, P., Roman, L. J., Masters, B. S. S., Jones, C. L., Weissman, B. A., Lane, P., Liu, Q., and Gross, S. S. (1997) J. Biol. Chem. 272, 29769–29777
28. Daff, S., Sagami, I., and Shimizu, T. (1999) J. Biol. Chem. 274, 30589–30595
29. Nishida, C. R., and Ortiz de Montellano, P. R. (1999) J. Biol. Chem. 274, 14692–14698
30. 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
31. Scheele, J. S., Bruner, E., Kharitonov, V. G., Martasek, P., Roman, L. J., Masters, B. S. S., Sharma, V. S., and Magde, D. (1999) J. Biol. Chem. 274, 15105–15110
32. Roman, L. J., Martasek, P., Miller, R. T., Harris, D. E., de la Garza, M. A., Shea, T. M., Kim, J. J. P., and Masters, B. S. S. (2000) J. Biol. Chem. 275, 29225–29232