Chimeras of Nitric-oxide Synthase Types I and III Establish Fundamental Correlates between Heme Reduction, Heme-NO Complex Formation, and Catalytic Activity*

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Neuronal nitric-oxide synthase (nNOS or NOS I) and endothelial NOS (eNOS or NOS III) differ widely in their reductase and nitric oxide (NO) synthesis activities, electron transfer rates, and propensities to form a heme-NO complex during catalysis. We generated chimeras by swapping eNOS and nNOS oxygenase domains to understand the basis for these differences and to identify structural elements that determine their catalytic behaviors. Swapping oxygenase domains did not alter domain-specific catalytic functions (cytochrome c reduction or H2O2-supported Nω-hydroxy-L-arginine oxidation) but markedly affected steady-state NO synthesis and NADPH oxidation compared with native eNOS and nNOS. Stopped-flow analysis showed that reductase domains either maintained (nNOS) or slightly exceeded (eNOS) their native rates of heme reduction in each chimera. Heme reduction rates were found to correlate with the initial rates of NADPH oxidation and heme-NO complex formation, with the percentage of heme-NO complex attained during the steady state, and with NO synthesis activity. Oxygenase domain identity influenced these parameters to a lesser degree. We conclude: 1) Heme reduction rates in nNOS and eNOS are controlled primarily by their reductase domains and are almost independent of oxygenase domain identity. 2) Heme reduction rate is the dominant parameter controlling the kinetics and extent of heme-NO complex formation in both eNOS and nNOS, and thus it determines to what degree heme-NO complex formation influences their steady-state NO synthesis, whereas oxygenase domains provide minor but important influences. 3) General principles that relate heme reduction rate, heme-NO complex formation, and NO synthesis are not specific for nNOS but apply to eNOS as well.

Nitric oxide (NO)1 is generated by nitric-oxide synthases (NOSs) and has multiple functions in physiology and pathology

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1 The abbreviations used are: NO, nitric oxide; NOS, NO synthase; CaM, calmodulin; H2B, (6R)-5,6,7,8-tetrahydro-L-biopterin; nNOS, neuronal NOS; eNOS, endothelial NOS; Arg, L-arginine; FMN, flavin mononucleotide; NOHA, Nω-hydroxy-L-arginine; Nω-FeNO, a chimera containing an nNOS oxygenase domain, an eNOS reductase domain, and a CaM binding site; EωNω-FeNO, a chimera containing an eNOS oxygenase domain, an nNOS reductase domain, and a CaM binding site; PCR, polymerase chain reaction; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.
nNOS and characterized their steady-state NO synthesis, cytochrome c reduction, and NADPH oxidation in response to Arg and H₂B. The authors concluded that the reductase domain controlled the rates of NO synthesis and cytochrome c reduction, whereas oxygenase domains controlled NADPH oxidation in response to Arg and H₂B. In our case, we hoped the chimeras would reveal how heme reduction, NO complex formation, and NO synthesis are related in eNOS and help us gauge to what extent reductase and oxygenase domains control these parameters in either NOS isoform. We examined flavin and heme reduction rates, heme-NO complex formation, and initial and steady-state catalytic behaviors of each chimera and compared these to data obtained with eNOS and nNOS. The results show how individual reductase and oxygenase domains regulate heme reduction and NO complex formation in eNOS and nNOS and how these two factors combine to regulate catalysis.

**Experimental Procedures**

**Materials**—All reagents and materials were obtained from Sigma or sources reported previously (21, 23).

**Molecular Biology**—Restriction digestions, cloning, bacterial growth, and restriction and isolation of DNA fragments were performed using standard procedures. Rat nNOS DNA and bovine eNOS DNA were inserted into the 5′-EcoRI and 3′-XbaI sites of the pCWori vector (23, 24). To create the chimeras we used site-directed mutagenesis to generate a unique restriction site between the end of the oxygenase domain and the beginning of the CaM binding domain in both bovine eNOS and rat nNOS. The unique restriction site Eco47III was incorporated at S°356–A°357 of eNOS and H°714–V°715 of nNOS. This created a silent mutation in eNOS and an His-Val→Ser-Ala mutation in nNOS. Sequence alignment using MacVector revealed that both Eco47III sites were located in identical positions in nNOS and eNOS. For making the Eco47III restriction site in eNOS, we used the QuikChange™ site-directed mutagenesis kit from Stratagene. The oligonucleotides used to construct the Eco47III site (underlined) in eNOS were synthesized by Integrated DNA Technologies, and their corresponding oligonucleotides were as follows: S°356-A°365-Eco47III sense, TGAAAGGGGCGCCCTACCAAGGGGCGCCGGCATTACA and S°356-A°365-Eco47III antisense, TGATGGCGCGCCCTTGATGCCGTCCCTTCCATCA. A RoboCycler gradient 96 from Stratagene was employed. The standard PCR cycling parameters were 5 min for denaturing of the template at 95 °C and 16 cycles for amplification (30 s for melting at 95 °C, 1 min for annealing at 60 °C, and 18 min for extension at 68 °C) followed by a 7-min extension at 68 °C. The protocol used – 50 ng of template, 20 pmol of each primer, 2 μl of 10 mM dNTPs, and 1.5 μl 2.5-unit Pfu polymerase in a final volume of 100 μl. The PCR product was digested by 1 μl of DpnI endonuclease and then transformed into Epicurian ColI XL1-Blue supercompetent cells. The Eco47III restriction site in the nNOS cDNA was constructed by exon shuffling of the generated fragments using a 3′-oligo containing a newly engineered Eco47III site. The nNOS fragment was obtained by PCR amplification using Pfu Turbo DNA polymerase (Stratagene), which possesses higher fidelity than other polymerases. The nNOS cDNA fragment coding from the Bpi unique restriction site 622 to the SanDI restriction site 2162 was amplified using the following primers: primer 1, CCTGGTCTGAGCATCCTCAA; primer 2, TGGGGGCTTCGGTTGGCTCCAAAGGGGCGCCGGCATCA. The PCR cycling parameters were 3 min for denaturing of the template at 95 °C and 28 cycles for amplification (30 s for melting at 95 °C, 1 min for annealing at 58 °C, and 6 min for extension at 68 °C) followed by a 60-min extension at 68 °C. The protocol used – 50 ng of template, 20 pmol of each primer, 2 μl of 10 mM dNTPs, and 1.5 μl 2.5-unit Pfu polymerase in a final volume of 100 μl. The PCR product and wild-type pCWori vector containing nNOS DNA were digested by both BpiI and SanDI restriction endonuclease enzymes, and fragments were isolated by 1% agarose gel. The double-digested fragment of wild-type NOS pCWori plasmid was replaced by the double-digested PCR fragment and transformed into JM109 cells to generate the recombinant plasmid. Both chimera proteins were expressed using the double restriction (NciI and Eco47III) digested fragments. Chimeric DNA constructs were confirmed by DNA sequencing at the Cleveland Clinic sequencing facility. Chimeric cDNAs in the pCWori plasmid were transformed into Escherichia coli strain BL21(DE3) for protein expression.

**Expression and Purification of Wild-type and Chimera Proteins**—Wild-type rat nNOS, bovine eNOS, and both chimera proteins (E₅₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁₆₋₃₋₆₋₁}
Reducase domain (25, 26) and indicates that, under this circumstance, reductase domain identity did not influence catalysis by the oxygenase domains.

Steady-state Catalysis—

NADPH-dependent cytochrome c reductase activity of each chimera in the presence or absence of CaM matched the activity of the NOS that provided its reductase domain (Table II). Thus, swapping oxygenase domains did not influence the reductase domain catalysis or response to CaM, which is consistent with previous results (12, 22). Steady-state NO synthesis activities of EoxNred and nNOS were identical and well coupled to NADPH oxidation (2.1 and 1.9 NADPH oxidized/NO formed, respectively) (Table II). In contrast, the steady-state NO synthesis activity of NoxEred was about one-third that of nNOS but was 33% greater than eNOS. NO synthesis by NoxEred and eNOS was also less coupled to their NADPH oxidation (4.4 and 4.0 NADPH oxidized/NO formed, respectively). Thus, the rates of NO synthesis and NADPH oxidation by each chimera equaled or approached the NOS isoform that provided its reductase domain.

Kinetics of Flavin and Heme Reduction—

We measured the rates of NADPH-dependent flavin and heme reduction in CaM-bound chimeras using stopped-flow spectroscopy under anaerobic conditions. Fig. 2 (left panels) depicts flavin reduction as an absorbance decrease at 485 nm versus time. Flavin reduction was biphasic in both chimeras and was somewhat faster in the chimera containing the eNOS reductase domain (Table III). This rate difference was also observed when comparing flavin and heme reduction in EoxNred and NoxEred. Stopped-flow traces for EoxNred and NoxEred are denoted by solid lines. The dotted lines are calculated lines of the best fit. Left panels, flavin reduction was followed at 485 nm under anaerobic conditions at 10 °C after mixing excess NADPH with CaM-bound EoxNred and NoxEred proteins. Right panels, heme reduction was detected by CO binding under anaerobic conditions, 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. The data shown are an average of 7–10 individual scans.

Fig. 1. SDS-polyacrylamide gel electrophoresis of chimeras and native eNOS and nNOS. Proteins were visualized with Coomassie stain. Lane 1, wild-type eNOS; lane 2, wild-type nNOS; lane 3, EoxNred after the final 2.5'-ADP purification step; lane 4, crude lysate of EoxNred; lane 5, NoxEred after the final 2.5'-ADP purification step; lane 6, crude lysate of NoxEred; lane 7, molecular weight markers.

Table I

| Enzyme | Nitrite/mole of enzyme |
|--------|-----------------------|
| nNOS   | 88 ± 2                |
| eNOS   | 30 ± 2                |
| EoxNred| 26 ± 2                |
| NoxEred| 90 ± 5                |

H$_2$O$_2$-dependent NOHA oxidation by wild-type NOSs and chimeras

Incubations were run for 10 min at 25 °C prior to quenching as described under “Experimental Procedures.” The values are the amount of product formed in 10 min and are the mean and S.D. for three measurements each.

Fig. 3. Light absorbance spectra recorded before and during NO synthesis at 15 °C. Three spectra in each panel were recorded in the following sequence: ferric enzyme in the presence of CaM, excess EDTA, 1 mm Arg, and 20 μM H$_2$B (dashed lines); after the addition of 160 μM NADPH to cause flavin reduction (dotted lines); and after the addition of excess Ca$^{2+}$ to initiate heme reduction and NO synthesis (solid lines). The insets show the difference spectra obtained by subtracting the final spectrum from the middle spectrum. Results are representative of three similar experiments.
Catalytic Regulation in eNOS and nNOS

Activity measurements were performed at 25 °C in the absence or presence of CaM as described under “Experimental Procedures.” Activities are expressed as moles of product formed/mole of heme/min. Values represent the mean and S.D. for three measurements.

| Protein          | Cytochrome c reduction | NO synthesis | NADPH oxidation |
|------------------|------------------------|--------------|-----------------|
|                  | +CaM -CaM              | +CaM -CaM   | +CaM            |
| Wild-type nNOS   | 5600 ± 500 452 ± 40    | 57 ± 2 0     | 110 ± 10        |
| Wild-type eNOS   | 500 ± 30 60 ± 2        | 15 ± 1 0     | 60 ± 6          |
| EoxNred          | 6000 ± 500 500 ± 50    | 56 ± 4 0     | 120 ± 8         |
| NoxEred          | 700 ± 100 80 ± 10      | 20 ± 2 0     | 88 ± 8          |

reduction in eNOS and nNOS (13). In contrast, heme reduction (as measured by CO binding) in NoxEred was 380 times slower than in EoxNred (Fig. 2, right panels). The heme reduction rate observed for EoxNred closely matched that reported for nNOS, and the rate seen with NoxEred was twice as fast as eNOS (Table III).

Heme-NO Complex Buildup—We compared heme-NO complex buildup in the chimeras during steady-state NO synthesis. Fig. 3 contains wavelength scans of nNOS, eNOS, and the two chimeras before and during NO synthesis at 15 °C. nNOS and EoxNred exhibited strong Soret absorbance positioned near 436 nm during steady-state NO synthesis, indicating their significant partitioning into a heme-NO complex. In contrast, NoxEred had a less prominent Soret absorbance at 436 nm in the steady state, indicating it formed less heme-NO complex, and eNOS showed very little absorbance gain in this region of the spectrum. Difference spectroscopy (Fig. 3, insets) confirmed that the heme-NO complex had a Soret peak at 436 nm and a broad visible absorbance at 560 nm in all cases, indicating that the heme-NO complex was predominantly ferrous. The estimated percentage of ferrous heme-NO complex present at steady state was ~70% in nNOS and EoxNred, ~25% in NoxEred, and ~12% in eNOS.

Kinetics of NADPH Oxidation and Heme-NO Complex Formation—We next utilized stopped-flow spectroscopy to investigate the kinetics and extent of heme-NO complex formation and their relationship to NADPH oxidation during the initial and steady-state phases of NO synthesis. In Fig. 4, absorbance changes at 436 and 340 nm were monitored versus the time to follow heme-NO complex formation and NADPH oxidation, respectively, in reactions run at 10 °C. In all cases, heme-NO complex buildup was best described as a biphasic process (Table IV, k1, k2). The rates of heme-NO complex formation were essentially the same in nNOS and EoxNred, whereas they were somewhat faster in NoxEred compared with eNOS. The apparent k1 values for nNOS and EoxNred were four and six times faster than k1 values for NoxEred and eNOS, respectively. The apparent k2 values for nNOS and EoxNred were 14 and 8 times faster than k2 values for NoxEred and eNOS, respectively. The percentage of heme-NO complex at steady state was estimated from the stopped-flow data and displayed the rank order nNOS > EoxNred > NoxEred > eNOS (Table IV).

The initial rates of NADPH oxidation during heme-NO complex formation (Table IV, m1 values) followed a rank order of nNOS > EoxNred > NoxEred > eNOS. Subsequent heme-NO complex buildup in nNOS and in EoxNred was associated with a slowing down of their NADPH oxidation rates (Fig. 4) such that they were ~seven and five times slower than the initial rates, respectively, once reaching the steady state (Table IV, m2 values). In contrast, NoxEred showed no discernable change in its NADPH oxidation rate between initial and steady-state phases of NO synthesis, and for eNOS the NADPH oxidation rate slightly increased (Table IV; Fig. 4).

**DISCUSSION**

Although nNOS and eNOS are both expressed constitutively and become activated by Ca2+/CaM binding, they differ markedly in their reductase and NO synthesis activities, electron transfer rates, and propensities to form a heme-NO complex during catalysis (12–14, 17, 18, 27). We generated nNOS-eNOS chimeras with swapped oxygenase domains to understand how electron transfer, heme-NO complex formation, and NO synthesis activities are related in eNOS and nNOS and to identify structural features that underpin their different catalytic behaviors.

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*The initial absorbance decrease at 444 nm seen in the EoxNred trace is caused by flavin reduction (14).*
Catalytic Regulation in eNOS and nNOS

TABLE III

Observed rate constants for NADPH-dependent flavin and heme reduction

| Enzyme | Flavin reduction | Heme reduction | Reference |
|--------|------------------|----------------|-----------|
|        | k1 (s⁻¹) | k2 (s⁻¹) | kr (s⁻¹) |                              |
| eNOS   | 85 ± 10 | 3.4 ± 0.3 | 0.005 ± 0.001 | 13 |
| nNOS   | 23 ± 2.1 | 3.8 ± 0.3 | 3.9 ± 0.3 | 21 |
| EoxNred | 22 ± 2.2 | 2.6 ± 0.3 | 0.011 ± 0.001 | In text |
| NoxNred | 44 ± 3.2 | 2.6 ± 0.3 | In text |

TABLE IV

Kinetics of heme-NO complex formation and NADPH oxidation after initiating NO synthesis

| Enzyme | NADPH oxidation | Heme-NO complex formation | Heme-NO complex |
|--------|-----------------|---------------------------|-----------------|
|        | m1 (min⁻¹) | m2 (min⁻¹) | k1 (s⁻¹) | k2 (s⁻¹) | % |
| nNOS   | 203 ± 36 | 29 ± 5 | 7.3 ± 1 (35%) | 1.6 ± 0.2 (65%) | 65 ± 3 |
| eNOS   | 7 ± 1 | 16 ± 2 | 1.3 ± 0.3 (33%) | 0.2 ± 0.03 (67%) | 12 ± 2 |
| EoxNred | 155 ± 20 | 29 ± 6 | 7.5 ± 1 (30%) | 2.0 ± 0.3 (70%) | 59 ± 4 |
| NoxNred | 34 ± 8 | 34 ± 8 | 2.0 ± 0.2 (50%) | 0.14 ± 0.02 (50%) | 25 ± 5 |

Steady-state cytochrome c reductase and NO synthesis activities of each chimera most closely matched the NOS isoforms that provided its reductase domain. This implies that each reductase domain maintained its native catalytic functions and was the primary determinant of NO synthesis activity. Nishida and Ortiz de Montellano (12, 22) reached identical conclusions using eNOS-nNOS chimeras that were similar but not identical to ours.3 Our study extends their work by providing data on electron transfer rates, steady-state behaviors, and heme-NO complex formation, which considered together can explain the catalytic profiles of nNOS, eNOS, and their chimeras.

A central finding is that eNOS and nNOS reductase domains essentially maintained their native electron transfer rates to NOS ferric heme in both chimeras. Thus, in this regard reductase domains of eNOS and nNOS can interact equally well with either oxygenase domain. In eNOS the reductase domain catalyzes slow electron transfer from its FMN group to the ferric heme, whereas the nNOS reductase domain is much faster (13, 17). Heme reduction is not limited by slow flavin reduction in either eNOS (13, 17), nNOS (27), or the chimeras (see Table III) when CaM is bound. Together, this establishes that no distinct structural or electronic features exist in the oxygenase domains of eNOS and nNOS to control their different heme reduction rates. Our data support the concept of a common docking site for the FMN module being present on nNOS and eNOS oxygenase domains. A putative docking site has been suggested on the basis of surface homology mapping and is made up of basic and nonpolar surface residues (28, 29). Our data also establish that electron transfer from FMN to heme is controlled almost exclusively by structural and/or electronic features inherent in each NOS reductase domain. Conceivably, these could include nonconserved regions both in and away from the FMN module. We are making second generation chimeras to help identify the key structural elements.

Our previous work (13–15) showed that NO synthesis by nNOS causes significant heme-NO complex buildup, whereas in eNOS very minor heme-NO complex formation is observed. The chimeras help to identify what factors control different heme-NO complex formation in nNOS and eNOS. First, we found that the four enzymes had the same rank order regarding their rates of heme reduction and rates of heme-NO complex formation (nNOS = EoxNred > NoxNred > EoxNred > eNOS; see Tables III and IV). This is consistent with nNOS single turnover experiments that show its ferric heme binds newly formed NO before releasing it (20) and suggests that this process also occurs for the eNOS heme. The data also provide a direct indication that ferric heme reduction limits the rate of NO synthesis in all four proteins. Indeed, the speeds of biosynthetic steps that follow ferric heme reduction (O₂ binding, transfer of a second electron, chemical transformations, and product release), when measured individually, have all been faster than this initial step (19).

The percentage of heme-NO complex observed at steady state also correlated with the rate of ferric heme reduction in all four proteins. The percentages calculated from stopped-flow traces ranged from 12% of total enzyme for eNOS to 65% for nNOS, with the chimeras falling in between. These percentages generally agree with estimates we derived from our steady-state spectra. The difference in chimera heme-NO complex formation can be interpreted based on a recent model we developed for nNOS catalysis (Fig. 5) (19). As the ferric heme reduction rate increases (Fig. 5, kr), the NOS proteins generate NO faster, and the rate of ferric heme-NO complex formation increases as discussed above. However, faster heme reduction also increases the probability that the ferric heme-NO product will become reduced (kr⁻¹) before NO can dissociate (kd). Reduction generates a ferrous heme-NO complex from which NO dissociates very slowly (19). The steady-state level of the ferrous heme-NO species depends on its relative rate of formation versus O₂-dependent decay (Fig. 5, kox). In nNOS, the different rates are set such that in an air-saturated buffer a majority of enzyme is present as a ferrous heme-NO complex during the steady state. Previous work with nNOS (21, 27) and model simulations (19) shows that the percentage of its ferrous

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3 Nishida and Ortiz de Montellano (12, 22) swapped both oxygenase domains and CaM binding sites in their eNOS and nNOS chimeras, whereas we only swapped the oxygenase domains.
heme-NO complex varies in proportion to the rate of ferric heme reduction ($k_r$, $k_{r'}$) within the range of 0–4 s$^{-1}$. This is precisely what we observed for the chimeras. $N_{ox}E_{red}$ had slower ferric heme reduction than nNOS and displayed less heme-NO complex during the steady state. On the other hand, the faster ferric heme reduction in $E_{ox}N_{red}$ relative to eNOS was associated with greater heme-NO complex accumulation. Our results with $E_{ox}N_{red}$ are the first to indicate how speeding eNOS ferric heme reduction will affect its catalytic profile. The data predict that eNOS will behave more like nNOS under this circumstance, increasing both its NO synthesis rate and degree of heme-NO complex buildup. Thus, different rates of ferric heme reduction seem to primarily determine the catalytic profiles of eNOS, nNOS, and the chimeras.

The different NO synthesis activities of eNOS, nNOS, and the chimeras can best be appreciated when one also considers how heme-NO complex formation affects steady-state NO synthesis. Fast heme reduction caused a majority of nNOS and $E_{ox}N_{red}$ to be present as their ferric heme-NO complex during steady-state NO synthesis. This slowed down their activities to about one seventh and one fifth of the initial rates, respectively, as inferred from their initial versus steady-state NADPH oxidation rates (Table IV, m1 and m2 values). Multiplying their steady-state NO synthesis activities in Table II by factors of seven and five eliminates the effect of enzyme partitioning and provides a better estimate of their true activity (400 and 280 NO/min, respectively, at 25 °C). On the other hand, eNOS and $N_{ox}E_{red}$ had minor heme-NO complex formation, and thus their steady-state activities (15–20 NO/min, respectively, at 25 °C in Table II) are decent estimates of their intrinsic activities. The analysis suggests that the intrinsic NO synthesis activities of nNOS and $E_{ox}N_{red}$ are actually 26 and 14 times greater than those for eNOS and $N_{ox}E_{red}$, respectively, instead of the 3–4-fold difference indicated by steady-state NO synthesis measurements. This reveals how heme-NO complex formation can blunt intrinsic differences in NO activity.

In CaM-bound NOS, cytochrome c competes with the NOS oxygenase domain for electrons from the FMN group. However, the estimated differences in intrinsic NO synthesis rates as described above are somewhat greater than our measured rate differences in cytochrome c reduction (11-fold greater for nNOS versus eNOS, and 9-fold greater for $E_{ox}N_{red}$ versus $N_{ox}E_{red}$; see Table II). This may reflect some inprecision in the values we used for calculating estimates. Alternatively, it may reflect inherent differences in kinetics or mechanisms that control electron transfer to cytochrome c versus the NOS oxygenase domain. Indeed, chimeras of CaM and cardiac troponin C differentially activate cytochrome c reduction and NOS heme reduction (27). Thus, the two processes diverge in some aspects and cannot be presumed equivalent.

Although heme reduction rate is the major parameter distinguishing catalytic behaviors of eNOS, nNOS, and the chimeras, it is not the sole parameter. Related work indicates that the rate at which O$_2$ reacts with the ferrous heme-NO complex (Fig. 5, $k_{ox}$) differs between NOS isofoms and is solely a function of the oxygenase domain. This parameter helps to control the proportion of enzyme that is present as a ferrous heme-NO complex during the steady state and becomes more important as the heme reduction rate in the system increases (Fig. 5, $k_{r'}$) (19). For example, a faster $k_{ox}$ for eNOS could explain why $N_{ox}E_{red}$ accumulates a somewhat smaller amount of heme-NO complex than nNOS despite their identical steady-state NADPH consumption and NO synthesis rates (see Tables II and IV). It might also explain why $E_{ox}N_{red}$ and nNOS display equivalent NO synthesis in the steady state although the initial rate of NADPH consumption by $E_{ox}N_{red}$ is only 75% that of nNOS (see Table IV). NOS oxygenase domains also differ in their extent of NADPH-dependent heme reduction, with eNOS being the poorest (7, 13, 30). This may help explain why $N_{ox}E_{red}$ is faster than eNOS regarding its initial rate of NADPH oxidation (Table IV) and its steady-state rates of NADPH consumption and NO synthesis (Table II). These and other differences probably also underpin the different rates of H$_2$O$_2$-promoted NOHA oxidation for eNOS and nNOS in Table I. However, it is apparent from our analysis that oxygenase domain-specific effects only “fine tune” the catalytic behavior of eNOS and nNOS. The most dramatic effects are controlled by the heme reduction rate, which is a function of their reductase domains.

There is an interesting difference between eNOS and nNOS regarding the effect of O$_2$ on heme reduction. When measured under anaerobic conditions, even in the presence of CO, heme reduction in eNOS is much slower than the rate that can be inferred from the initial rates of NADPH oxidation or NO synthesis in oxygenated buffer (compare Tables III and IV). This discrepancy does not occur in nNOS (19) or in iNOS. We also observed an O$_2$ effect on heme reduction for $N_{ox}E_{red}$ but not for $E_{ox}N_{red}$. Thus, the behavior seems specific for the eNOS reductase domain and operates independent of oxygenase domain identity.

To summarize, chimeras of nNOS and eNOS help to show how their reductase and oxygenase domains support different heme reduction, heme-NO complex formation, and NO synthesis. The heme reduction rate is controlled almost exclusively by the reductase domain and is the major parameter controlling heme-NO complex formation and NO synthesis, with oxygenase domains providing minor but measurable influences. Increasing the heme reduction rate in a chimera containing the eNOS oxygenase domain resulted in a catalytic profile approaching nNOS, whereas slowing the heme reduction in a chimera containing the nNOS oxygenase domain resulted in a catalytic profile approaching eNOS. Thus, general principles

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4 J. Santolini and D. J. Stuehr, manuscript in preparation.
governing heme-NO complex formation and NO synthesis activity in nNOS apply to eNOS as well.

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