We studied steps that make up the initial and steady-state phases of nitric oxide (NO) synthesis to understand how activity of bovine endothelial NO synthase (eNOS) is regulated. Stopped-flow analysis of NADPH-dependent flavin reduction showed the rate increased from 0.13 to 86 s⁻¹ upon calmodulin binding, but this supported slow heme reduction in the presence of either Arg or N⁶-hydroxy-L-arginine (0.005 and 0.014 s⁻¹, respectively, at 10 °C). O₂ binding to ferrous eNOS generated a transient ferrous dioxy species (Soret peak at 427 nm) whose formation and decay kinetics indicate it can participate in NO synthesis. The kinetics of heme-NO complex formation were characterized under anaerobic conditions and during the initial phase of NO synthesis. During catalysis heme-NO complex formation required buildup of relatively high solution NO concentrations (>50 nM), which were easily achieved with N⁶-hydroxy-L-arginine but not with Arg as substrate. Heme-NO complex formation caused eNOS NADPH oxidation and citrulline synthesis, which decreased 3-fold and the apparent \( K_m \) for O₂ increased 6-fold. Our main conclusions are: 1) The slow steady-state rate of NO synthesis by eNOS is primarily because of slow electron transfer from its reductase domain to the heme, rather than heme-NO complex formation or other aspects of catalysis. 2) eNOS forms relatively little heme-NO complex during NO synthesis from Arg, implying NO feedback inhibition has a minimal role. These properties distinguish eNOS from the other NOS isoforms and provide a foundation to better understand its role in physiology and pathology.

Nitric-oxide synthases (NOSs) catalyze a stepwise oxidation of L-arginine (Arg) to citrulline and nitric oxide (NO) (1–3). In mammals, three NOSs are expressed that differ in their primary sequence, post-translational modifications, cellular location, and tissue expression (4–6), consistent with their participating in a range of physiologic and pathologic systems. Two NOSs (neuronal, nNOS or NOS-I; and endothelial, eNOS or NOS-III) are constitutively expressed and participate in signal cascades by synthesizing NO in response to Ca²⁺-dependent CaM binding. A third NOS (cytokine-inducible, iNOS or NOS-II) is primarily regulated by transcriptional mechanisms, binds CaM irrespective of the Ca²⁺ concentration to be always active, and functions as both a regulator and effector of the immune response.

Although NO synthesis activities of the NOS isoforms differ considerably, how and why this occurs is unclear. A comparison of published steady-state rates shows that eNOS is about four to eight times slower than either nNOS or iNOS (7–14). Because NO synthesis is actually the result of many steps, it is imperative to identify which steps limit the activity of a particular NOS isoform. Work with NOS chimeras containing swapped reductase domains has suggested heme reduction could be responsible for the low activity of eNOS (30). However, it seems that NOS catalysis is comprised of two parts (15, 16): an active component that includes all steps involved in NO generation and an inactive component that includes NO binding to the NOS heme and subsequent dissociation or oxidation of the heme-NOS complex to regenerate active enzyme. For iNOS and nNOS, evidence suggests that their activities are significantly decreased in a number of settings because a majority of enzyme partitions into an NO-bound inactive form (17, 18). This also increases their \( K_m \) for O₂, which has physiologic consequences (19, 20). In nNOS, a residue that controls enzyme partitioning between the active and NO bound forms has recently been identified (16). In contrast, the extent by which heme-NOS complex formation may limit eNOS NO synthesis is still unknown. To address this issue, we analyzed several steps involved in NO synthesis by eNOS and its propensity to partition between active and inactive forms during catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—NO gas was purchased from Matheson Gas Products, Inc., and O₂ gas was purchased from the Ohio Gas Company and used without further purification. N⁶-Hydroxy-L-arginine was a generous gift from Dr. Bruce King of Wake Forest University. All other materials were obtained from Sigma or from sources reported previously (11, 13, 16, 18).

**Enzyme Purification**—Bovine eNOS with a six-histidine tag at its N terminus was expressed in *Escherichia coli* BL21(DE3) using the pC-Wori expression vector and purified in the presence of \( (6R)-5,6,7,8 \)-tetrahydrobiopterin (H₄B) by ammonium sulfate precipitation and sequential nickel-nitrilotriacetic acid and \( 2′,5′ \)-ADP affinity chromatography as described previously (11). The purified enzyme was homogeneous as judged by SDS-polyacylamide gel electrophoresis. The eNOS was quantitated based on its \( P₄₅₀ \) hem content which was determined using an extinction coefficient of 74 mm⁻¹ cm⁻¹ for its dithionite-reduced, CO bound form (11).
Electrochemical Detection of NO—NO concentration was monitored using an ISO-NO mark II NO meter equipped with an ISO-NOP 200 sensor (World Precision Instruments, Inc., Sarasota, FL). Applied voltage was +585 mV. Measurements were made in a water-jacketed and stirred 4-ml cell at 15 °C. Eletrode calibration involved the consecutive addition of 15 ml of a 25 °C NO solution to 3 ml of argon-deoxygenated Hepes buffer (40 mM, pH 7.4) in a rubber-sealed cell at 15 °C. The NO solution was made in argon-deoxygenated buffer, and its NO concentration was determined with oxygenhemoglobin. The electrode current was proportional to NO concentration up to 10 μM, and sensitivity was typically 410 nM NO/Ha at 15 °C.

Measurement of NO Synthesis and NADPH Oxidation at Different O2 Concentrations—Concentrated eNOS was placed in septum-sealed cuvettes and diluted with various ratios of N2-, O2-, or O2-saturated buffer solutions that contained 40 mM BisTris propane, pH 7.4, 1 mM Arg, 15 μM Ca2+, 1.2 mM Ca2+, 0.9 mM EDTA, 1 mM dithiothreitol, 5–10 μM oxyhemoglobin, and 4 μM each of FAD, FMN, and H4B (final volume 1 ml). Final eNOS concentrations for the NADPH oxidation and NO synthesis rate measurements were 300 and 80 nM, respectively. The initial O2 concentration in each reaction was calculated based on the solution mixing ratio and the O2 concentration of air- or O2-saturated buffer at 25 °C and initiated by injecting 10 μl of NADPH solution to give 100 μM final concentration. The rate of NO synthesis was determined by monitoring the NO-mediated conversion of oxyhemoglobin to methemoglobin at 401 nm (11), whereas the initial rate of NADPH oxidation was determined at 340 nm in the presence or absence of Arg and in the absence of oxyhemoglobin. Km values for O2 were estimated from double reciprocal plots of the data.

Optical Spectroscopy and Stopped-Flow Measurements—Optical spectra were recorded on a Hitachi 3101 UV-visible spectrophotometer equipped for anaerobic work. Rates of NO and O2 binding to ferrous eNOS at different ligand concentrations were obtained as described previously (22). Experiments were carried out at 10 °C and initiated by rapidly mixing an anaerobic buffered solution that contained 2 μM ferrous eNOS (generated in a cuvette by titrating in an anaerobic dithionite solution), 10 μM H4B, 1 mM dithiothreitol, and 0.15 mM NaCl with a buffered solution that contained 0.15 mM NaCl and different concentrations of O2 or NO. Dithionite reduction was omitted when studying ferric eNOS. All NO and O2 binding rates were measured in the presence or absence of Arg. In some cases, O2 binding to ferrous eNOS was monitored using a rapid scanning diode array detector (Hi-Tech MG-6000) designed to collect 96 complete spectra in a specific time frame. The diode array detector was calibrated relative to five reference absorbance wavelengths of holmium oxide filter (HY-1) at 362, 420, 446, 460, and 536 nm. Spectra were collected after rapidly mixing anaerobic ferrous eNOS with air-equilibrated buffer at 10 °C. In all cases, 6–10 replica scans were collected and utilized to derive mean kinetic values. Solution compositions were as described above.

Rates of NADPH-dependent flavin and heme reduction were measured at 10 °C. Similar stopped-flow conditions as described above, except that the anaerobic ferric eNOS solution also contained 0.9 mM EDTA with or without 1.2 mM Ca2+ and 3 μM CaM and was mixed with an anaerobic buffered solution that contained 0.2 mM NADPH and 0.15 mM NaCl. Flavin reduction was monitored at 485 nm, and heme reduction was monitored at 400 nm. Signal-to-noise ratios were improved by averaging 6–10 individual traces. The time courses were fit using a nonlinear least-squares method provided by the instrument manufacturer.

RESULTS

Extent and Kinetics of Flavin and Heme Reduction—We first characterized UV-visible transitions that occur during reduction of eNOS flavin and heme centers by NADPH. As shown in the upper panel of Fig. 1, an anaerobic sample of ferric eNOS containing H4B displayed a broad Soret absorbance peak centered near 400 nm with flavin absorbance peaks ranging from 445 to 550 nm, consistent with earlier reports (8, 9, 23). Adding NADPH in the absence of bound CaM caused flavin reduction as judged by the disappearance of the flavin visible absorption bands. No heme reduction occurred as judged by our observing only a slight decrease in heme Soret absorbance, which could be attributed to flavin reduction in this region of the spectrum, and no change in ferric heme absorbance at 640 nm. Adding Ca2+ to promote CaM binding led to partial heme reduction, as judged by an intermediate decrease and red shift in Soret absorbance. Upon adding Arg to the sample, heme reduction became complete. The lower panel of Fig. 1 shows spectra from a similar experiment that initially contained ferric eNOS saturated with both H4B and Arg. Under this condition, Ca2+-promoted CaM binding led to complete heme reduction as judged by the decrease in Soret absorbance and its shift to 414 nm. Heme reduction was blocked when N-nitro-L-arginine methyl ester replaced Arg in the experiment (data not shown), as also occurs in eNOS isolated from mammalian cells (23). These results confirm that CaM triggers heme reduction in CaM-bound eNOS, indicate that Arg facilitates heme reduction, and provide wavelengths to monitor the kinetics of flavin and heme reduction.

We studied the kinetics of flavin and heme reduction using stopped-flow spectroscopy under anaerobic conditions at 10 °C. Based on spectra in Fig. 1 and our previous work with nNOS (24), we followed flavin and heme reduction at 485 and 400 nm, respectively. Reduction of flavins was studied by rapidly mixing CaM-free or CaM-bound eNOS with a solution containing excess NADPH. Fig. 2 shows flavin reduction in CaM-free eNOS was very slow and required about 25 s to reach completion (left panel), whereas flavin reduction in CaM-bound eNOS was fast and reached completion in less that 0.1 s (right panel). In both cases, the spectral change was essentially monophasic and fit well to a single exponential function with rate constants of 0.13 and 85 s−1, respectively.

Replica experiments were run to monitor heme reduction in eNOS at 400 nm. In CaM-free eNOS, the spectral change at 400 nm was monophasic with a rate of 0.15 s−1, essentially identical to that observed at 485 nm and consistent with only flavin reduction occurring in this circumstance. In contrast, for CaM-bound eNOS that contained Arg and H4B the absorbance change was biphasic. The first phase was attributed to flavin reduction with a rate constant identical to that obtained at 485 nm, whereas the slow phase was attributed to heme reduction. As shown in Fig. 3, heme reduction in the presence of Arg had a rate constant of 0.005 s−1 at 10 °C and was about three times faster (0.014 s−1) with NOHA. The slow phase attributed to heme reduction at 400 nm was absent when eNOS contained the heme reduction inhibitor N-nitro-L-arginine methyl ester (data not shown). Adding Arg, NOHA, or N-nitro-L-arginine methyl ester did not alter the rate of flavin reduction (data not shown).

Oxygen Binding Kinetics—We next characterized O2 binding to ferrous eNOS. We used rapid-scanning stopped-flow spectroscopy to identify species that form upon mixing a pre-reduced, anaerobic solution of H4B-saturated eNOS with air.
equilibrated buffer. As shown in Fig. 4, the starting spectrum recorded after 3 ms is characteristic of ferrous eNOS, which displays a Soret absorbance peak at 414 nm and almost no absorbance at 630 nm. This was followed by buildup of a transient species whose spectrum was characterized by absorbance peaks at 427, 560, and a shoulder at 600 nm, identical to the ferrous-dioxy complex of nNOS obtained under similar temperature and buffer conditions (25, 26). This transient species decayed to form ferric eNOS, as judged by a shift in Soret absorbance to 396 nm and buildup of visible absorbance centered near 630 nm. The eNOS ferrous-dioxy complex formed and decayed at sufficiently different rates such that the kinetics of both steps could be studied using single wavelength stopped-flow methods. We therefore examined the rates of ferrous-dioxy formation and decay as a function of O2 concentration. We studied H4B-bound eNOS in the presence and absence of Arg or NOHA and monitored the change in absorbance at 400 nm. For all six conditions tested, plots of $k_{obs}$ versus O2 concentration were linear with positive intercept at the y axis (Fig. 7), indicating that NO binding is reversible and follows a one step mechanism. The kinetic parameters for O2 binding were estimated from the slope and y intercept of each plot and are listed in Table I, along with the rates of ferrous-dioxy complex decay, which were independent of O2 concentration under each circumstance (data not shown).

**NO Binding Kinetics**—As shown in Fig. 6, adding excess NO to H4B-bound ferric or ferrous eNOS formed stable 6-coordinate nitrosyl complexes under anaerobic conditions in the absence of Arg. The ferric-NO complex displayed a Soret absorbance peak at 440 nm and two absorbance bands centered at 549 and 580 nm, whereas the ferrous-NO complex had a Soret peak at 436 nm and a broad visible band centered at 580 nm. These spectral features are essentially identical to NO complexes of iNOS, nNOS, and eNOS (17, 18, 27). Kinetics of NO binding were studied at 10 °C in the presence or absence of Arg or NOHA. Reaction of NO solutions of different concentration with ferric or ferrous eNOS was monitored at 440 or 436 nm, respectively. For all six conditions tested, plots of $k_{obs}$ versus NO concentration were linear with positive intercept at the y axis (Fig. 7), indicating that NO binding is reversible and follows a simple one step mechanism. Kinetic constants for NO binding estimated from these plots are listed in Table I.
recorded prior to, during, and after NO synthesis with Arg as the substrate. NADPH was limiting in the reaction. The spectra clearly show that the majority of eNOS molecules contained reduced flavins and oxidized (ferric) heme during the steady state, with very little or no NO complex present. This is similar to the state in which eNOS exists when it oxidized NADPH in the absence of substrate (upper panel inset). We then examined if a heme-NO complex would form during NO synthesis from NOHA, which for eNOS supports a higher rate of NO synthesis compared with Arg (23). Again, spectra were recorded prior to, during, and after NO synthesis. As shown in the lower panel of Fig. 8, the spectrum recorded during NO synthesis from NOHA had less ferric heme character (absorbance at 400 nm) as compared with Arg and displayed a shoulder above 420 nm. As the inset shows, the shoulder is actually a gain in absorbance at 430 nm. Thus, some heme-NO complex built up in the initial fast rate. As shown in the lower panel of Fig. 8, the spectrum recorded during NO synthesis from NOHA compared with Arg (23). Again, spectra were recorded prior to, during, and after NO synthesis. As shown in the lower panel of Fig. 8, the spectrum recorded during NO synthesis from NOHA had less ferric heme character (absorbance at 400 nm) as compared with Arg and displayed a shoulder above 420 nm. As the inset shows, the shoulder is actually a gain in absorbance centered near 430 nm. Thus, some heme-NO complex built up during NO synthesis from NOHA.

We next examined the kinetics of heme-NO complex formation and decay during NO synthesis from NOHA using the stopped-flow method. The reaction was initiated by rapid mixing an NADPH solution with a solution of CaM-bound eNOS that contained H4B and NOHA. The upper panel of Fig. 9 shows, the shoulder is actually a gain in absorbance at 430 nm. Thus, some heme-NO complex built up in the initial fast rate. As shown in the lower panel of Fig. 9, best fit to a single exponential function and gave an observed rate of 0.065 s⁻¹. This absorbance increase at 436 nm was actually preceded by a more rapid absorbance decrease (lower panel inset), which best fit to a single exponential function with an apparent rate constant of 94 s⁻¹ and can be attributed to flavin reduction. This initial drop also explains why the trace at 436 nm in the upper panel appears not to return to its initial level after the reaction terminated and flavins become oxidized. The relationship between Solution NO Concentration and eNOS Activity—We next utilized an electrode to monitor NO concentrations during NO synthesis from Arg or NOHA to see how these levels correlate with rates of NADPH oxidation and citrulline formation. Experiments were carried out by immersing a NO-selective electrode in a reaction vial that contained eNOS and all the necessary substrates and cofactors. Aliquots were removed for citrulline analysis at timed intervals after initiating the reaction with NADPH, and replica experiments were run in a cuvette to monitor concurrent NADPH oxidation by eNOS under each condition. As shown in the upper panel of Fig. 10, the NO concentration rose after initiating NO synthesis from Arg, achieved a maximum of 61 nM after 1 min, fell as NADPH continued to be consumed, and then fell more rapidly after all NADPH was oxidized. The rate of NADPH consumption was approximately linear during the reaction and in- increased only slightly when the NO scavenger oxyhemoglobin was present.

When NOHA was used in place of Arg (Fig. 10, middle panel), the NO concentration rose to a much higher level during the reaction (840 nM) and then gradually fell as in the Arg reaction. NADPH consumption by eNOS was slowed about 3 times as the NO concentration built up. This effect was NO-dependent, because NADPH oxidation in a replica reaction that contained oxyhemoglobin as an NO scavenger continued at the initial fast rate. As shown in the lower panel of Fig. 10, there may also be a deflection in the rate of citrulline formation from NOHA but not from Arg.

Apparent $K_m$ for O₂ and Effect of NO Synthesis—For nNOS and iNOS, heme-NO complex formation causes a shift in the

![Image 63x564 to 282x729]

**FIG. 4.** Representative spectra of ferrous, ferrous-dioxy, and ferric eNOS observed during O₂ binding experiments. An anaerobic solution of ferrous eNOS (6 μM) containing H4B was rapidly mixed at 10 °C with an air-saturated buffer solution, and 96 spectra were collected using a diode array detector. The solid, dotted, and dashed lines were observed at 0.003, 0.08, and 120 s after mixing, respectively, and represent the spectrum of ferrous, ferrous-dioxy, and ferric eNOS. The inset magnifies these traces in the absorbance range 450 to 700 nm.

![Image 68x303 to 278x479]

**FIG. 5.** Rate of ferrous-dioxy complex formation versus O₂ concentration and effect of substrate. eNOS ferrous-dioxy complex formation at 10 °C was followed at 430 nm to determine an observed association rate at each indicated O₂ concentration. Experiments contained H4B-bound eNOS either in the absence of substrate (○) or in the presence of 1 mM Arg (●) or 1 mM NOHA (□). The lines are a least squares fit for each data set.

| Species  | Ligand | $k_{on} \text{ M}^{-1} \text{s}^{-1}$ | $k_{off} \text{ s}^{-1}$ | $k_{dissociation} \text{ s}^{-1}$ |
|----------|--------|---------------------------------|----------------|-----------------|
| Ferrous eNOS | O₂ | $3.1 \times 10^5$ | 38 | 3.0 |
| Ferrous eNOS | Arg | $3.4 \times 10^5$ | 28 | 2.8 |
| Ferrous eNOS | NOHA | $2.6 \times 10^5$ | 18 | 2.0 |
| Ferric eNOS | NO | $6.1 \times 10^5$ | 93 | 3.0 |
| Ferric eNOS | Arg | $8.2 \times 10^5$ | 70 | 3.0 |
| Ferric eNOS | NOHA | $2.8 \times 10^5$ | 100 | 3.0 |
| Ferrous eNOS | NO | $1.1 \times 10^6$ | 45 | |
| + Arg | $1.1 \times 10^6$ | 70 | |
| + NOHA | $8.9 \times 10^5$ | 50 | |
Km for O₂ to higher values (19, 20). We therefore examined the O₂ concentration versus activity response for eNOS during NO synthesis. The upper panel of Fig. 11 plots enzyme activity (rate of NADPH oxidation) versus O₂ concentration when NOHA or Arg served as substrate. The O₂ response curve for NOHA was shifted to the right relative to the Arg curve, although they both approached a similar Vmax. Double reciprocal plots (Fig. 11, middle panel) were linear and gave apparent Km values for O₂ of 25 μM with NOHA versus 4 μM with Arg. Repeating the NOHA experiment in the presence of the NO scavenger oxyhemoglobin (Fig. 11, lower panel) caused the O₂-response curve to shift back to the left. This indicates the increase in Km for O₂ was because of NO.

**DISCUSSION**

NO synthesis involves many steps including NADPH and substrate binding, electron transfer between flavins and heme, O₂ binding and reduction, proton transfer, bond making and breaking, product release, and associated protein conformational changes. In addition, enzyme-generated NO can bind to the NOS heme and influence subsequent catalysis. Here we examined steps involved in the initial and steady-state phases of NO synthesis to understand what regulates eNOS activity.

**Flavin and Heme Reduction**—Flavin reduction differs in eNOS compared with other NOS. In CaM-free eNOS, flavin reduction was much slower than in nNOS (Table II). However, it had a greater fold increase upon CaM binding such that the rate became slightly greater than CaM-bound nNOS or iNOS. This implies that CaM stimulates NADPH reduction of bound FAD to a greater extent in eNOS than in nNOS. Although the mechanism is still unclear, our related work shows that CaM binding to nNOS speeds NADPH reduction of FAD by relieving a repressive effect of the FMN module on this “upstream” electron transfer event (13). If a similar mechanism operates in eNOS, then the repressive effect of its FMN module must be stronger. Recent results with an eNOS deletion mutant (28) suggest that an autoinhibitory loop contained in its FMN module may be involved in this process.

Heme reduction in eNOS also differs from other NOSs. In the absence of substrate about half of the heme was reduced when excess NADPH was added to anaerobic, CaM-bound eNOS,
whereas heme reduction was nearly complete with substrate present. This differs from nNOS or iNOS, where excess NADPH caused almost complete heme reduction in the presence or absence of substrate (29). If the thermodynamics of flavin-mediated heme reduction are less favorable in substrate-free eNOS compared with other NOSs, this could help explain why eNOS NADPH consumption is so slow in the absence of substrate (8, 9, 23). Heme reduction was also much slower in eNOS than in nNOS or iNOS (Table II). Given that CaM induced a 600-fold increase in flavin reduction rate that brought it to a level comparable with other NOSs, it is astounding that CaM so poorly stimulated electron transfer to the eNOS heme. CaM also is a poor stimulator of electron transfer from the eNOS reductase domain to external acceptors like Fe(CN)$_6^3$ or cytochrome c (8, 9, 11). Conceivably, slow heme reduction in eNOS could involve structural elements in both its reductase and oxygenase domains. However, poor Fe(CN)$_6^3$ and cytochrome c reduction by the CaM-bound reductase domain (11), along with the fact that a NOS chimera comprised of an nNOS reductase and eNOS oxygenase domain had faster NO synthesis than native eNOS (30), suggest that structural elements responsible for slow heme reduction reside in the reductase domain. From our present work we conclude that slow heme reduction is not because of slow flavin reduction in eNOS, which is actually quite fast in the presence of CaM.

Our heme reduction rates (Table II) differ considerably from those reported by Miller et al. (31). Although both studies followed change in heme Soret absorbance, our measurements were made anaerobically at 10 °C, whereas theirs were made aerobically under catalytic conditions at room temperature. In their system, absorbance change at 397 nm actually reflects the combination of flavin reduction, heme reduction, O$_2$ binding, and conversion to a heme-NO complex. Taking measurements under anaerobic conditions avoids this complication.

O$_2$ and NO Binding—Our stopped-flow study showed that O$_2$ binding in eNOS is generally similar to what has been reported for nNOS. The eNOS ferrous-dioxy intermediate had a Soret absorbance at 427 nm and other absorbance bands that were identical to the ferrous-dioxy intermediate of nNOS (25, 26) and iNOS$^2$ obtained under similar conditions. Thus, all NOSs appear to form a ferrous-dioxy intermediate with similar electronic characteristics.$^3$ Association and dissociation of O$_2$ to

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$^2$ H. M. Abu-Soud and D. J. Stuehr, manuscript in preparation.
$^3$ The spectral characteristics of the NOS ferrous-dioxy complex ap-
eNOS and decay of its ferrous-dioxo complex were about three times slower than rates observed with the nNOS oxygenase domain under identical conditions (25). This may reflect that different NOS isoforms were studied or that a difference exists between full-length and oxygenase domain proteins (26). Ferric and ferrous eNOS displayed similar NO binding kinetics, suggesting NO affinity is not strongly influenced by the reduction state of the eNOS heme. However, our stopped-flow method may overestimate the NO dissociation rate from ferrous NOS (32). Estimated rate constants for NO binding were similar to those observed with ferric and ferrous iNOS oxygenase domain by stopped-flow under the same conditions (22). Because O₂ and NO binding appear to be relatively similar among the three NOS isoforms, the lower activity of eNOS is not likely because of differences in O₂ or NO binding.

Neither Arg nor NOHA strongly affected O₂ or NO binding to eNOS, as was also reported for Arg in iNOS, eNOS, and nNOS (22, 27, 32). However, the NOHA result is surprising because a recent crystal structure of the iNOS oxygenase domain with NOHA has its N-hydroxy group positioned above the ferric heme close enough to constrain the binding geometry of a model ferrous-dioxo complex (33). Although the same binding geometry would exist in eNOS (34), our kinetic results imply it does not impede O₂ or NO binding to the heme.

NO Synthesis and Heme-NO Complex Formation—We detected little or no heme-NO complex during NO synthesis from Arg, even though a measurable concentration of NO built up in solution. This distinguishes eNOS from iNOS and nNOS, which both predominantly convert to a heme-NO complex during NO synthesis under similar conditions (17, 18). In contrast, with NOHA as substrate eNOS did partition between a heme-NO complex and its ferric form during the steady state. Heme-NO complex formation was associated with a greater buildup of solution NO and a decrease in the rate of NADPH consumption. This was not seen in the Arg reaction and was prevented in the NOHA reaction by adding oxyhemoglobin as a NO scavenger. Because the deflection in NADPH oxidation occurred as the heme-NO complex formed, it likely results from eNOS molecules partitioning into the NO-bound form. Thus, under appropriate conditions eNOS behaves like iNOS and nNOS in forming an inactive heme-NO complex during NO synthesis. That oxyhemoglobin prevented heme-NO complex formation in eNOS shows that it is dependent on the external NO concentration. This makes eNOS similar to iNOS (17) but distinguishes it from nNOS, whose heme-NO complex formation occurs independent of the external NO concentration (18).

A far greater NO concentration build up during NO synthesis from NOHA than from Arg. This is consistent with eNOS catalyzing a 3-fold faster rate of NO synthesis from NOHA in the presence of the NO scavenger oxyhemoglobin (8, 9, 21). The different rates may arise because the two substrates differ in their ratio of NADPH oxidized to product formed (21). Specifically, NOHA conversion to NO requires transfer of only one electron (0.5 NADPH equivalent) from the reductase domain to the heme, whereas Arg conversion to NO requires transfer of three electrons (1.5 NADPH equivalents). Given that the rate of NADPH oxidation was approximately the same when NOHA

Like eNOS, the level of heme-NO complex observed in iNOS is dependent on the external NO concentration.

**Fig. 11. Activity versus O₂ concentration with Arg or NOHA as substrate.** The upper panel plots rate of NADPH oxidation (absorbance change at 340 nm × 10³/min) observed at each O₂ concentration during NO synthesis from Arg (○) or NOHA (●) in the absence of oxyhemoglobin. The middle panel contains the same data plotted in double reciprocal form (O₂ concentrations are in µM), with a least squares line of best fit through each data set. The lower panel plots the rate of NO synthesis from NOHA (absorbance change at 401 nm × 10³/min) observed at each O₂ concentration in the presence of oxyhemoglobin.

| Enzyme | Flavins | Heme |
|--------|---------|------|
|        | CaM-free | CaM-bound |
| eNOS   | 0.13 ± 0.01 | 85 ± 10 | 0.005 ± 0.001 | This work |
| nNOS   | 2.5      | 41     | 4.0           | 24       |
| iNOS   | 10       | 2.5    |               | =        |

* H. M. Abu-Soud and D. J. Stuehr, unpublished data.

**TABLE II**

Rates of NADPH-dependent flavin and heme reduction in three NOS

All rates were determined using anaerobic stopped flow spectroscopy at 10 °C. Reactions were started by rapidly mixing eNOS containing H₄B and Arg with an excess of NADPH.

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eNOS and decay of its ferrous-dioxo complex were about three times slower than rates observed with the nNOS oxygenase domain under identical conditions (25). This may reflect that different NOS isoforms were studied or that a differences exist between full-length and oxygenase domain proteins (26). Ferric and ferrous eNOS displayed similar NO binding kinetics, suggesting NO affinity is not strongly influenced by the reduction state of the eNOS heme. However, our stopped-flow method may overestimate the NO dissociation rate from ferrous NOS (32). Estimated rate constants for NO binding were similar to those observed with ferric and ferrous iNOS oxygenase domain by stopped-flow under the same conditions (22). Because O₂ and NO binding appear to be relatively similar among the three NOS isoforms, the lower activity of eNOS is not likely because of differences in O₂ or NO binding.

Neither Arg nor NOHA strongly affected O₂ or NO binding to eNOS, as was also reported for Arg in iNOS, eNOS, and nNOS (22, 27, 32). However, the NOHA result is surprising because a recent crystal structure of the iNOS oxygenase domain with NOHA has its N-hydroxy group positioned above the ferric heme close enough to constrain the binding geometry of a model ferrous-dioxo complex (33). Although the same binding geometry would exist in eNOS (34), our kinetic results imply it does not impede O₂ or NO binding to the heme.

**Regulation of Endothelial NO Synthase**

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or Arg served as substrate in the presence of oxyhemoglobin, the difference in NADPH stoichiometry specifies a 3-fold faster rate for NO synthesis from NOHA.

In the absence of the NO scavenger oxyhemoglobin, the initial rate of NADPH oxidation was only maintained for the first 30–50 s in the NOHA reaction, after which a 2–3-fold slower rate was observed. Thus, after the rate deflection occurred eNOS synthesized NO from NOHA at a rate that was approximately equivalent to the Arg reaction. This is consistent with the NOHA reaction continuing almost three times longer than the Arg reaction in Fig. 10. The “initial burst” kinetic pattern (i.e. a fast phase of NO synthesis followed by a slower phase) helped achieve and maintain a higher NO concentration in the NOHA reaction. Although this explanation is consistent with the data, pathways for NO loss also helped determine the NO concentration achieved under each circumstance. Because superoxide dismutase was not added to the reactions, any superoxide produced by eNOS (35) would be expected to lower the NO concentration. Because eNOS heme reduction is slow, uncoupled NADPH oxidation may be greater during NO synthesis from Arg compared with NOHA, resulting in greater superoxide production and a lower NO concentration.

Relation between Heme-NO Complex Formation and O₂ Response—Because more heme-NO complex formed with NOHA as substrate, this provided an opportunity to test how NO complex formation would affect eNOS activity versus O₂ concentration response. Heme-NO complex formation increased the apparent $K_m$ for O₂ 6-fold. This confirms our previous work with nNOS and iNOS that suggests NO complex formation can have major impact on NOS O₂ response (18–20). When little or no heme-NO complex formed (i.e. either with Arg as substrate or with NOHA in the presence of oxyhemoglobin), the apparent $K_m$ values for O₂ were similar to other enzymes that contain thiolate-ligated heme (36). This suggests that O₂ affinity toward the ferrous heme primarily determines eNOS $K_m$ for O₂ when a heme-NO complex does not form.

A Model for eNOS Catalysis—Our results fit within a hypothetical model for NOS catalysis (16, 19) (Scheme 1). NOS molecules can partition between two cycles during steady-state NO synthesis: an active cycle that generates NO and an inactive cycle that involves formation of a heme-NO complex. At the start of NO synthesis all NOS molecules are active. However, if heme-NO complex formation occurs, the ratio between NO-free and NO-bound NOS determines activity during the steady state.

The spectrum of eNOS during NO synthesis from Arg showed that the majority of enzyme was in a flavin-reduced, ferric form during the steady state. This is consistent with flavin reduction being relatively fast and identifies heme reduction as the slow step in the active cycle. Because ferrous eNOS does not build up, O₂ binding and subsequent steps must be faster than heme reduction. With Arg as substrate almost all of the enzyme molecules were in the active cycle during NO synthesis. This explains why the rates of citrulline synthesis and NADPH oxidation remained constant. In contrast, the spectrum we collected during NO synthesis from NOHA showed some of the eNOS molecules were present in their NO-bound form. Thus, both active and inactive cycles were operative with slow steps being heme reduction and NO complex decay, respectively. Such partitioning explains why NADPH oxidation and associated citrulline synthesis from NOHA became attenuated after an initial burst phase.

The rate of NO synthesis and degree of heme-NO complex formation may both depend on the O₂ concentration. For NO synthesis the relevant reaction is O₂ binding to ferrous heme, whereas the level of heme-NO complex can be affected by a reaction between O₂ and the ferrous heme-NO complex (as occurs for nNOS; Ref. 19) or any O₂-dependent reaction that lowers the solution NO concentration. Our data indicate that NO binding and dissociation from the ferric heme occur much faster in eNOS than heme reduction. Thus, for eNOS the external NO concentration and affinity toward the ferric heme likely determine the amount of heme-NO complex that builds up during the steady state. Indeed, the solution NO concentration that would be required for 50% heme-NO complex formation as calculated from our NO binding constants is well above those actually achieved in the reaction. Because heme-NO complex formation shifts the eNOS apparent $K_m$ for O₂ to the right, reactions limiting heme-NO complex buildup must be more broadly related to O₂ concentration than is NO synthesis itself. This also appears to be the case for nNOS (19).

The different activities of eNOS and nNOS are best appreciated in the context of Scheme I. For nNOS, heme-NO complex formation is intrinsic to catalysis and causes a majority of enzyme to partition into the NO-bound form. This slows down activity to about 10–20% of the initial value within the first two catalytic turnovers. The slower rate is commonly considered the $V_{max}$ activity of nNOS (about 75 NO/min at 25 °C). Multiplying by a factor of 5 minimizes the effect of enzyme partitioning and provides an estimate of nNOS activity prior to NO complex formation (375 NO/min). For eNOS, its slow rate of NO synthesis from Arg is not due to NO complex formation. Thus, its steady-state activity (~15 NO/min at 25 °C) is a good estimate of its intrinsic activity. The analysis reveals that nNOS is actually 25 times more active than eNOS, which is remarkable from a structure-function standpoint.

Why does so little heme-NO complex build up during eNOS NO synthesis from Arg? Our results suggest that a sufficient NO concentration is simply not achieved. This likely reflects slow catalysis, which in turn reflects a particularly slow electron transfer between flavins and heme in eNOS. Perhaps this characteristic evolved to minimize NO feedback regulation in eNOS. Do NO concentrations that support heme-NO complex formation ever build up in tissues? This is important to consider because of the effect on eNOS O₂ response. Our electrode results suggest that a NO level above 50 nM would be needed. Under what circumstances does this occur is unclear. However, in human and pig lung NO levels are apparently sufficient to support significant iNOS heme-NO complex formation with a strong resultant effect on that enzyme’s O₂ concentration-activity response (20, 43). Because eNOS is coexpressed in human lung, it may be exposed to the same NO level. Several mechanisms also boost eNOS activity in cells. These include eNOS interaction with the membrane or proteins like HSP-90 (37, 38) and phosphorylation within its reductase domain (39, 40). How these might alter the connection between NO concentration in tissues, NO feedback regulation, and the O₂ response of eNOS can now be explored.
