Mammalian NO synthases catalyze the monoxygenation of L-arginine (L-Arg) to N-hydroxyarginine (NOHA) and the subsequent monoxygenation of this to NO and citrulline. Both steps proceed via formation of an oxyferrous heme complex and may ultimately lead to a ferrous NO complex, from which NO must be released. Electrochemical reduction of NO-bound neuronal nitric-oxide synthase (nNOS) oxygenase domain was used to form the ferrous heme NO complex, which was found to be stable only in the presence of low NO concentrations, due to catalytic degradation of NO at the nNOS heme site. The reduction potential for the heme-NO complex was approximately -140 mV, which shifted to 0 mV in the presence of either L-Arg or NOHA. This indicates that the complex is stabilized by 14 kJ mol⁻¹ in the presence of substrate, consistent with a strong H-bonding interaction between NO and the guanidino group. Neither substrate influenced the reduction potential of the ferrous heme CO complex, however. Both L-Arg and NOHA appear to interact with bound NO in a similar way, indicating that both bind as guanidinium ions. The dissociation constant for NO bound to ferrous heme in the presence of L-Arg or NOHA was determined electrochemically to be 0.17 nM, and the rate of dissociation was estimated to be 10⁻⁴ s⁻¹, which is much slower than the rate of catalysis. Stopped-flow kinetic analysis of oxyferrous formation and decay showed that both L-Arg and NOHA also stabilize the ferrous heme dioxy complex, resulting in a 100-fold decrease in its rate of decay. Electron transfer from the active-site cofactor tetrahydrobiopterin (H₄B) has been proposed to trigger the monoxygenation process. Consistent with this, substitution by the analogue/inhibitor 4-amino-H₄B stabilized the oxyferrous complex by a further two orders of magnitude. H₄B is required, therefore, to break down both the oxyferrous and ferrous nitrosyl complexes of nNOS during catalysis. The energetics of these processes necessitates an electron donor/acceptor operating within a specific reduction potential range, defining the role of H₄B.

Different isoforms of nitric-oxide synthase (NOS)¹ are found in specific cell types and locations, where NO is required to act, for example, as a post synaptic signaling agent, in immune response or to regulate blood flow and pressure. The NOSs share a common domain architecture consisting of a structurally unique dimeric oxygenase domain (2–5), which binds heme and tetrahydrobiopterin (H₄B), and a reductase domain, which binds FMN and FAD and is related to cytochrome P₄₅₀ reductase (6). The reductase domain catalyzes NADPH dehydrogenation and supplies electrons, via FAD and FMN, to the heme of the other enzyme subunit (7, 8). FMN to heme electron transfer limits the overall rate of catalysis and is activated by calcium-dependent calmodulin binding in the neuronal and endothelial NOS isoforms, by a mechanism based largely within the reductase domain (9–13). The active site for NO synthesis is based around Cys-ligated heme, which hydrogen bonds via a propionate group to the H₄B cofactor. The substrate, L-arginine (L-Arg), binds above the heme plane by forming a salt bridge with a glutamate residue and is ~4 Å away from the heme iron (4, 5). The bound L-Arg initially undergoes monoxygenation to N-hydroxy-L-arginine (NOHA), consuming two electron equivalents and one molecule of dioxygen. During the second step, NOHA remains bound in the enzyme active site and is converted to citrulline and NO in an unusual reaction consuming one electron equivalent and one molecule of dioxygen. The first step is believed to follow a typical P₄₅₀-type mechanism, in which L-Arg reacts with an oxyferryl heme complex. Several mechanisms have been proposed for the second step, these can involve reaction of the ferrous-dioxygen complex with NOHA to form NO⁺ or reaction of the oxyferryl species to generate NO⁻ (14–16). Recent single-turnover studies have shown that, when the ferrous oxygenase domain reacts with an equivalent of molecular oxygen in the presence of bound H₄B, monoxygenation of either substrate can occur (16, 17). In both cases, H₄B free radical formation and decay has been observed, suggesting that the H₄B cofactor acts as a single electron donor/acceptor during catalysis. The penultimate species formed in these experiments is the ferric NO complex, from which NO is then released (16, 18). NO has been shown to bind to the ferric enzyme in the absence of substrate and to the ferrous enzyme in the presence of substrate (19, 20). The latter complex is believed to inhibit NO synthesis, particularly in neuronal NOS (nNOS), resulting in the enzyme cycling at a sub-optimal rate (16). X-ray crystal structures of the eNOS

The production of nitric oxide in mammals is linked to numerous physiological control mechanisms and diseases (1, 2).

¹ The abbreviations used are: NOS, nitric-oxide synthase; nNOS, iNOS, and eNOS, neuronal, induced, and endothelial NOS, respectively; NOS₁, dimeric oxygenase domain of NOS; P₄₅₀, cytochrome P₄₅₀; H₄B, (6R)-5,6,7,8-tetrahydro-L-β-pterin; aH₄B, 4-aminotetrahydrobiopterin; NOHA, N-hydroxy-L-arginine; OTTLE, optically transparent thin layer electrochemistry.
oxygenase domain with bound NO (21) shows the substrate guanidinium terminal N positioned within H-bonding range of the NO oxygen. EPR (22) and Resonance Raman spectroscopy (19) also suggest an interaction between bound NO and substrate. Given the similarity of the NO and O₂ ligands, it is likely that the ferrous dioxygen complex of NOS forms similar O₂-substrate interactions prior to oxygen activation. The interactions between substrate and diatomic ligand are therefore important to both the stability of the ferrous nitrosyl complex and to the oxygen activation process, influencing both key catalytic steps and the product inhibition mechanism.

In this study, redox potentiometry was used to probe the thermodynamic properties of neuronal NOS heme domain (nNOSoxy) in the presence of the substrates (L-Arg and NOHA) and the diatomic ligands, CO and NO. Stopped-flow spectrophotometry was also used to follow the formation and decay of the ferrous nNOSoxy-dioxygen complex. The two approaches lead to new insights into the roles of substrates and cofactors in the oxygen activation processes leading to NO synthesis.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Procedures**—All chemicals were obtained from Sigma-Aldrich (Poole, Dorset) unless otherwise stated. Preparation of nNOS oxygenase domain (nNOSoxy): The nNOSoxy dimer (residues 1–720 with an N-terminal His tag) was expressed in E. coli BL21 cells also containing the gProESL plasmid as described previously (8). The cells were lysed in 50 mM Tris-HCl (pH 7.5), containing 1 mM EDTA, 10% glycerol, 20 μM H₂B (Schircks Lab., Switzerland) 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor tablet (Complete, Roche Applied Science) and centrifuged for 40 min at 20,000 × g. The supernatant was applied to a nickel-nitrilotriacetic acid-agarose column, washed with 50 mM potassium phosphate, pH 7.5, containing 1 mM EDTA, 10% glycerol, 20 μM H₂B, 1 mM dithiothreitol, and 20 μM imidazole, and eluted with the same buffer containing 500 μM H₂B. The enzyme solution was diluted into a 50% saturated buffer solution of the appropriate protein liquid chromatography gel filtration (Amersham Biosciences). Enzyme samples prepared in this way were >90% pure according to SDS-PAGE and >90% dimeric according to Superdex S200 fast protein liquid chromatography gel filtration (Amersham Biosciences). The concentrations of nNOSoxy were determined optically from the ferrous CO difference spectrum using ε(444–467 nm) = 55 M⁻¹ cm⁻¹ (24).

**Optically Transparent Thin Layer Electrochemical Potentiometry**—Spectroelectrochemical analysis of nNOSoxy was conducted in an OTTLE cell from a modified quartz EPR cell with a 208 μm path length, containing a platinum/Rh (95/5) gauze working electrode, a platinum wire counter electrode, and an argon/AgCl reference electrode (25). Enzyme samples (0.5 ml × 100–200 μM) were eluted through a G25 column pre-equilibrated with 0.1 M Tris, pH 7.5, containing 10 mM KCl, 10 μM H₂B in an anaerobic glove-box. The mediators, pyocyanine (10 μM), 2-hydroxy-1,4-naphthoquinone (20 μM), FMN (5 μM), benzyl viologen (10 μM), and methyl viologen (10 μM), were then added. Spectroelectrochemical titrations were performed at 25 ± 2 °C using an Autolab PGSTAT10 potentiostat and a Cary 50 UV-visible spectrophotometer. The argon/AgCl reference electrode employed in the OTTLE cell was calibrated against indigotrisulfonic acid (Eₒ = −99 mV versus the standard hydrogen electrode) and FMN (Eₒ = −220 mV versus the standard hydrogen electrode) in the same buffer conditions. All electrode potentials were corrected relative to the standard hydrogen electrode. The potential of the working electrode was decreased in 30-mV steps until the enzyme was fully reduced and increased stepwise until reoxidation was complete. After each step the current and UV-visible absorption spectrum were monitored until no further change occurred. This equilibration process typically lasted 15 min. Absorbance changes were plotted against the potential of the working electrode and analyzed by non-linear regression via the Nernst equation using Origin (MicroCal). For experiments in the presence of CO or NO, the enzyme solution was diluted into a 50% saturated buffer solution of the appropriate gas in the anaerobic chamber immediately prior to use.

**Pre-steady-state Kinetics**—All pre-steady-state measurements were performed at 10 °C using an Applied Photophysics stopped-flow spectrophotometer (SX.17MV) contained within an anaerobic glove box (Belle Technology; [O₂] < 5 ppm) using either single-wavelength or diode-array detectors (12). The buffer used was 50 mM Tris/HCl, pH 7.5, containing either 10 μM H₂B or 50 μM aH₂B. L-Arg or NOHA were also added to 10 mM when required. nNOSoxy (~10 μM) with either H₂B or aH₂B bound was pre-reduced by addition of 40 μM sodium dithionite. Excess dithionite was removed prior to experimentation by elution through a pre-equilibrated 10 ml of G25 gel-filtration column. Reduction was confirmed by UV-visible spectrometry on a Varian Cary 50 Bio spectrophotometer, also contained within the glove-box. Formation of the oxyferrous complex was achieved by mixing the reduced enzyme with buffer containing various concentrations of oxygen. The oxygen concentration was measured by direct reaction with electrochemically generated reduced methyl viologen (εₒ = 13,000 M⁻¹ cm⁻¹) using a stoichiometry of 4 to 1. Rate constants (kₒ) for the formation of the oxyferrous complexes of nNOSoxy were determined by monitoring the absorbance increase at 426 nm with time. The resultant traces (averages of three or more) were fitted to single exponential functions. The values were plotted against oxygen concentration and fitted by linear regression analysis using Origin (MicroCal) to give second order rate constants (gradient) for oxyferrous complex formation (kₒ) and oxygen dissociation (intercept, kₐ). For the H₂B-bound enzyme in the absence of substrate, insufficient absorbance change occurred at 426 nm to enable analysis via this method. Decay of the oxyferrous complex to ferric nNOSoxy, was monitored at 426 nm. Absorbance traces (averages of three or more) were fitted to single exponential functions. The values at higher oxygen concentrations (above 100 μM) were invariant; these were averaged to give the oxyferrous decay rate constants (kₐ). For the H₂B-bound enzyme in the absence of substrate, this was not the case. These values are plotted in Fig. 4A and were fitted to a hyperbolic function giving a saturation rate constant (kₒ) and apparent Kₒ values. In this case the second order rate constant (kₒ) was defined by kₒ/Kₒ.

**Determination of NO Dissociation Constants**—nNOSoxy, 1 ml of 0.5 μM in 50 mM Tris/HCl, pH 7.5, containing 10 mM L-Arg or NOHA as required was titrated against a saturated solution of NO in an anaerobic glove-box. Changes in the visible absorbance spectrum of the enzyme were monitored as the titration progressed. The concentration of NO was fitted against the absorbance change and fitted using non-linear regression analysis to a single binding-site model to give the Kₒ. The stock NO concentration was determined by titration with 5 μM P450 BM3 heme domain (26) in an analogous experiment (Kₒ < 0.1 μM, i.e. tight binding) using ε₉₀ = 108,000 M⁻¹ cm⁻¹ for the low spin ferric P450.

**RESULTS**

**nNOSoxy Reduction Potentials**—Dimeric nNOSoxy purified in the presence of H₂B was reduced to the ferrous form and reoxidized within an anaerobic OTTLE cell by stepping the potential of the working electrode in 30-mV increments. After equilibration UV-visible absorption spectra were recorded (Fig. 1B). This was repeated in the presence of L-Arg (Fig. 2B), NOHA, and L-citrulline at 10 mM concentration. For each experiment, the proportion of enzyme reduced, calculated from the change in absorption spectrum, was plotted against the electrode potential and fitted to the Nernst equation to determine the heme reduction potential (Table I and Figs. 1A and 2A). L-Arg and L-citrulline caused a small increase in potential (10 mV), whereas for NOHA this was a more significant 38 mV, indicating that NOHA specifically binds more tightly to the ferrous enzyme than to the ferric by a factor of 4. The potentials are 60 mV more negative than those reported previously (27), but the shifts observed are in broad agreement.

In the presence of CO, the heme reduction potential was increased by 86 mV, consistent with CO binding solely to the ferrous enzyme. None of the substrates affected this value by a statistically significant amount, indicating that the substrates and bound CO do not interact, despite being in very close proximity within the enzyme’s active site. Note that because CO binds only to the ferrous form of the enzyme, the reduction potential is dependent on the concentration of CO (as observed). In these experiments the concentration was ~0.5 mM, as measured by titration with reduced P450 BM3. Each solution was prepared in the same way using buffer saturated with...
CO at room temperature and diluted by half, and this led to reproducible results.

Determining reproducible reduction potentials in the presence of NO was more problematic. Although NO solutions are known to be stable for days at room temperature in the absence of oxygen, NO can be reduced to NO/\text{H}_2\text{O}_2 at low potential electrodes, or oxidized to nitrite at high potentials (28). It is impossible therefore to be sure of the NO concentration within the OTTLE cell. Furthermore, the nitrosyl complexes of nNOS\textsubscript{oxy} are known to have very different levels of stability. The ferrous nitrosyl complex has been reported to be stable only in the presence of substrate (19), whereas in the absence of substrate NO was found to bind more tightly to the ferric enzyme. NO was added to the enzyme solution to 50% saturation, as described for CO above. In both the absence and presence of substrate (10 mM L-Arg or NOHA) the ferric NO complex formed. In the absence of substrate, as the potential of the working electrode was decreased, increasing levels of current flowed through the solution with no apparent reduction of the enzyme. This behavior was typical in the −50 to −150 mV (versus the standard hydrogen electrode) potential range. Reduction at below −300 mV led to formation of the ferrous enzyme after a prolonged surge of current, in which case all the NO had been consumed. However, stepping the potential slowly in the −50 to −200 mV range led to formation of the substrate-free ferrous nitrosyl complex, which was only stable once all excess NO had been consumed. At fixed potentials of around −200 mV the complex was stable for several hours. At lower potentials the complex reduced to ferrous nNOS\textsubscript{oxy}, and at higher potentials it was readily oxidized to the ferric enzyme, with the NO ligand being lost in both cases. These events were evident from the distinct UV-visible spectra of the different enzyme species. It appears therefore, that the ferrous NO complex is destroyed by reduction at low potential and that it is oxidized by excess NO at higher potentials. The UV-visible spectrum of substrate-free ferrous nitrosyl nNOS\textsubscript{oxy} is shown.

**Fig. 1.** Determination of reduction potentials for nNOS\textsubscript{oxy} in the absence of substrate. A, proportion of enzyme reduced versus potential for nNOS\textsubscript{oxy} (●), in the presence of CO (●) and NO (○), all fitted to the Nernst equation. B–D are visible spectra recorded during electrochemical reduction of nNOS\textsubscript{oxy} in the absence of ligand, presence of CO, and presence of NO, respectively. Insets are absorbance × 5. Data collected by OTTLE potentiometric titration in 100 mM Tris/\text{HCl}, pH 7.5, 0.5 M KCl, 25 °C in the presence of 10 μM \text{H}_2\text{B}.  

- \text{Proportion Reduced}  
- \text{A}  
- \text{λ (nm)}  
- \text{Applied potential vs SHE (mV)}  
- nNOS\textsubscript{oxy} Fe\textsuperscript{2+}  
- nNOS\textsubscript{oxy} Fe\textsuperscript{3+}  
- nNOS\textsubscript{oxy} Fe\textsuperscript{2+} + CO  
- nNOS\textsubscript{oxy} Fe\textsuperscript{3+} + CO  
- nNOS\textsubscript{oxy} Fe\textsuperscript{2+} + NO  
- nNOS\textsubscript{oxy} Fe\textsuperscript{3+} + NO  

Oxygen Activation in NO Synthase
Determination of reduction potentials for nNOS$_{oxy}$ in the presence of l-Arg. A, proportion of enzyme reduced versus potential for nNOS$_{oxy}$ (●), in the presence of CO (○) and NO (□), all fitted to the Nernst equation. B–D are visible spectra recorded during electrochemical reduction of nNOS$_{oxy}$, in the absence of ligand, presence of CO, and presence of NO, respectively. Insets are absorbance × 5. Data collected by OTTLE potentiometric titration in 100 mM Tris/HCl, pH 7.5, 0.5 M KCl, 25 °C in the presence of 10 μM H$_4$B, 10 mM l-Arg.

### Table I

nNOS$_{oxy}$ reduction potentials

Determined from OTTLE potentiometric titrations in 100 mM Tris/HCl, pH 7.5, 0.5 M KCl, 25 °C in the presence/absence of 10 mM substrate.

| Ligand | No substrate, $E_0$ | l-Arg | NOHA | l-Citrulline |
|--------|---------------------|-------|------|-------------|
|        | $E_0$ | $\Delta G_{sb}^*$ | $E_0$ | $\Delta G_{sb}^*$ | $E_0$ | $\Delta G_{sb}^*$ |
| CO     | –316 ± 5 | –306 ± 4 | –1 | –278 ± 6 | –4 | –308 ± 4 | –1 |
| NO     | –230 ± 6 | –229 ± 4 | 0 | –236 ± 6 | +1 | –229 ± 4 | 0 |
| NOHA   | –140 ± 30 | –2 ± 6 | –14 | +6 ± 5 | –14 | ND | – |

$\Delta G_{sb} = \Delta G$ for substrate binding to ferric heme-$\Delta G$ for substrate binding to ferrous heme.

* CO gas was 50% saturation, for [NO] see “Results.” NOHA impairs CO binding $K_d = 40.6 \pm 4$ measured directly in the presence of 10 mM NOHA.

* ND, not determined. The ferrous NO citrulline complex was unstable.

in Fig. 1D and is similar to that of the substrate-bound complex (Fig. 2D). Given the complications, determining the reduction potential of the nNOS$_{oxy}$ NO complex from these experiments is difficult. The data presented in Fig. 1 (A and D) show oxidation of the ferrous nitrosyl complex after formation by reduction at a fixed potential of –280 mV. The ferric enzyme is formed by increasing the potential in five steps of 30 mV with 15-min equilibration between each step. Reductive Nernst curves were also observed in the same range, but only after most of the NO had been depleted, as judged by loss of the
spectral signature of the ferric NO complex. Because the system is never fully at equilibrium during reduction/oxidation, the reduction potential of the complex can only be estimated. On the basis of numerous reduction/oxidation experiments, this must be in the range ~110 to ~170 mV (Table I).

In the presence of either substrate (l-Arg or NOHA), the ferric nitrosyl complex was reduced to the ferrous nitrosyl complex at around 0 mV. However, NO was consumed during this process, probably by similar mechanisms observed in the absence of substrate, and reoxidation always resulted in less NO-bound ferric enzyme. Note that NO binds more weakly to the ferric enzyme in the presence of substrate. Once the NO concentration had dropped, however, the electrochemical reduction and oxidation processes were generally reversible, with a steady depletion of the remaining NO being observed. Fig. 2D depicts a typical reduction/oxidation experiment in which the mainly ferric enzyme is converted to the ferrous nitrosyl complex and back. The reduction potentials observed were largely independent of the concentration of NO and are given in Table I. In the presence of citrulline the ferrous nitrosyl complex was unstable, its formation led to irreversible changes to the UV-visible spectrum of the heme, indicative of cofactor modification or loss of the axial ligand.

Dissociation Constants for NO—The $K_d$ values for NO binding to ferric nNOS<sub>oxy</sub> were determined by titrating NO directly into an anaerobic solution of the enzyme at $-1 \mu M$ (Table II). In the presence of substrate NO binds relatively tightly ($K_d = 1.9 \mu M$), but binding is weaker in the presence of substrate, as observed previously. l-Arg appears to compete with NO in the active site, but does not exclude the ligand completely. Even at saturation (10 mM l-Arg) NO still binds with a $K_d$ of 23.3 $\mu M$. NOHA impedes NO binding to ferric nNOS<sub>oxy</sub> by a factor of 7 more than l-Arg does. Using the reduction potential shifts observed on NO binding, it is also possible to estimate $K_d$ values for NO with ferrous nNOS<sub>oxy</sub>. The large reduction potential shifts observed in all cases led to very low values. In the presence of l-Arg, the $K_d$ is 0.17 nM. Even substrate-free nNOS<sub>oxy</sub> has a $K_d$ value of 4.3 nM indicating very slow rates of NO dissociation from both complexes.

SF of Oxyferrous Formation and Decay—Ferrous nNOS<sub>oxy</sub> was mixed with O<sub>2</sub>-saturated buffer in a stopped-flow spectrophotometer, and the reaction was monitored by both multichannel diode array and single wavelength detectors (Figs. 3 and 4). To ensure the enzyme retained its dimeric structure and to maintain homogeneity, one of two pterin cofactors were used in all the experiments. H<sub>4</sub>B is the natural cofactor of the enzyme and is essential for catalytic NO production. 4-AminoH<sub>4</sub>B (aH<sub>4</sub>B) is an inactive analogue, which still promotes dimerization of the enzyme (29). The effect of the substrates on oxyferrous formation and decay was also studied by including either l-Arg or NOHA at 10 mM in the reaction mixture. Varying the oxygen concentration between ~50 and 600 $\mu M$ enabled second order rate constants for oxyferrous formation to be derived (Table III).

Mixing ferrous nNOS<sub>oxy</sub> with oxygen has been reported to proceed via formation of an oxyferrous complex, which decays via release of superoxide or peroxide to the ferric enzyme. The oxyferrous complexes of nNOS (30–34), eNOS (35–40), and iNOS (17, 41–43) oxygenase domains have been studied previously, with some differences in the kinetics and spectral properties being observed. In our study, the reaction observed for enzyme prepared with H<sub>4</sub>B in the absence of substrate proceeded without accumulation of the oxyferrous complex. This is best illustrated by the kinetic traces shown in Fig. 3. At 410 nm (the Soret peak position for ferrous enzyme), at maximum O<sub>2</sub> concentration, the absorbance simply increases as a first-order process. Using diode-array scanning, the Soret peak did not shift significantly but broadened to that of mixed-spin ferric nNOS<sub>oxy</sub>. This contrasts with the reaction observed for enzyme prepared in the presence of aH<sub>4</sub>B (Fig. 3). In this case, the absorbance at 419 nm, consistent with it being an oxyferrous complex (Fig. 3B). Its rate of decay was 8.3 s<sup>-1</sup> and independent of O<sub>2</sub> concentration, while the second order rate constant for its formation was 0.47 $\mu M$ s<sup>-1</sup>, derived as shown in Fig. 5. The kinetic traces observed in the presence of H<sub>4</sub>B were O<sub>2</sub> concentration dependent up to the limit of O<sub>2</sub> buffer saturation in the experiments. This indicates that decay of the oxyferrous species in the presence of H<sub>4</sub>B is faster than the rate of O<sub>2</sub> binding, preventing accumulation of the complex in the reaction mixture. This was not observed for nNOS previously (30) but corresponds with recent results obtained for eNOS (39, 40). However, the rate constants derived from fitting the data to single exponential decay functions were not linearly dependent on O<sub>2</sub> concentration and showed some evidence of saturation. Fig. 5 shows the data fitted to a hyperbolic function, in which the saturation rate constant is 160 s<sup>-1</sup>, and the second order rate constant for O<sub>2</sub> binding is 0.46 $\mu M$ s<sup>-1</sup>, consistent with that observed in the presence of aH<sub>4</sub>B. The validity of this approach is dependent on the rate of dissociation of O<sub>2</sub> from ferrous nNOS<sub>oxy</sub> being fast enough for pre-equilibration to occur. For aH<sub>4</sub>B-bound nNOS<sub>oxy</sub>, this was determined to be ~40 s<sup>-1</sup> (Table III), which is slower than the decay rate in the presence of H<sub>4</sub>B, although all the traces fitted well to single exponential functions. The saturation rate constant should therefore be viewed as a lower estimate of the oxyferrous decay rate, as indicated in Table III. Regardless, it is clear that H<sub>4</sub>B destabilizes the oxyferrous intermediate in nNOS<sub>oxy</sub>, accelerating its rate of decay by more than 20-fold.

In the presence of l-Arg, the oxyferrous complex is stabilized and has a Soret peak position at 429 nm (Fig. 4B), as observed previously (30). For both H<sub>4</sub>B- and aH<sub>4</sub>B-bound nNOS<sub>oxy</sub>, a decrease in absorbance at 410 nm followed by a slow increase corresponds to oxyferrous complex formation and decay (Fig. 4). The second order rate constants for O<sub>2</sub> binding were observed to be the same for H<sub>4</sub>B- and aH<sub>4</sub>B-bound enzyme and were double the values observed in the absence of substrate. Therefore, although O<sub>2</sub> binding is faster in the presence of substrate, the substitution of H<sub>4</sub>B by aH<sub>4</sub>B has little effect. The rates of decay in the presence of l-Arg were at least 8-fold slower for the H<sub>4</sub>B-bound enzyme and 100-fold slower for aH<sub>4</sub>B-bound nNOS<sub>oxy</sub>.

In the presence of NOHA, the second order rate constants for O<sub>2</sub> binding were similar to those obtained in the presence of l-Arg, whereas the decay rates were somewhat slower (Table III). The reactions progressed via an oxyferrous complex with a Soret maximum at 424 nm. In the presence of H<sub>4</sub>B some evidence for the ferric-NO complex was observed, but this did not accumulate. When reactions were run in the presence of a
small excess of dithionite, the ferrous NO complex was generated and characterized by the appearance of a Soret band at 436 nm, which persisted. The oxyferrous decay rate quoted in Table III is based on the decay as observed at 390 nm (i.e. ferric heme formation) and may therefore be dependent partially on the rate of NO release from the ferric enzyme.

In the presence of aH4B and NOHA, the oxyferrous decay traces were biphasic. This may be due to a side reaction between the oxyferrous complex and NOHA providing an additional breakdown route. The rates obtained were broadly similar to that obtained in the presence of L-Arg, i.e. they were slow enough to lead to a very stable oxyferrous complex.

The rates of O2 dissociation determined from the O2 concentration dependence data all lie in the range of 0–50 s−1. It is not possible to distinguish between values determined under different conditions within the accuracy of the experiment. However, it is interesting to note that O2 dissociation may be slower than the rate of decay of the oxyferrous complex under turnover conditions, that is, in the presence of H4B and substrate.

### DISCUSSION

NO synthesis from L-arginine and dioxygen is a multistep process catalyzed by the mammalian NOS isoenzymes in a unique active site constructed around heme and H4B cofactors. Scheme 1 shows a catalytic cycle for this process, based on proposals made by the Stuehr laboratory (16). It is generally accepted that the first steps (1–4) of NO synthesis involve P450-type monooxygenation of L-arginine to form the NOHA intermediate (14, 15, 44). However, the role played by H4B during this sequence of events is highly unusual. Monooxygenation of NOHA leads to the release of the NO free radical and requires just one electron equivalent. This sets it apart from a typical P450 reaction. P450s use two electron equivalents and a molecule of dioxygen to generate an unstable oxyferryl intermediate (compound 1), which reacts with the substrate (44). This would be impossible if only one electron were supplied. Several alternatives have been proposed (14, 15), many involving reaction between the oxyferrous species and NOHA. How-
ever, this would require that the two substrates (L-Arg and NOHA) react in very different ways, despite being chemically very similar. The model shown in Scheme 1 avoids this problem by using H$_4$B as an electron donor/acceptor during monooxygenation. Essentially, the enzyme performs two sequential P450-type monooxygenations to generate NO$^*$, which binds to the ferric heme (note that the involvement of an oxyferryl species, the active intermediate formed in P450s, has not been demonstrated for NOS). In Step 10 of the cycle, an electron is reclaimed by the H$_4$B radical to enable NO release (16). Recent freeze-quench experiments report the formation of the H$_4$B radical on reaction of ferrous NOS$_{oxy}$ with dioxygen. In the presence of NOHA, this decays away more rapidly as NO is produced (16, 17). Formation of the ferrous NO complex has also been observed during turnover (45).

The role of H$_4$B as a single electron donor/acceptor is unprecedented (46). P450s derive electrons from reductase systems based on low potential flavins and iron-sulfur clusters (47), whereas some peroxidases use aromatic acid residues as high potential electron donors (48). In Scheme 1, H$_4$B is responsible for donating the second electron required for oxygen activation during Steps 3 and 8, and the H$_4$B radical must also reclaim an electron from the ferrous nitrosyl complex during Step 10. Reaction of ferrous NOS$_{oxy}$ with dioxygen using stopped-flow spectrophotometry models Steps 2–4 and 7–9 of the catalytic cycle (30, 39, 41). Formation of the oxyferryl complex generally occurs rapidly after decay to the ferric enzyme. However, for substrate-free H$_4$B-bound nNOS$_{oxy}$, accumulation of the oxyferryl species did not occur. This is clear from the effects of substituting H$_4$B with aH$_4$B (Fig. 3). It appears that H$_4$B causes the oxyferryl complex to decay so rapidly that even at 50% O$_2$ saturation (the limit of our experiment), the rate of oxidation of the heme is partially limited by the rate of O$_2$ binding (Fig. 5B). This effect was not reported in previous studies on nNOS$_{oxy}$ (30). The substitution of H$_4$B by aH$_4$B stabilized the oxyferryl complex by at least 20-fold in the absence of substrate, as observed with eNOS (39), similarly resulting in the accumulation of an oxyferryl complex with a Soret maximum at around 420 nm.

The structure of aH$_4$B-bound iNOS$_{oxy}$ is essentially identical to that with H$_4$B bound (49). The bioppterin cofactors are too far from the iron to have a direct effect on oxygen binding or activation, so presumably the inability of aH$_4$B to transfer an electron to the oxyferryl heme complex to trigger its decay is responsible for its lack of activity, although aH$_4$B appears to have similar redox properties to H$_4$B (38, 39, 50). The rate of O$_2$ dissociation from ferrous heme ($k_{on}$) is $\sim 40$ s$^{-1}$ as determined for the aH$_4$B-bound enzyme. This is slower than the rate of decay in the presence of H$_4$B ($\sim 160$ s$^{-1}$). Consequently, in the absence of substrate, O$_2$ binds and reacts before dissociation can occur. This may also be the case in the presence of substrate, although the decay rate is slower (22 s$^{-1}$). Unfortunately, the rate constants for O$_2$ dissociation are small compared with the association rates, which compromises the accuracy of these values. Consequently, it is difficult to ascertain whether substrate affects the rate of O$_2$ dissociation or not, although this may well be the case. The second order rate constants for O$_2$ binding ($k_{on}$) are dependent on substrate and double with either L-Arg or NOHA bound. Interestingly, results with eNOS$_{oxy}$ were reported to be very different (39), with an 8-fold decrease in $k_{on}$ observed in the presence of either substrate (from 2.5 to 0.3 $\mu$M$^{-1}$ s$^{-1}$). For nNOS, both substrates appear to facilitate O$_2$ binding to similar extents. Likewise, both substrates stabilize the oxyferryl complex. Accumulation of the oxyferryl heme complex was observed in the presence of H$_4$B and either substrate but not in the absence of substrate. This anomaly is caused by an increase in the rate of decay and a decrease in the second order rate constant for O$_2$ binding in substrate-free conditions. Previous studies reporting similar rates for oxyferryl decay in the presence and absence of substrate may be limited by the rate of O$_2$ binding (particularly in the latter case), rather than reduction of the heme oxyferryl complex (30). For eNOS$_{oxy}$, recent reports give rates of oxyferryl decay and H$_4$B free radical formation that are both substrate dependent and similar in magnitude to our nNOS$_{oxy}$ results (39, 40). As expected, the most stable oxyferryl complexes are formed in the presence of substrate and aH$_4$B. These decay 1000-fold more slowly than those formed in the absence of substrate, presence of H$_4$B. For both nNOS and eNOS (39, 40) H$_4$B appears to counteract the effect of substrate, which increases the activation barrier for the decay process, whether this is via superoxide release or electron transfer. Steps 3 and 8 in Scheme 1 represent reduction of the oxyferryl complex by H$_4$B. The one-electron reduction potential of unbound H$_4$B has been estimated to be $+0.27$ mV using cyclic voltammetry, and although this need not reflect the enzyme-bound potential, it is clear that H$_4$B is not a low potential reductant (it is unable to spontaneously reduce the ferric heme). Given that H$_4$B and heme are within hydrogen bonding distance, it is unlikely that the intrinsic rate of electron transfer is slow. Oxyferryl decay must therefore be limited by the events leading to scission of the dioxygen bond or by peroxide release from the enzyme. As in P450s, protonation may affect these steps (44). The remarkable stability of the oxyferryl-substrate complex formed by nNOS$_{oxy}$ clearly impedes the oxygen activation process. In P450 BM3, mutants with stabilized oxyferryl complexes were also shown to have slower rates of turnover, indicating that reduction of the oxyferryl complexes or subsequent oxygen activation steps had been slowed (25). The role of H$_4$B in Steps 3 and 8 may therefore be to activate the particularly stable oxyferryl complexes formed in the presence of L-Arg and NOHA. In Scheme 1, Steps 3 and 8 are given as reversible electron transfer events in which the back reaction is favored, although there are other possibilities. Note also that the monooxygenation reactions shown in Steps 4 and 9 are composed of multiple steps, which are discussed for NOS and P450 systems at length elsewhere (14, 15, 44). The heme reduction steps (Steps 1 and 6) are slow for all the NO synthases and partially limit the overall rate of enzyme turnover (16). It has been proposed that the main reason for the slow heme reduction rate lies in the possible formation of the ferrous nitrosyl complex.
(Step 12) through the supply of an electron at an inappropriate time (16). In support of this, the ferrous nitrosyl (dead-end) complex has been shown to be the dominant enzyme form under turnover conditions for nNOS (19). It is also possible that the intrinsically slow rate of electron transfer from FMN to heme makes it difficult for the reductase domain to reduce the stabilized oxyferrous complex and trigger oxygen activation in the normal P450 manner.

The second key role of H4B is in Step 10 of the cycle, during which the bioppterin radical must reclaim an electron from the ferrous nitrosyl complex. The reduction potentials of the bioppterin and heme at this point are critical to the viability of this step. In the presence of substrate, the potential of the ferrous nitrosyl complex is 0 mV. Oxidation therefore requires a high potential electron acceptor. Neuronal NOS supplies electrons at a potential of $-300 \text{ mV}$ from NADPH (51, 52), therefore the oxidant must be generated transiently during catalysis, i.e. as the H4B radical is. The NO is otherwise unable to dissociate from ferrous heme. The shift in the potential of the heme on NO binding from $-300 \text{ mV}$ to 0 mV in the presence of substrate indicates that the dissociation constant for NO binding to ferrous nNOS$_{oxy}$ is 0.17 nM, which is consistent with flash-photolysis data collected on the nNOS$_{oxy}$-NO complex (53). This is extremely tight binding. Given an association rate constant of $10^7 \text{ M}^{-1} \text{s}^{-1}$, the dissociation rate constant can be estimated to be less than $10^{-13} \text{s}^{-1}$, consistent with the results from NO scavenging experiments (53). The substrate-bound ferrous nitrosyl complex is therefore unable to release NO fast enough to support catalytic turnover and can be considered to be a dead-end complex (16). In the absence of substrate the dissociation rate constant is likely to be less than $10^{-2} \text{s}^{-1}$, so the release of NO before substrate binding (during turnover) would also be impossible. The most likely mechanism for breakdown of the dead-end ferrous nitrosyl complex is reaction with dioxygen to form either nitrate or peroxynitrite (45). It is also interesting to note that excess NO is also able to breakdown the ferrous nitrosyl complex, albeit slowly. The substrate-free enzyme appears to be particularly susceptible to this reaction, although the ferrous nitrosyl complex itself is stable in the absence of O$_2$ or excess NO. The mechanism of oxidation may be similar to the action of other heme proteins, which catalyze N$_2$O generation (19).

The model shown in Scheme 1 is based on the postulation that both monooxygenation Steps (4 and 9) occur via similar mechanisms. Decay of the oxyferrous complexes formed with both NOHA and l-Arg is dependent on H$_2$B. In addition, both l-Arg and NOHA stabilize the oxyferrous complex with respect to autooxidation by similar amounts, suggesting that they interact with heme-bound oxygen in a similar way. The crystal structure of the l-Arg-bound nitrosyl complex of eNOS$_{oxy}$ (21) shows the guanidinium group of l-Arg within H-bonding distance of the heme-bound NO. In the oxyferrous complex, the bound dioxygen is believed to form a similar interaction. The difference in the reduction potentials of the nNOS$_{oxy}$ nitrosyl complexes formed in the presence and absence of substrate gives an indication of the strength of interaction between substrate and bound NO. For both l-Arg and NOHA the free energy difference is $-14 \text{ kJmol}^{-1}$, which is consistent with a strong hydrogen bond. The fact that NOHA has an identical effect to l-Arg confirms that it is bound as the guanidinium ion under these circumstances. The reduction potential values indicate that there is no interaction between bound CO and either substrate, probably due to the weaker H-bonding ability of the CO, although NOHA does impede the binding of CO and NO to the heme. The interaction between dioxygen and the bound substrates is likely to reflect that of NO and involve a strong H-bonding interaction. If this is the case, both substrate-oxyferrous complexes will be energetically very similar and likely to follow the same oxygen activation mechanism. The fact that their rates of decay are also similar suggests that the same step limits the rate of oxygen activation in both cases. By analogy with P450 systems, the likely intermediate is the oxy-
ferryl radical heme complex (compound 1) which, in this case, H-bonds to the H$_2$B radical. This would be an extraordinary diradical species. The alternative involves reaction between a peroxo-heme complex and the substrates (14, 15, 16, 23). However, currently there is insufficient evidence to distinguish between the two.

CONCLUSIONS

The shift in reduction potential of the heme-NO complex of nNOS$_{oxy}$ on substrate binding indicates that the H-bonding interaction between NO and the guanidinium group of either L-Arg or NOHA is around 14 kJ mol$^{-1}$. A similar interaction between substrate and heme-bound dioxygen increases the kinetic stabilization of the o xoferrous complex, slowing the rate of decay via electron transfer from H$_2$B or via superoxide release. The results reinforce recent models of the NOS kinetic mechanism, which define the role of H$_2$B as a high potential one-electron donor/acceptor.

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