Ability of Tetrahydrobiopterin Analogues to Support Catalysis by Inducible Nitric Oxide Synthase: Formation of a Pterin Radical Is Required for Enzyme Activity†

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ABSTRACT: Pterin-free inducible nitric oxide synthase (iNOS) was reconstituted with tetrahydrobiopterin (H_4B) or tetrahydrobiopterin analogues (5-methyl-H_4B and 4-amino-H_4B), and the ability of bound 5-methyl-H_4B and 4-amino-H_4B to support catalysis by either full-length iNOS (FLiNOS) or the isolated heme domain (HDiNOS) was examined. In a single turnover with HDiNOS, 5-methyl-H_4B forms a very stable radical, 5-methyl-$H_3B^\bullet$, that accumulates in the arginine reaction to ∼60% of the HDiNOS concentration and decays ∼400-fold more slowly than $H_3B^\bullet$ (0.0003 vs 0.12 s^{-1}). The amount of radical (5-methyl-$H_3B^\bullet$ or $H_3B^\bullet$) observed in the NHA reaction is very small (<3% of HDiNOS). The activity of 5-methyl-H_4B-saturated FLiNOS and HDiNOS is similar to that when H_4B is bound: arginine is hydroxylated to NHA, and NHA is oxidized exclusively to citrulline and •NO. A pterin radical was not observed with 4-amino-H_4B- or pterin-free HDiNOS with either substrate. The catalytic activity of 4-amino-H_4B-bound FLiNOS and HDiNOS resembles that of pterin-free iNOS: the hydroxylation of arginine is very unfavorable (<2% that of H_4B-bound iNOS), and NHA is oxidized to a mixture of amino acid products (citrulline and cyanoornithine) and NO^- rather than •NO. These results demonstrate that the bound pterin cofactor undergoes a one-electron oxidation (to form a pterin radical), which is essential to its ability to support normal NOS turnover. Although binding of H_4B also stabilizes the NOS structure and active site, the most critical role of the pterin cofactor in NOS appears to be in electron transfer.

Nitric oxide synthase (NOS,[1] EC 1.14.13.39) catalyzes the oxidation of L-arginine to citrulline and •NO (for reviews, see refs *1*−*3*). Hydroxylation at one of the terminal guanidino nitrogens of L-arginine is the first step of the NOS reaction, leading to the formation of N^G-hydroxy-L-arginine (NHA) as an intermediate (*4*, *5*). Subsequent oxidation of NHA at the oxime nitrogen yields citrulline and •NO. The overall reaction is a five-electron oxidation that utilizes NADPH and O_2 as cosubstrates in both steps and requires protoporphyrin IX heme (*6*−*8*), FMN, FAD (*9*−*11*), and H_4B (*11*−*13*) as enzyme-bound cofactors. Three isoforms of NOS have been characterized; the NOS isoforms are distinct gene products differing in subcellular localization, tissue distribution, and mode of regulation (*1*, *3*). All are homodimeric and probably share a common mechanism, although there are some isoform-specific differences in catalysis. NOSs have a two-domain structure—a heme (or oxygenase) domain and a flavoprotein reductase domain (*14*). The enzyme active site is contained entirely within the heme domain, which binds substrate (arginine or NHA) and the heme and pterin cofactors (*15*, *16*). The reductase domain binds NADPH and both flavin cofactors and serves to provide electrons to the active site for catalysis. The heme domains of all three isoforms have been expressed and characterized; these

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[1] Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS; nNOS, neuronal NOS; iNOS, inducible NOS; HDiNOS, heme domain of inducible NOS; FLiNOS, full-length inducible NOS; H_4B, (6*R*)-5,6,7,8-tetrahydro-L-biopterin or (6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; 5-methyl-H_4B, (6*R*)-*N*5-methyl-6,7,8-trihydro-L-biopterin or (6*R*)-*N*5-methyl-6,7,8-trihydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; 4-amino-H_4B, (6*R*)-4-amino-5,6,7,8-tetrahydro-L-biopterin, also (6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pteridine; 7,8-H_2B, (6*R*)-7,8-dihydro-L-biopterin; 4-amino-H_2B, (6*R*)-4-aminodihydro-L-biopterin; DTT, dithiothreitol; NHA, N^G-hydroxy-L-arginine; CN-orn, N^δ-cyanoornithine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; Tris-HCl, Tris[hydroxymethyl]aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; NDA, 2,3-naphthalenedicarboxaldehyde; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RFQ, rapid freeze-quench; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography.

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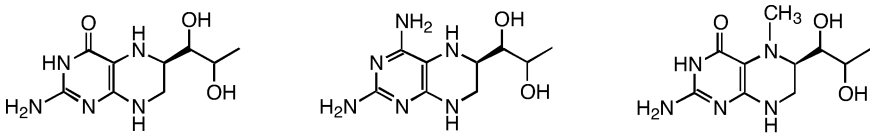
Scheme 1: Structure of Tetrahydrobiopterin Analogues



domains are dimeric and are catalytically active if provided with an exogenous source of reducing equivalents, such as sodium dithionite (*17*−*19*).

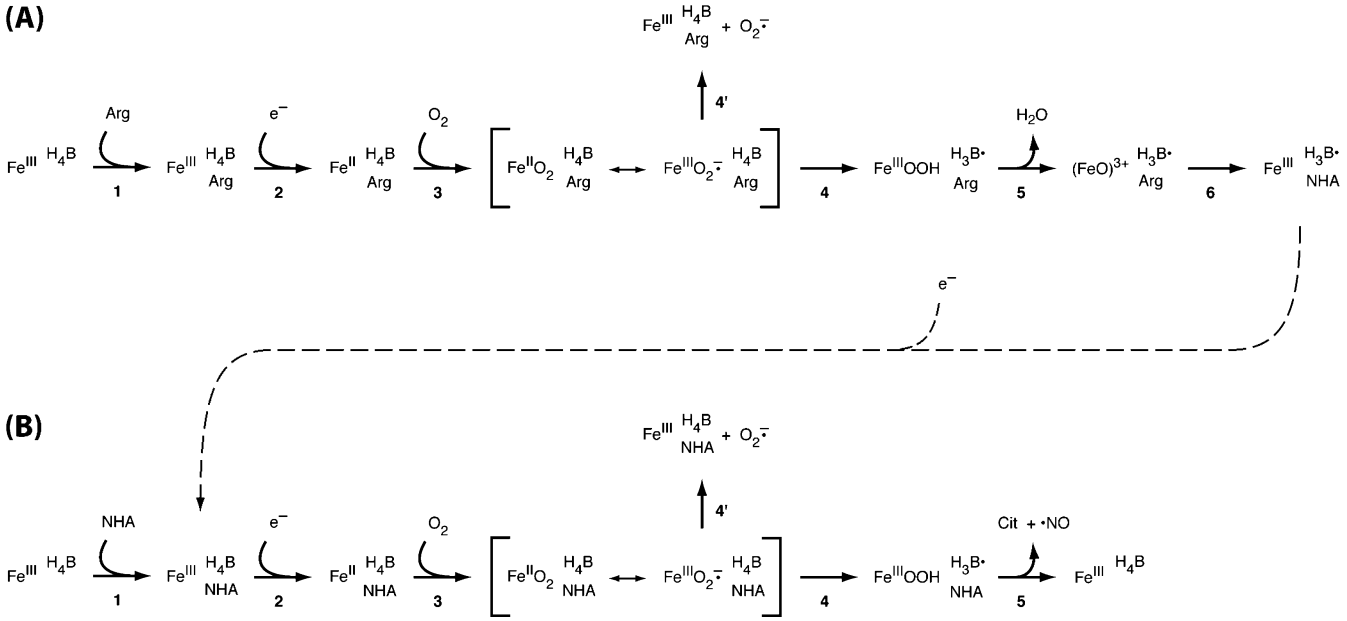
The roles of the enzyme-bound heme and H_4B cofactors in the NOS reaction mechanism have been the subject of extensive study. The NOS heme is bound with proximal ligation to a cysteinyl thiolate (*15*, *16*) and has spectral properties resembling those of cytochrome P450 (*6*−*8*). A catalytic role for the heme in both steps of the NOS reaction was suggested by CO-inhibition studies (*6*, *20*), and further evidence for a heme-based oxidant in the oxidation of NHA was provided by the ability of hydrogen peroxide to substitute for NADPH and O_2 (the peroxide-shunt pathway of cytochrome P450) in this step (*21*). The products of the peroxide-shunt reactions are consistent with a heme ferric-peroxide nucleophile intermediate in NHA oxidation (*21*, *22*). Direct biochemical evidence for the involvement of the heme in arginine hydroxylation has been more elusive since hydrogen peroxide does not support this reaction (*21*, *22*). However, information obtained from the crystal structures of NOS heme domains supports a catalytic role for the heme in both steps of the NOS reaction. Arginine binds in the active site with one of the terminal guanidino nitrogens immediately above the plane of the porphyrin ring and 3.8 Å from the heme iron (*15*, *16*), positioned to undergo hydroxylation by the heme—probably by a high valent iron−oxo species such as that proposed for P450-catalyzed hydroxylations. NHA binds in a manner similar to arginine, adjacent to the heme iron (*23*, *24*), consistent with heme-dependent catalysis in the oxidation of NHA as well.

H_4B is essential for NOS catalysis with either arginine or NHA as the substrate, and it has become clear that the function of the pterin cofactor in NOS is fundamentally different from that characterized for other enzymes that utilize pterin as a substrate or cofactor. Previous work has focused on the activity of pterin-free full-length iNOS (FLiNOS; refs *25* and *26*) or the isolated heme domain (HDiNOS; ref *19*). Elucidation of a direct chemical role for H_4B from experiments with pterin-free NOS is difficult because H_4B is also important to NOS structure, stability, and active site integrity. Specifically, binding of the pterin cofactor influences enzyme dimerization (*27*−*29*), binding affinity for substrate (*30*, *31*), spin state equilibrium of the heme (*32*, *25*), and heme midpoint potential (*33*). Although H_4B clearly stabilizes the NOS structure, the effect of the pterin cofactor is not completely explained by these structural effects. We have examined the ability of pterin analogues, including a redox-inactive, N5-deazapterin, to support catalysis by FLiNOS (*34*) and HDiNOS (*19*). These studies suggested that the redox properties of H_4B are essential to its function in NOS catalysis; however, interpretation of these results is complicated by the poor binding affinity of these analogues and the lack of structural information concerning the mode of binding of these pterins to NOS. Recent proposals for the involvement of H_4B in the NOS reaction have focused on a role for this cofactor in electron transfer, which would necessitate a redox-active pterin for NOS activity. These proposals speculated that a pterin radical might form in the NOS reaction, on the basis of differences in the spectral decay of the ferrous−dioxygen complex of nNOS in the presence and absence of H_4B (*35*) and on unique features of the pterin-binding site of NOS (*16*). Direct observation of a pterin radical ($H_3B^{\bullet}$) in reactions of reduced HDiNOS with oxygen (*17*) lends support to these proposals, namely, that H_4B provides the electron required to reduce the ferrous−dioxygen complex. Subsequent studies (*36*−*39*) have demonstrated the formation of a pterin radical in other NOS heme domains and in full-length nNOS and have shown that the formation of the pterin radical is kinetically coupled to the decay of the ferrous−dioxygen complex when arginine is the substrate (*37*, *40*, *41*).

In this paper, we have used two tetrahydrobiopterin analogues to further probe the involvement of the pterin radical in NOS catalysis. Both of these analogues, 5-methyl-H_4B[2] and 4-amino-H_4B, retain the 6-dihydroxypropyl side chain (Scheme 1) required for high-affinity binding to NOS (*42*). The 4-amino analogue is a potent inhibitor of NOS (*43*−*45*) that binds to nNOS with 20-fold higher affinity (K_i 13.2 nM; ref *43*) than the natural H_4B cofactor (K_d 250 nM; ref *30*). Although 4-amino-H_4B is unable to support NOS catalysis, it effects a complete spin-state conversion of the heme and stabilizes the dimeric structure of both nNOS and iNOS (*44*, *45*). The structure of an iNOS heme domain with bound 4-amino-H_4B shows that this analogue binds in the NOS active site in a manner indistinguishable from that of H_4B (*24*). The 5-methyl analogue can support NOS turnover (*46*, *47*), even though it is unreactive toward O_2 in solution (*46*). The binding affinity of nNOS for 5-methyl-H_4B (K_i ∼5 μM; ref *47*) is ∼20-fold lower than that for H_4B. A recent paper reports the structure of an iNOS heme domain with bound 5-methyl-H_4B (*41*), which demonstrates that the binding of 5-methyl-H_4B in the NOS active site is very similar to that of the native H_4B cofactor. Nearly all of the hydrogen-bonding interactions to the pterin are preserved (except with one water molecule, which hydrogen bonds to the pterin N5), and the differences in the conformation of the 5-methyl-H_4B-bound active site are minimal, including a slight tilt of the pterin ring to avoid collision of the N5-methyl group with Arg375 and small shifts in the position

[2] 5-Methyl-H_4B has a methyl substituent in place of the *N*5-hydrogen of H_4B and is thus actually a trihydro- rather than a tetrahydrobiopterin. However, we use the tetrahydro- nomenclature and 5-methyl-H_4B abbreviation to indicate that this compound is at the same level of oxidation as H_4B and 4-amino-H_4B. This nomenclature also serves to distinguish the fully reduced 5-methyl analogue from the one-electron oxidized trihydrobiopterin radicals, $H_3B^{\bullet}$ and 5-methyl-$H_3B^{\bullet}$ (formally a dihydrobiopterin species).

Scheme 2: Reaction Scheme for the Oxidation of Arginine and NHA by NOS[a]



[a] The hydroxylation of arginine (A) and the oxidation of NHA (B) by NOS is shown. With either substrate, the first electron (step 2) for single-turnover experiments with HDiNOS is provided by sodium dithionite; in the NADPH-dependent reaction of FLiNOS, this electron (step 2) is instead provided by the flavins of the reductase domain. For both HDiNOS and FLiNOS, H_4B directly reduces the ferrous−dioxygen species (steps 4A,B), resulting in the formation of a pterin radical. In the oxidation of NHA, $H_3B^\bullet$ subsequently acts as an electron acceptor (step 5B), leading to the formation of •NO and H_4B. Whereas $H_3B^\bullet$ (or 5-methyl-$H_3B^\bullet$) is observed during arginine hydroxylation by HDiNOS, the radical is apparently too short-lived to observe when NHA is the substrate. A complete turnover by FLiNOS starting with arginine proceeds through the intermediates shown in panel A and continues via the dashed line on to the path in panel B, ultimately forming citrulline and •NO. An additional electron from the reductase domain (dashed step) is required to reduce $H_3B^\bullet$ to H_4B prior to NHA oxidation. In the absence of a pterin that can participate in electron transfer, reduction of the ferrous-dioxygen complex is slow. Step 4′ then predominates, leading to essentially no product in the arginine reaction and to products off the normal catalytic pathway in the NHA reaction.

of Met114 and two active site waters. These reports suggested that both 5-methyl-H_4B and 4-amino-H_4B were good choices for mechanistic studies.

FLiNOS and HDiNOS can be expressed in *Escherichia coli* and purified in the absence of exogenous H_4B to obtain preparations of protein that have no detectable bound pterin. These pterin-free preparations can be readily reconstituted with either H_4B or tetrahydrobiopterin analogues to probe the function of the pterin cofactor in catalysis. Since both arginine and NHA can serve as substrates for NOS (Scheme 2), the effect of the pterin analogues on both steps of the NOS reaction can be studied. In this work, we have examined the ability of 4-amino-H_4B and 5-methyl-H_4B to form an enzyme-bound radical, and we show that this pterin radical is essential to NOS catalysis. These pterin analogue studies further our mechanistic understanding of the complex reaction catalyzed by NOS and more clearly define the role of H_4B in the enzyme reaction.

EXPERIMENTAL PROCEDURES

Materials and General Methods. H_4B, 5-methyl-H_4B, and 4-amino-H_4B were purchased from Schircks Laboratories (Jona, Switzerland), and stock solutions (∼10 mM) were prepared in 100 mM HEPES (pH 7.5) containing 100 mM DTT. For experiments requiring high concentrations of 5-methyl-H_4B, fresh stock solutions were made up in 100 mM HEPES with no DTT, to allow the addition of high concentrations of pterin without additional DTT; 5-methyl-H_4B is stable to oxidation in aerobic solutions (*46*). NHA was purchased from Cayman Chemical Co. (Ann Arbor, MI) and was found to contain less than 2% citrulline contamination as analyzed by HPLC. Sodium dithionite was purchased from Aldrich; solutions were prepared in 100 mM HEPES (pH 7.5) in an anaerobic chamber (Coy Laboratory Products) and were standardized against potassium ferricyanide before use. All other reagents were purchased from Sigma or from previously reported sources (*19*). UV−vis spectra were recorded on a Cary 3E spectrophotometer (Varian) equipped with a Neslab RTE-111 circulating water bath. All spectral experiments were carried out at 25 °C.

Purification of FLiNOS and HDiNOS. Expression and purification of pterin-free FLiNOS were as described previously (*34*). HDiNOS was expressed in JM109 cells transformed with the $pCWiNOS_{heme}$ expression vector and grown in Terrific Broth (*19*). Purification of HDiNOS was achieved in three steps and was carried out in the absence of exogenous H_4B, as previously described (*17*). Purified pterin-free HDiNOS was stored at −80 °C in 20 mM Tris-HCl (pH 8.0), with 2 mM imidazole and 300 mM NaCl. The purity of both proteins was greater than 95%, as judged by SDS−PAGE stained with Coomassie Blue R-250. Protein concentration was determined in one of two ways, depending on the experiment, and is shown as the concentration of FLiNOS or HDiNOS monomer: (1) all FLiNOS concentrations and those of HDiNOS used to calculate the stoichiometry of bound pterin or of product formation were determined by the Bradford protein assay with BSA as the standard. (2) For all other experiments, HDiNOS concentrations were determined spectrophotometrically, using the reported extinction coefficients (*19*).

Preparation of H_4B-Bound HDiNOS. Pterin-free HDiNOS was reconstituted with H_4B as follows: HDiNOS (as

purified, 50 μM) was incubated with 500 μM H_4B, 5 mM DTT, and 100 mM arginine for 1 h at 4 °C. The spectrum of HDiNOS following this incubation confirmed a complete conversion to high-spin heme (λ_{max} at 396 nm). H_4B-bound HDiNOS was concentrated to 0.1−1 mM and stored at −80 °C with 30% glycerol. Preparations of H_4B-bound HDiNOS (<300 μL) were desalted immediately prior to use on Sephadex G25M (PD10 prepacked columns, Pharmacia Amersham Biotech) into 100 mM HEPES (pH 7.5).

Reconstitution of HDiNOS with Tetrahydrobiopterin Analogues. Reconstitution with 5-methyl-H_4B or 4-amino-H_4B was carried out exactly as described above, except that 500 μM appropriate tetrahydrobiopterin analogue was substituted for H_4B. For both analogues, higher concentrations of arginine (250 mM for 5-methyl-H_4B and 170 mM for 4-amino-H_4B) were required to effect a complete spectral shift. Both HDiNOS preparations were stored as described above for H_4B-HDiNOS and were desalted immediately before use. For 5-methyl-H_4B-HDiNOS, desalting into 100 mM HEPES (pH 7.5) with no substrate resulted in a preparation of HDiNOS that was mostly low-spin (λ_{max} ~417 nm) and that did not undergo a complete spin-state conversion upon addition of saturating arginine (λ_{max} ~400 nm instead of 396 nm with 1 mM arginine), suggesting that some pterin loss might be occurring in the desalting step. The inclusion of substrate (1 mM arginine or NHA, depending on the experiment) in the desalting step prevented the shift in the heme Soret (λ_{max} ~398 nm).

Stoichiometry of Bound Pterin. HDiNOS samples were desalted into 100 mM HEPES (pH 7.5) containing 1 mM arginine. The pterin content of HDiNOS samples was determined by reverse-phase HPLC, as previously described (*19*). Retention times were as follows: H_4B, 4.3 min; 7,8-H_2B, 5.2 min; 5-methyl-H_4B, 8.0 min; 4-amino-H_4B, 5.5 min; 4-amino-H_2B, 6.5 and 8.0 min; and oxidized DTT, 10.4 min. Oxidized H_4B or 5-methyl-H_4B were not detected in either the standards or the protein samples. In contrast, oxidized species of 4-amino-H_4B were readily detected in 4-amino-H_4B standards and in samples reconstituted with this analogue. Spectrophotometric studies of stock solutions of 4-amino-H_4B were carried out and showed the reduced species to be stable for many hours in buffer at neutral pH. However, under conditions similar to those of the chromatography (stock solutions diluted into mobile phase containing guanidine, then passed through a Sep-Pak C_{18} cartridge), 4-amino-H_4B solutions were rapidly oxidized to the dihydro-species, with a $t_{1/2}$ <1 h. The spectral conversion of 4-amino-H_4B to 4-amino-H_2B had isosbestic points at 296 and 312 nm; the coincidence of the isosbestic point with the wavelength of detection (297 nm) allowed the total 4-amino-H_4B to be determined by summing the area of the reduced and oxidized pterin peaks for the standards and samples. Authentic standards of the appropriate pterin were used to quantify bound pterin in each HDiNOS sample; the standard curves obtained for the three pterins (H_4B, 5-methyl-H_4B, and 4-amino-H_4B) were indistinguishable.

Reconstitution of FLiNOS with H_4B and Tetrahydrobiopterin Analogues. Pterin-free FLiNOS (5−10 μM) was reconstituted with H_4B, 5-methyl-H_4B, or 4-amino-H_4B (50 μM) in the presence of DTT (500 μM) at 25 °C. The extent of pterin binding was monitored spectrophotometrically as a shift in the Soret peak to ~400 nm. Spectral experiments were also carried out with HDiNOS (5 μM) under identical conditions, to examine the kinetics and extent of pterin binding in the absence of substrate. For these experiments, the protein spectrum was recorded prior to and at various times following the addition of pterin and DTT. Difference spectra were calculated by subtracting the initial spectrum obtained after addition of pterin and DTT (defined as $t = 0$) from the subsequent spectra (t = 2.5−160 min). The absorbance changes in the difference spectra ($\Delta\Delta Abs = \Delta Abs_{max} - \Delta Abs_{min}$) were plotted as a function of time.

Rapid Freeze-Quench Reactions of HDiNOS. The reaction of reduced HDiNOS with oxygen was examined by rapid freeze-quenching as previously described (*17*), using an Update Instruments Model 100 unit with a custom-built quenching bath. Desalted HDiNOS preparations (H_4B-bound, 5-methyl-H_4B-bound, or 4-amino-H_4B-bound, containing 1 mM appropriate substrate) were concentrated to 200−300 μM, made anaerobic by 10 cycles of alternate evacuation and purging with purified argon gas, and reduced with sodium dithionite in an anaerobic cuvette. Sodium dithionite was added until the heme was completely reduced, as monitored spectrophotometrically (*17*); no additional dithionite was added. The reduced heme domain sample was transferred in an anaerobic chamber to the drive syringe of the freeze-quench instrument and mixed with oxygenated buffer (100 mM HEPES, pH 7.5, containing 1 mM arginine or 1 mM NHA) in a 2:1 (v/v) ratio. The buffer was oxygenated by 10 cycles of alternate evacuation and purging with oxygen gas and was allowed to equilibrate for >30 min on ice prior to transfer to the RFQ drive syringe, to give a solution concentration of ~2.2 mM O_2 (based on the solubility of O_2 at 0 °C; ref *48*). Reactions were carried out at 4 °C and were quenched at various times (15 ms to 40 min) after mixing. Final concentrations were 150−200 μM HDiNOS, 730 μM O_2, and 1 mM arginine or NHA. Oxidized and reduced controls were prepared for each experiment, as previously described (*17*).

Sample solutions remaining in the freeze-quench tubing (between the mixing chamber and the spray nozzle of the RFQ instrument) after each reaction were retrieved. All retrieved samples for a particular time course were pooled and stored at 4 °C until the end of the experiment (t >4 h), when they were frozen at −80 °C. Final concentrations were identical to those in freeze-quenched samples. Since these samples were not freeze-quenched, they represent very long reaction times (t >4 h) as compared to the time course of the RFQ experiments. These recovered samples were subsequently analyzed for the formation of amino acid products.

EPR Spectral Acquisition and Kinetic Analysis. EPR spectra were measured using a Bruker ER-200D-SRC spectrometer equipped with an Oxford Instruments ESR 910 continuous-flow cryostat. All spectra were obtained at T = 10 K with a microwave power of 2 mW, a frequency of 9.65 GHz, a modulation amplitude of 1 mT, and a modulation frequency of 100 kHz. Spin quantitations were performed by double integration using a 3 mM Cu-EDTA standard under nonsaturating conditions. Kinetic analysis of the $g = 2.0$ data was carried out by assuming two sequential irreversible first-order steps for the formation and decay of the radical species (A → B → C, where B is the radical).

Dithionite-Dependent HDiNOS Reactions. Desalted HDiNOS preparations (either pterin-bound or -free) were con-

centrated to 80 μM and made anaerobic in sealed 3 mL reaction vials by 10 cycles of alternate evacuation and purging with purified argon gas. Reactions were carried out in sealed 0.3 mL reaction vials. HDiNOS (40 μL) was transferred to each vial in an anaerobic chamber. Reactions contained 1 mM NHA and were reduced with 160 μM sodium dithionite (to provide 4 electrons per HDiNOS). Reactions were initiated by the addition of 40 μL of oxygenated buffer (100 mM HEPES, pH 7.5, containing 1 mM NHA and 100 μM phenylalanine, oxygenated by purging with pure oxygen gas for 30 min on ice to give a solution concentration of ~2.2 mM O_2; ref *48*) in a gastight syringe to each reaction vial. After mixing, reactions contained 40 μM HDiNOS, 1 mM NHA, 80 μM dithionite, 50 μM phenylalanine, and 1.1 mM O_2 in 100 mM HEPES (pH 7.5) in a total volume of 80 μL. Reaction mixtures were analyzed for the formation of •NO and for amino acid products.

•NO Detection. A Sievers Model 270 •NO chemiluminescence detector (Boulder, CO) was used to detect •NO, as previously described (*25*). For these reactions, 100 μL of headspace gas from each reaction was analyzed within 1 min after the addition of oxygenated buffer to the sealed reaction vials. The detection limit of the instrument reported by the manufacturer is ~10 fmol of •NO.

Assays of FLiNOS Activity. (A) The initial rate of •NO formation was measured by the oxidation of oxyhemoglobin as previously described (*49*), using an extinction coefficient ($\Delta\epsilon_{401}$) of 60 000 M^{-1} cm^{-1} for the appearance of methemoglobin. Assays (total volume 500 μL) contained 1 mM substrate (arginine or NHA), 60 μM NADPH, 4.8 μM oxyhemoglobin, 0−8 μM pterin, 0−80 μM DTT, and 16 nM FLiNOS (1 μg) in 100 mM HEPES, pH 7.5. Reactions were initiated by the addition of enzyme, and the increase in absorbance at 401 nm was monitored on a Beckman DU 640 spectrophotometer at 37 °C.

(B) Endpoint product analysis (amino acid and NO_2^-/NO_3^-) was also carried out for a parallel set of FLiNOS reactions. These assays (total volume 50 μL) contained 0.5 mM substrate (arginine or NHA), 1 mM NADPH, 0−100 μM pterin, 0 or 100 μM DTT, and 160 nM FLiNOS (1 μg) in 100 mM HEPES, pH 7.5. Reactions were initiated by the addition of enzyme and were allowed to proceed for 2 h at 25 °C. Samples were diluted prior to analysis (3-fold for amino acid analysis, up to 10-fold for NO_2^-/NO_3^-); 50 μM phenylalanine was added as an internal standard prior to amino acid analysis.

Quantitation of Amino Acid Products. The amino acid products of the FLiNOS and HDiNOS reactions were derivatized with NDA and analyzed by reverse-phase HPLC. Two different separation methods were used, the details of which were previously described (*19*). For most of the samples in this study, derivatized amino acids were separated using a methanol gradient, which gives optimal resolution of citrulline and CN-orn; for reactions of HDiNOS with arginine as the substrate (retrieved RFQ samples only), a gradient of acetonitrile was used instead for greater resolution of arginine and NHA. NHA and citrulline standards were used to quantify the reaction products, using phenylalanine as an internal standard.

Quantitation of Nitrite and Nitrate. Total NO_x^- (NO_2^- and NO_3^-) formed in the FLiNOS reactions was quantified using the colorimetric assay kit from Cayman Chemical Co. (Ann Arbor, MI), as previously described (*25*).

RESULTS

Reconstitution of HDiNOS with H_4B and Tetrahydrobiopterin Analogues. As previously reported for H_4B (*17*, *19*), HDiNOS can be reconstituted with tetrahydrobiopterin analogues (Scheme 1). H_4B binding to HDiNOS effects a shift in the heme spin-state from low- to high-spin; consequently, binding of the pterin cofactor can be monitored by the resulting spectral changes. Reconstitution with any of the pterins in the presence of substrate results in a complete shift of the heme to the high-spin state (λ_{max} 396 nm). After desalting to remove the unbound pterin, H_4B-HDiNOS and 4-amino-H_4B-HDiNOS remained mostly high-spin (λ_{max} ~400 nm) even in the absence of substrate. For 5-methyl-H_4B-HDiNOS, the inclusion of substrate in the desalting step was necessary to prevent conversion of the heme to low-spin (λ_{max} ~417 nm). This observed spectral shift cannot be attributed only to the dissociation of arginine since the desalted 5-methyl-H_4B-HDiNOS has a decreased binding affinity for arginine (in the presence of saturating arginine, only a partial spin-state conversion is obtained, λ_{max} ~400 nm instead of 396 nm) and also binds DTT (DTT only binds to pterin-free iNOS; see ref *50*). Together, these observations suggest that 5-methyl-H_4B dissociates during the desalting step and that the presence of arginine increases the affinity of HDiNOS for 5-methyl-H_4B, as observed for H_4B (*30*−*32*). To minimize the dissociation of pterin from reconstituted HDiNOS samples, desalting into buffer containing either arginine or NHA was carried out immediately prior to experiments.

Time Dependence of Pterin Binding to HDiNOS. To further examine pterin binding to HDiNOS, reconstitutions were carried out in the absence of substrate, and the progress of the reactions was monitored spectrophotometrically. Figure 1 shows the spectra obtained following the addition of H_4B or tetrahydrobiopterin analogues to HDiNOS. For these experiments, pterin-free HDiNOS was first desalted to remove the bound and excess imidazole. The starting spectrum is that of the 6-coordinate (water-bound), low-spin ferric complex of HDiNOS (λ_{max} ~421 nm) and is identical in Figure 1, panels A−C. The first intermediate in the reconstitution reaction has a characteristic split Soret spectrum (λ_{max} 378 and 458 nm) typical of a bis-thiolate complex (*51*). This intermediate arises from binding of DTT to the heme, as previously observed in the reconstitution of full-length NOS (*34*, *52*−*54*), and is observed immediately after addition of the pterin/DTT solution to HDiNOS. At longer time points, the split Soret of the bis-thiolate disappears concomitantly with the appearance of high-spin HDiNOS (λ_{max} ~400 nm). This conversion occurs with distinct isosbestic points at 430, 490, 543, 620, and 670 nm. The slow spectral conversion, complete in ~60 min, reflects a complex process involving dissociation of DTT from the active site, binding of the pterin cofactor, and a spin-state conversion of the heme cofactor (*34*, *53*, *54*). The final spectrum obtained upon addition of either H_4B (Figure 1A) or 4-amino-H_4B (Figure 1C) is essentially identical and indicates a mixture of ~85% high-spin and ~15% low-spin heme. Complete conversion of the heme to the high-spin state (λ_{max} 396 nm) in either of these samples is obtained

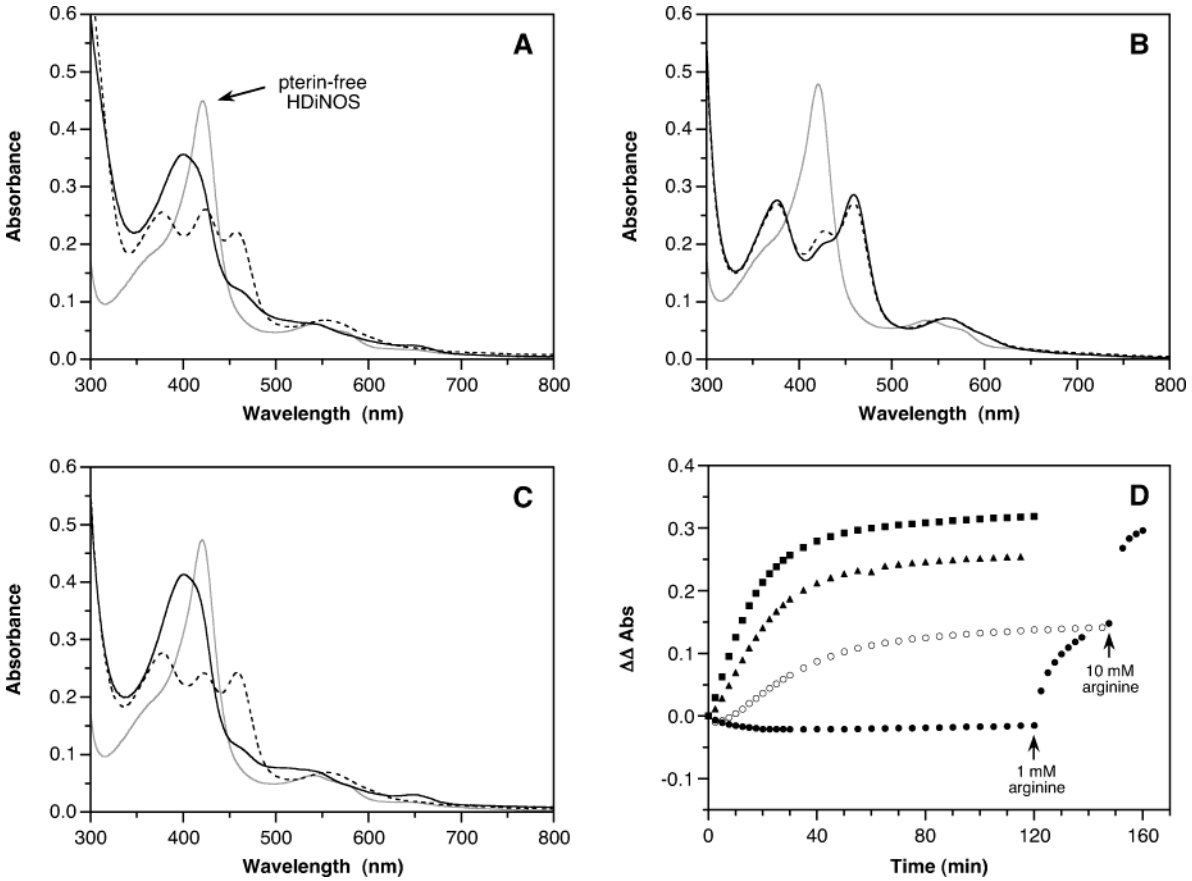


FIGURE 1: Reconstitution of HDiNOS with H_4B and tetrahydrobiopterin analogues. HDiNOS (~5 μM) was incubated at 25 °C with 50 μM H_4B (A), 5-methyl-H_4B (B), or 4-amino-H_4B (C) in the presence of 500 μM DTT. No substrate was present during the reconstitution. Pterin-free HDiNOS (shaded spectra; labeled in panel A) has a λ_{max} around 421 nm, characteristic of a low-spin heme. The initial spectrum obtained after the addition of pterin/DTT (dashed spectra; λ_{max} 378 and 458 nm) is consistent with the binding of DTT to the heme to form a bis-thiolate complex. The spectrum recorded after 2 h of incubation is shown for each sample (solid spectra). In the presence of H_4B or 4-amino-H_4B (A and C, respectively), the final spectrum is that of the pterin-bound, mostly high-spin heme (λ_{max} ~400 nm). In the presence of 5-methyl-H_4B (B), however, the bis-thiolate complex remains, even after 2 h of incubation. (D) Difference spectra were calculated by subtracting the initial spectrum ($t = 0$) from the subsequent spectra ($t = 2.5-160$ min), and the absorbance changes in the difference spectra ($Abs = Abs_{400} - Abs_{359}$) were plotted as a function of time. Solid symbols represent reconstitutions carried out in the absence of substrate and are derived from the experiment shown in panels A−C. The spectral changes observed with H_4B (▲) and 4-amino-H_4B (■) occur at a similar rate and are complete within ~60 min. The negative Abs observed in the 5-methyl-H_4B incubation (●) indicates further conversion to the bis-thiolate complex. Addition of 1 mM arginine at $t = 120$ min results in a partial shift of HDiNOS to a high-spin heme. The endpoint reached is essentially the same as that obtained when 1 mM arginine is added to HDiNOS simultaneously with 5-methyl-H_4B and DTT (○, spectra not shown). Higher concentrations of arginine (10 mM arginine added at $t = 150$ min) are required to reach a similar endpoint as that observed with H_4B and 4-amino-H_4B in the absence of substrate.

upon addition of 1 mM arginine (not shown). Reconstitution of HDiNOS with 5-methyl-H_4B, in contrast, appears to require the presence of substrate. Figure 1B shows the spectra obtained upon the addition of 5-methyl-H_4B. The DTT, bis-thiolate complex forms immediately on addition of 5-methyl-H_4B/DTT, but in this case, no further spectral changes are observed, even after 2 h of incubation time. The addition of arginine, however, initiates the conversion of the heme spectrum to high-spin (spectra not shown). These observations suggest that the binding of 5-methyl-H_4B to HDiNOS is dependent on the presence of arginine; however, we cannot exclude the possibility that the pterin analogue does in fact bind to HDiNOS but is unable to effect a spin-state shift of the heme once bound. A similar dependence on the presence of arginine for the binding of 5-methyl-H_4B to an iNOS heme domain was recently reported (*41*). Difference spectra were calculated for each time point ($t = 2.5-160$ min). Maximal changes in the difference spectra are observed at 400 nm (peak) and 459 nm (trough). ΔΔAbs values ($\Delta Abs_{400} - \Delta Abs_{459}$) represent the extent of the spectral conversion, and when plotted as a function of time, can be used to determine the rate of the conversion (Figure 1D). Reconstitution of HDiNOS with H_4B and 4-amino-H_4B is essentially complete within 60 min and occurs with a similar $t_{1/2}$, ~15 min. In contrast, 5-methyl-H_4B appears not to bind at all to HDiNOS in the absence of substrate; the negative ΔΔAbs values observed indicate that HDiNOS is instead further converted to the DTT bis-thiolate complex in the first 10 min of incubation, with no additional changes at longer time points. Upon addition of 1 mM arginine, a fast but only partial conversion to the high-spin state is observed; 10 mM arginine is required to reach the same endpoint as that observed in the presence of H_4B or 4-amino-H_4B with no substrate. The simultaneous addition of 1 mM arginine and 5-methyl-H_4B/DTT also results in partial reconstitution (spectra not shown), to the same extent as that observed when 1 mM arginine is added after 120 min of incubation with 5-methyl-H_4B/DTT alone. The spectral conversion with 5-methyl-H_4B in the presence of 1 mM arginine has a $t_{1/2}$ of ~25 min.

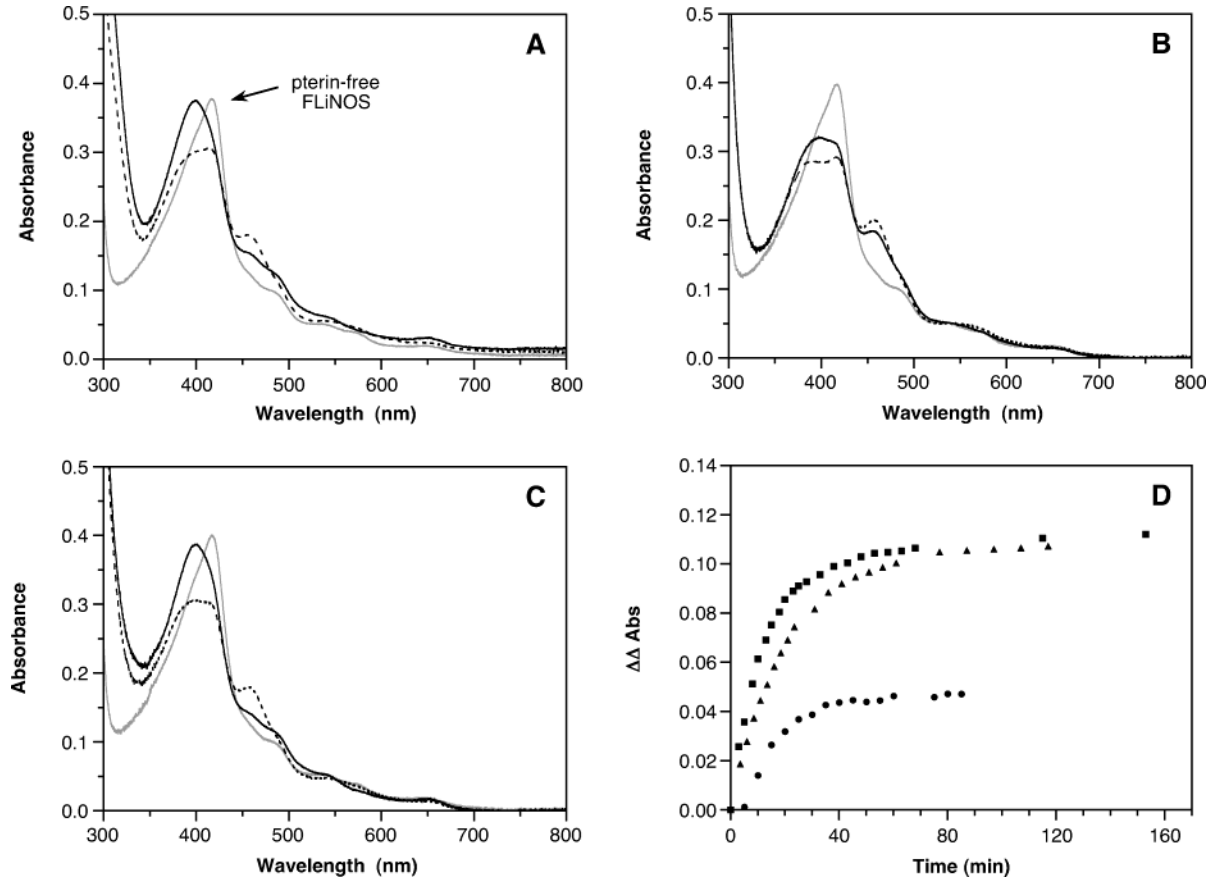


FIGURE 2: Reconstitution of FLiNOS with H_4B and tetrahydrobiopterin analogues. FLiNOS (~5 μM) was reconstituted at 25 °C with 50 μM H_4B (A), 5-methyl-H_4B (B), or 4-amino-H_4B (C) with 500 μM DTT and no substrate. Pterin-free FLiNOS (shaded spectra; labeled in panel A) has a λ_{max} around 417 nm, indicative of a predominantly low-spin heme. The initial spectrum obtained after the addition of pterin/DTT (dashed spectra) is consistent with partially DTT-bound FLiNOS (λ_{max} 378 and 458 nm). The spectrum recorded after 2 h of incubation is shown for each sample (solid spectra). In the presence of H_4B or 4-amino-H_4B (A and C, respectively), the final spectrum is that of the pterin-bound, mostly high-spin heme (λ_{max} ~400 nm). In the presence of 5-methyl-H_4B (B), the final spectrum is a mixture of high-spin heme and the bis-thiolate complex. (D) Difference spectra were calculated for each time point (t = 2.5−160 min), and the absorbance changes (Abs = $Abs_{400} - Abs_{359}$) were plotted as a function of time. The observed spectral changes for all three samples are complete within ~60 min, with $t_{1/2}$ of 10−15 min. The extent of the spectral conversion with 5-methyl-H_4B (●) is only ~40% of that observed with either H_4B (▲) or 4-amino-H_4B (■).

Reconstitution of FLiNOS with H_4B and Tetrahydrobiopterin Analogues. FLiNOS was also reconstituted with H_4B, 5-methyl-H_4B, and 4-amino-H_4B in the absence of substrate (Figure 2). Similar results are obtained as those described above for HDiNOS. Although pterin-free FLiNOS is predominantly low-spin, the λ_{max} around 417 nm indicates that there is some high-spin heme present in this preparation prior to reconstitution with pterin. Addition of any of the pterins in the presence of DTT results in a spectrum consistent with DTT binding to the heme to form a bis-thiolate complex. This intermediate spectrum has a broad absorbance band centered ~390 nm and a λ_{max} at 457 nm, reflecting that only a portion of the FLiNOS is present as the DTT-bound species. This difference between FLiNOS and HDiNOS is consistent with a faster rate of pterin reconstitution of the full-length enzyme as compared to the heme domain. The ensuing conversion of FLiNOS to high-spin involves the dissociation of DTT and the binding of pterin. Distinct isosbestic points are detected at ~430, 482, 552, and 620 nm. The rate and extent of reconstitution are assessed from absorbance changes in the calculated difference spectra (ΔΔAbs; see Figure 2D). The spectral conversion is complete within 60 min for all of the pterins studied.

The rate of reconstitution with H_4B and 4-amino-H_4B is slightly faster than that with 5-methyl-H_4B ($t_{1/2}$ of ~10 min, as compared with ~15 min). More importantly, the extent of the spectral conversion in the presence of 5-methyl-H_4B is only ~40% of that with H_4B and 4-amino-H_4B. This probably indicates that the affinity of FLiNOS for 5-methyl-H_4B is lower than that for the other pterins; however, this result contrasts with that obtained for HDiNOS, where no spectral shift is observed with 5-methyl-H_4B in the absence of substrate (compare with Figure 1).

Stoichiometry of Pterin Binding and Affinity for Tetrahydrobiopterin Analogues. The pterin content of several preparations of H_4B-, 5-methyl-H_4B-, and 4-amino-H_4B-HDiNOS was determined. The stoichiometry of H_4B binding in these samples is 0.83 ± 0.18 (n = 4), similar to that previously reported (0.86 ± 0.08; see ref *19*). The stoichiometry of analogue binding is 0.38 ± 0.19 (n = 6) and 0.79 ± 0.15 (n = 6) for 5-methyl-H_4B and 4-amino-H_4B, respectively. 4-amino-H_4B apparently binds to HDiNOS to approximately the same extent as the natural H_4B cofactor, suggesting that the affinity of HDiNOS for these two pterins is similar. In contrast, the binding of 5-methyl-H_4B to HDiNOS is weaker and more variable. This is consistent

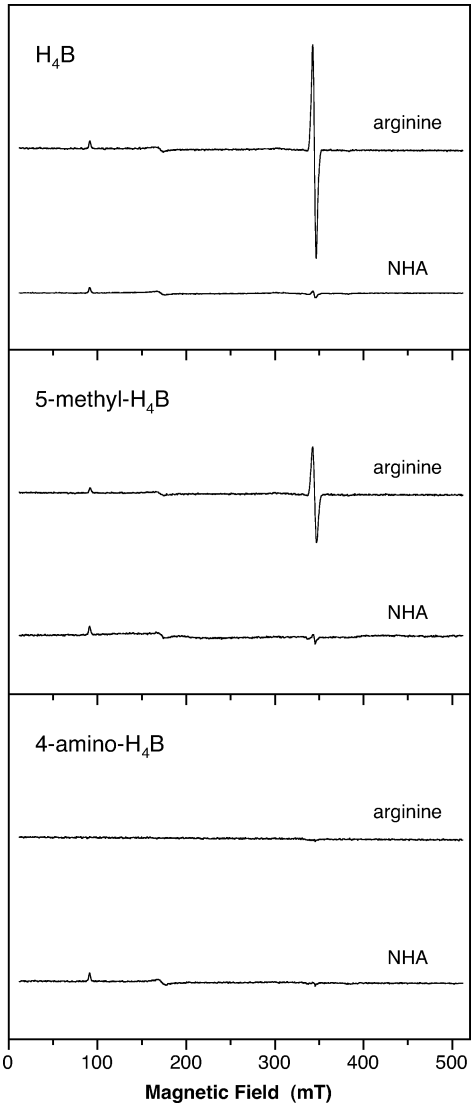


FIGURE 3: EPR spectra of rapid freeze-quenched samples. Spectra are shown for samples quenched 127 ms after mixing of reduced HDiNOS with oxygen. Final concentrations after mixing were 150−200 μM HDiNOS, 730 μM O_2, and either 1 mM arginine or 1 mM NHA, as indicated. Each spectrum was normalized to a HDiNOS concentration of 200 μM, to allow direct comparison among the samples. A ferric high-spin heme signal ($g = 7.5$, 4.1, and 1.8) of similar intensity is seen in all the spectra, except in that of 4-amino-H_4B-HDiNOS in the presence of arginine. The $g = 2.0$ signal is attributed to a pterin radical ($H_3B^{\bullet}$), observed only in samples of H_4B- and 5-methyl-H_4B-HDiNOS in the presence of arginine. EPR conditions were as described in Experimental Procedures.

with the observations reported above, that higher concentrations of pterin and arginine are required to effect a complete spectral shift of HDiNOS by 5-methyl-H_4B and that the desalting of 5-methyl-H_4B-HDiNOS in the absence of arginine results in a reversion of the heme to the low-spin state. Even when substrate is present, desalting apparently results in a significant loss of the bound pterin cofactor, as reflected in the calculated stoichiometry for 5-methyl-H_4B. Further evidence for pterin loss during the desalting step is that a bis-thiolate complex forms upon the addition of 5-methyl-H_4B/DTT to desalted 5-methyl-H_4B-HDiNOS. All of these observations together suggest that the affinity of HDiNOS for 5-methyl-H_4B is decreased as compared to that for H_4B and 4-amino-H_4B, consistent with the reported K_i values for these two analogues with nNOS (*43*, *47*). Although

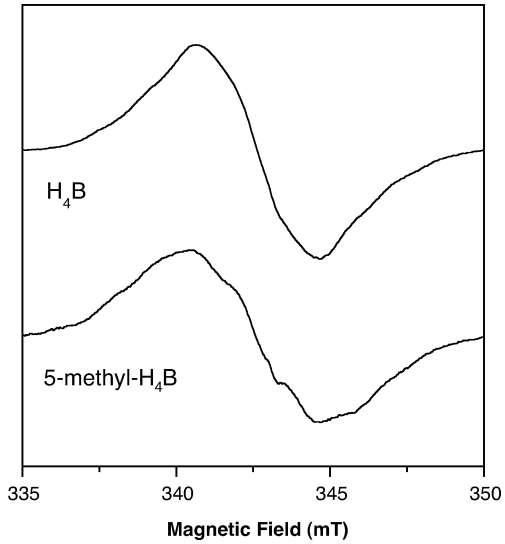


FIGURE 4: Comparison of the $g = 2.0$ signal of H_4B-HDiNOS and 5-methyl-HDiNOS. Expanded spectra of the $g = 2.0$ region of samples quenched at 127 ms are shown (full spectra of same samples in Figure 3). Final concentrations after mixing were 190−195 μM HDiNOS, 730 μM O_2, and 1 mM arginine. Each spectrum was normalized to a spin concentration of 150 μM for comparison.

the stoichiometries of pterin binding were not determined for FLiNOS, it is likely that the affinity of FLiNOS for 5-methyl-H_4B is also lower than that for H_4B and 4-amino-H_4B. The smaller spectral shift observed on the addition of 5-methyl-H_4B to FLiNOS (see Figure 2), as well as the dependence of FLiNOS activity on added 5-methyl-H_4B (vide infra), support this conclusion.

Rapid Freeze-Quench Reactions of HDiNOS. Figure 3 shows EPR spectra of HDiNOS after reaction of reduced HDiNOS with oxygen. The spectra of H_4B-, 5-methyl-H_4B-, and 4-amino-H_4B-HDiNOS are compared, in the presence of either 1 mM arginine or 1 mM NHA. Features at $g =$ 7.6, 4.1, and 1.8 are characteristic of ferric, high-spin heme. The spectra shown are for samples quenched 127 ms after mixing, which corresponds to the time point where the maximum signal at $g = 2.0$ is observed. This signal was previously characterized for the reaction of H_4B-HDiNOS and was shown to arise from a pterin radical (*17*). As previously observed for H_4B-HDiNOS (and repeated here for comparison), a radical species is observed with 5-methyl-H_4B-HDiNOS in the presence of arginine. Figure 4 shows the expanded spectra of the $g = 2.0$ region of the H_4B- and 5-methyl-H_4B-HDiNOS samples with arginine. The peak-to-trough line width of the radical in either sample measures ~40 G. Although the signals obtained with both proteins are similar in overall shape and width, there are small differences in the hyperfine structures of the radical signals. These differences suggest an altered electronic structure of the radical, providing further evidence that the signal does in fact arise from the pterin cofactor. With either pterin, only a very small radical signal is observed in the presence of NHA. No $g = 2.0$ signal was observed in reactions of 4-amino-H_4B-HDiNOS with either amino acid substrate at any time point. The spectrum at 127 ms of 4-amino-H_4B-HDiNOS in the presence of arginine is devoid of any paramagnetic signal—no pterin radical is observed, and furthermore, no oxidized heme signal is apparent. Oxidation of the heme cofactor is observed at longer time points (>5

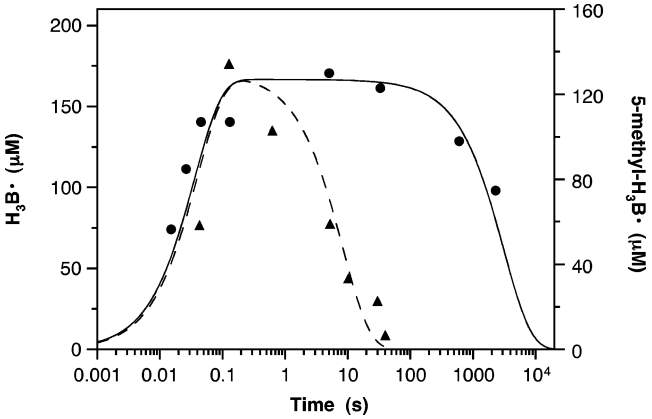


FIGURE 5: Time course of pterin radical formation and decay. Reactions of reduced HDiNOS and oxygen were quenched at various times after mixing (15 ms to 40 min). The concentration of radical present at each time point was determined by double integration of the $g = 2.0$ signal, using Cu-EDTA as the standard. The time course of pterin radical formation and decay is shown for reactions of H_4B-HDiNOS ($H_3B^{\bullet}$, ▲) and 5-methyl-H_4B-HDiNOS (5-methyl-$H_3B^{\bullet}$, ●) in the presence of 1 mM arginine. The final protein concentration in these reactions was 225 μM for H_4B-HDiNOS and 191 μM for 5-methyl-H_4B-HDiNOS. The data were fit by assuming two sequential irreversible first-order steps for the formation and decay of the radical. The calculated rates for the reaction of H_4B-HDiNOS are ~20 s^{-1} for the formation of $H_3B^{\bullet}$ and ~0.12 s^{-1} for its decay (dashed line; previously published data (*17*) shown for comparison). The fit to the 5-methyl-H_4B-HDiNOS data is shown as the solid line, giving rates of ~30 s^{-1} for the formation of 5-methyl-$H_3B^{\bullet}$ and ~0.0003 s^{-1} for its decay. The data are plotted on a double *y*-axis to emphasize that the rate of radical formation is similar in both samples.

s; $t_{1/2}$ ~25 s, data not shown). This result is similar to that previously described for pterin-free HDiNOS, where no heme oxidation is observed at reaction times up to 5 s in the presence of arginine (*17*). In both cases, this observation suggests that ultimate heme oxidation in the arginine reaction is coupled to the formation of a pterin radical. A ferric heme signal is seen at 127 ms in the reaction of 4-amino-H_4B-HDiNOS with NHA, indicating that heme oxidation is ~1000-fold faster ($t_{1/2}$ ~25 ms) when NHA rather than arginine is the substrate with this protein.

Quantitation and Time Dependence of the Pterin Radical Signal. As described above, the amount of pterin radical varies with the identity of the bound pterin as well as the substrate. The radical signal is also time-dependent, with maximal intensity of the signal at 127 ms (Figure 5). With H_4B-HDiNOS in the presence of 1 mM arginine, the concentration of pterin radical at 127 ms is ~80% of the concentration of the HDiNOS monomer. The radical of 5-methyl-H_4B-HDiNOS accumulates to a maximum of ~60% (120 μM) of the HDiNOS concentration in the presence of 1 mM arginine. When compared to the pterin stoichiometry determined for these samples, these results suggest that in both proteins all of the bound pterin is present as the radical species at 127 ms (although the average 5-methyl-H_4B stoichiometry is 0.38, this particular sample had the highest pterin content, 0.54). The rates of formation and decay of the 5-methyl-H_4B radical were calculated from these data. The $g = 2.0$ signal appears at a rate of ~30 s^{-1} and decays at a rate of ~0.0003 s^{-1} ($t_{1/2}$ ~2300 s or 40 min). Although the rate of formation of the two radicals is similar (k ~15−20 s^{-1} for $H_3B^{\bullet}$), the rate of decay of 5-methyl-$H_3B^{\bullet}$ is ~400-fold slower than that of $H_3B^{\bullet}$ (k ~0.12 s^{-1} or $t_{1/2}$ ~6 s). For both proteins, the amount of pterin radical observed in the NHA reaction is much lower than that observed with arginine, <3% of the HDiNOS, as previously reported for H_4B (*17*).

Dithionite-Dependent Reactions of HDiNOS. Samples retrieved after mixing in the RFQ instrument (not freeze-quenched, t >4 h; see Experimental Procedures for details) were analyzed for product formation. NHA is formed in the reaction of arginine with 5-methyl-H_4B-HDiNOS. The stoichiometry of product formed is 0.47 NHA per HDiNOS monomer ($n = 1$) for 5-methyl-H_4B-HDiNOS (same sample as in the previous paragraph, with a pterin content of 0.54), as compared to 0.78 obtained with H_4B-HDiNOS (*17*). In contrast, no product was observed in the reaction of arginine with 4-amino-H_4B-HDiNOS, giving a calculated product stoichiometry of 0 NHA per HDiNOS monomer ($n = 1$). A mixture of amino acid products is formed in the oxidation of NHA, as previously described for the reaction of pterin-free HDiNOS (*19*). The stoichiometry of total product formed in the NHA reactions is 0.55 and 0.65 per HDiNOS monomer for 5-methyl-H_4B-HDiNOS and 4-amino-H_4B-HDiNOS, respectively ($n = 1$ for each), where the total product represents the sum of citrulline, CN-orn, and an unidentified product in these samples. These stoichiometries are similar to that reported for H_4B-HDiNOS (0.63; ref *17*); however, citrulline is the sole product of NHA oxidation by H_4B-HDiNOS.

Additional reactions, using dithionite as the source of reducing equivalents, were carried out to determine the identity of the inorganic product of NHA oxidation for each of these proteins. An excess of dithionite (to provide 4 electrons per HDiNOS monomer) was included in these reactions to ensure full reduction of HDiNOS and to maximize product formation. Analysis of the headspace of these reactions by chemiluminescence, a very sensitive and specific detection method for $^{\bullet}NO$, indicates that only H_4B-HDiNOS and 5-methyl-H_4B-HDiNOS form $^{\bullet}NO$ in the reaction with NHA. Reactions of 4-amino-H_4B-HDiNOS and pterin-free HDiNOS gave no detectable signal on the $^{\bullet}NO$ analyzer; the inorganic product of these reactions is probably NO^- rather than $^{\bullet}NO$, as previously discussed (*19*, *25*). These same reactions were also analyzed for amino acid formation. A mixture of amino acid products is obtained in these samples, but the relative distribution of products depends on which pterin is bound. Although citrulline is the sole product of NHA oxidation reported for H_4B-HDiNOS (*17*, *19*), a small amount of CN-orn (13.5 ± 1.5%, $n = 4$) was detected in the H_4B-HDiNOS reaction here, likely reflecting a low stoichiometry of bound H_4B in this sample. Citrulline is the predominant product of NHA oxidation in the tetrahydrobiopterin analogue reactions, representing 44.1 ± 2.9% ($n = 4$) and 46.1 ± 7.9% ($n = 4$) of the total product for 5-methyl-H_4B-HDiNOS and 4-amino-H_4B-HDiNOS, respectively. In the pterin-free reaction, citrulline represents only 20.9 ± 9.3% ($n = 4$) of the total product, and the predominant product is CN-orn, accounting for 53.4 ± 6.6% ($n = 4$) of the total product. Although the distribution of products differs, the amount of product obtained in all of the reactions is similar, with calculated product stoichiometries (relative to HDiNOS concentration) of 1.12 ± 0.05 ($n = 4$), 1.04 ± 0.04 ($n = 4$), 1.07 ± 0.27 ($n = 4$), and 1.01

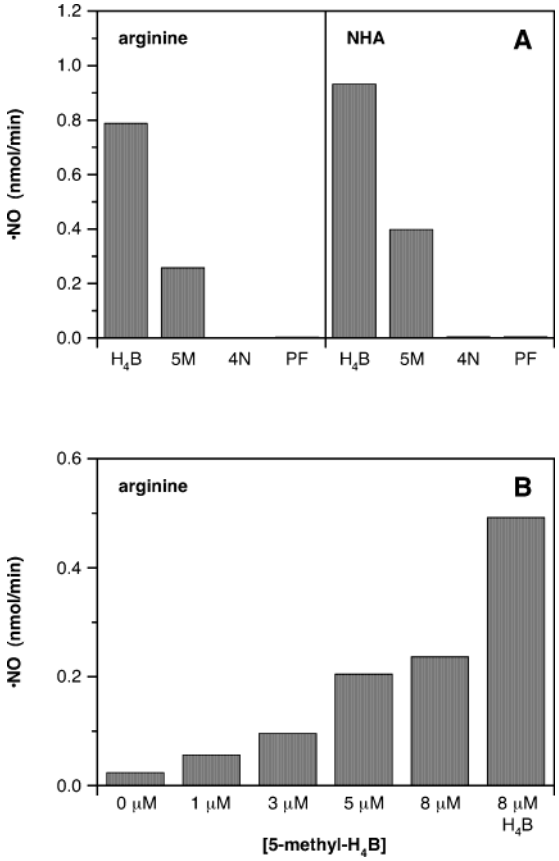


FIGURE 6: •NO formation by FLiNOS reconstituted with H_4B and tetrahydrobiopterin analogues. The initial rate of •NO formation was measured by the oxyhemoglobin assay at 37 °C, and results are shown for a representative experiment. Reactions (500 μL total volume) contained 1 mM substrate (arginine or NHA), 60 μM NADPH, 4.8 μM oxyhemoglobin, 0−8 μM pterin, 0−80 μM DTT, and 16 nM FLiNOS (1 μg) in 100 mM HEPES, pH 7.5. (A) Comparison of the activity of FLiNOS reconstituted with H_4B (H_4B), 5-methyl-H_4B (5 M), or 4-amino-H_4B (4 N) and pterin-free FLiNOS (PF). Bars represent the initial rate of •NO formation in the presence of 8 μM appropriate pterin (H_4B for H_4B-FLiNOS; 5-methyl-H_4B for 5-methyl-H_4B-FLiNOS, and 4-amino-H_4B for 4-amino-H_4B-FLiNOS) and 80 μM DTT; assays of pterin-free FLiNOS contained no exogenous pterin or DTT. Each protein was assayed in the presence of 1 mM arginine (left panel) or 1 mM NHA (right panel). (B) Dependence of the activity of 5-methyl-H_4B-FLiNOS on additional pterin. The rate of •NO formation by 5-methyl-H_4B-FLiNOS as a function of the concentration of pterin added to the assay is shown. All assays contained 1 mM arginine.

$\pm$ 0.26 ($n = 4$) for H_4B-, 5-methyl-H_4B-, 4-amino-H_4B-, and pterin-free HDiNOS, respectively.

Activity of FLiNOS Reconstituted with Tetrahydrobiopterin Analogues. The ability of the tetrahydrobiopterin analogues to catalyze turnover of arginine and NHA by FLiNOS was investigated by several methods.

(A) Oxyhemoglobin Oxidation Assay for •NO. The activity of FLiNOS as measured by the oxidation of oxyhemoglobin is summarized in Figure 6A. Data from different experiments were not directly comparable because of differences in specific activity between samples; however, the data shown are representative of the trends observed in each experiment. H_4B- and 5-methyl-H_4B-FLiNOS catalyze the formation of •NO, using either arginine or NHA as the substrate. In contrast to these two active proteins, 4-amino-H_4B- and pterin-free FLiNOS are both essentially inactive as measured by this assay. The activity of both H_4B- and 5-methyl-H_4B-FLiNOS is increased by the inclusion of additional pterin in the assay. For H_4B-FLiNOS, the addition of 8 μM H_4B to the assay results in a 10−20% increase in enzyme activity (data not shown). The activity of 5-methyl-H_4B-FLiNOS is far more dependent on the addition of pterin to the assay, consistent with the apparently decreased affinity of FLiNOS for this analogue. In the absence of additional pterin, 5-methyl-H_4B-FLiNOS has very little activity (Figure 6B), and the addition of 8 μM 5-methyl-H_4B results in a 10-fold increase in enzyme activity. Even when assayed in the presence of 8 μM additional pterin, the rate of product formation by 5-methyl-H_4B-FLiNOS is slower than that observed for H_4B-FLiNOS.

(B) Amino Acid Products. Parallel reactions of FLiNOS were set up to measure endpoint product formation and to examine the identity of the amino acid products formed under these conditions. A representative experiment, illustrating the amount and distribution of amino acids formed in each of these reactions, is shown in Figure 7A. Regardless of the substrate used, H_4B is the most active pterin, followed by 5-methyl-H_4B, and then 4-amino-H_4B (see Table 1 for relative activity with both substrates). Pterin-free FLiNOS is completely inactive with respect to the hydroxylation of arginine under these conditions but is able to catalyze the oxidation of NHA, as previously reported (*25*). Catalysis by H_4B- and 5-methyl-H_4B-FLiNOS results in the formation of citrulline from either arginine or NHA. Furthermore, some NHA is also observed as a product in the reaction with arginine with either pterin. In contrast to both these proteins, 4-amino-H_4B-FLiNOS is virtually inactive in the reaction with arginine, forming only 1.6 $\pm$ 0.7% ($n = 3$) of the citrulline observed in reactions with H_4B-FLiNOS. All of the FLiNOS preparations catalyze the oxidation of NHA, but the identity of the amino acid product depends on the bound pterin. While H_4B-FLiNOS forms only citrulline in this reaction, CN-orn is observed with both tetrahydrobiopterin analogues and in the pterin-free reaction. The relative amount of CN-orn was somewhat variable, depending on the experiment; CN-orn represents 1−2, 10−30, and 30−40% of the total product in the reactions with 5-methyl-H_4B-, 4-amino-H_4B-, and pterin-free FLiNOS, respectively (see Table 1 for average percent citrulline formed for each sample). The unidentified amino acid observed in some HDiNOS reactions (vide supra) is not a product of NHA oxidation by FLiNOS, even in the absence of pterin. Figure 7B,C shows the dependence of product formation by 5-methyl-H_4B-FLiNOS on the addition of 5-methyl-H_4B to the assay. As reported above for the oxyhemoglobin assays, the inclusion of additional 5-methyl-H_4B in the enzyme reactions results in higher activity with both arginine (Figure 7B) and NHA (Figure 7C). Furthermore, higher concentrations of added 5-methyl-H_4B in the reaction with NHA decrease the fraction of CN-orn formed to essentially 0% of the total product. DTT alone has only a small effect on the amount or identity of the amino acid products in either reaction. The effect of added pterin on the reaction catalyzed by 4-amino-H_4B-FLiNOS was also examined (Figure 7D). The addition of 10 μM 4-amino-H_4B has a small effect on the amount of CN-orn formed in the NHA reaction; higher concentrations of 4-amino-H_4B (up to 100 μM) had no additional effect. Figure 7D also shows that product formation with either substrate is dependent on the concentration

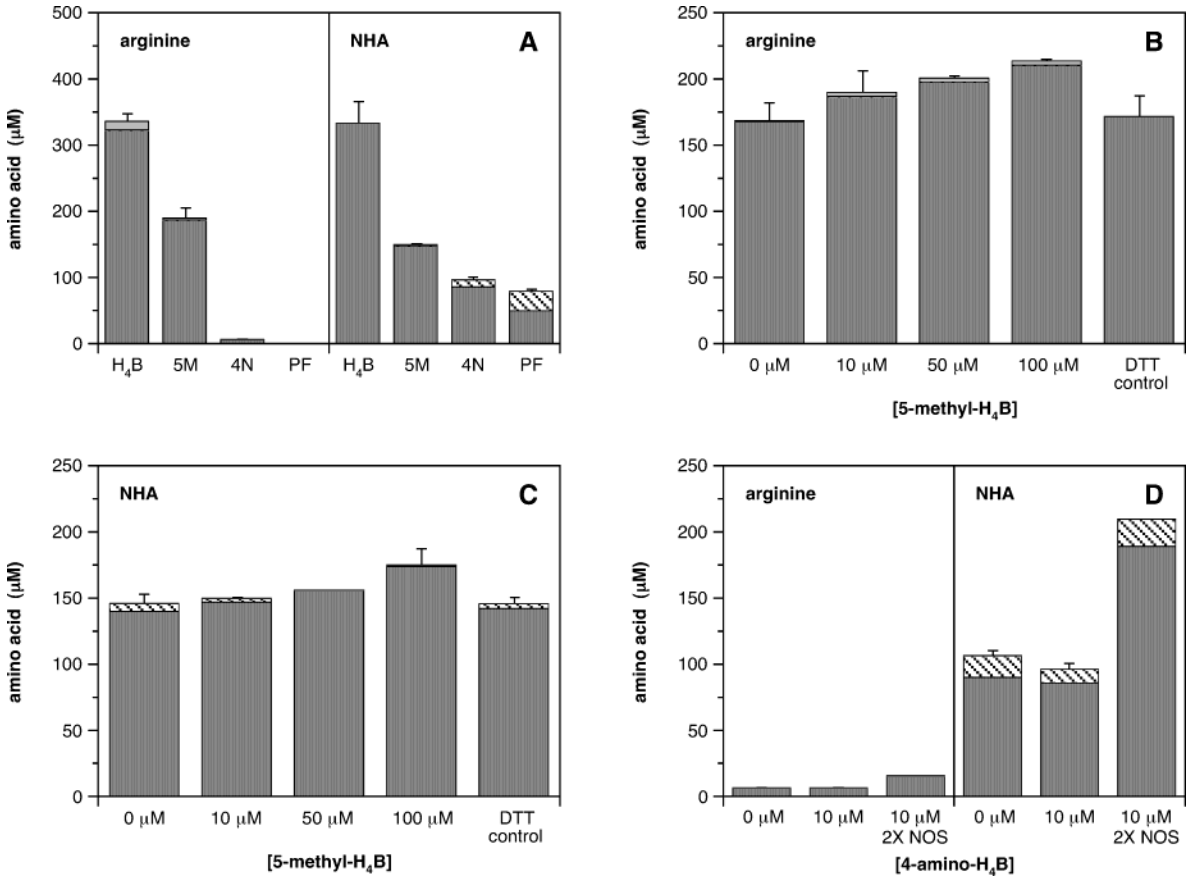


FIGURE 7: Endpoint product analysis of FLiNOS reactions. Reactions (total volume 50 μL) contained 0.5 mM substrate (arginine or NHA), 1 mM NADPH, 0−100 μM pterin, 0 or 100 μM DTT, and 160 nM FLiNOS (1 μg) in 100 mM HEPES, pH 7.5. After 2 h of incubation at 25 °C, reactions were analyzed for amino acid products (citrulline, dark gray bars; NHA, light gray bars; CN-orn, hashed bars), as detailed in the Experimental Procedures. Results are shown for a representative experiment; each bar is the average of duplicate assays, with error bars representing the standard deviations on the total amino acid product. (A) Comparison of the activity of FLiNOS reconstituted with H_4B (H_4B), 5-methyl-H_4B (5 M), or 4-amino-H_4B (4 N) and pterin-free FLiNOS (PF). FLiNOS was assayed in the presence of 0.5 mM arginine (left panel) or 0.5 mM NHA (right panel). Assays contained 10 μM appropriate pterin (H_4B for H_4B-FLiNOS; 5-methyl-H_4B for 5-methyl-H_4B-FLiNOS, and 4-amino-H_4B for 4-amino-H_4B-FLiNOS) and 100 μM DTT; assays of pterin-free FLiNOS contained no exogenous pterin or DTT. (B and C) Dependence of the activity of 5-methyl-H_4B-FLiNOS on additional pterin. Reactions contained 0.5 mM arginine (B) or 0.5 mM NHA (C). The amount of product formed is shown as a function of 5-methyl-H_4B added to the assay. The sample labeled 0 μM contained 5-methyl-H_4B-FLiNOS with no added pterin or DTT; all other assays contained additional 5-methyl-H_4B (10, 50, or 100 μM) in the presence of 100 μM DTT or 100 μM DTT alone (DTT control). (D) Dependence of the activity of 4-amino-H_4B-FLiNOS on additional pterin and on 4-amino-H_4B-FLiNOS concentration. 4-amino-H_4B-FLiNOS was assayed in the presence of 0.5 mM arginine (left panel) or 0.5 mM NHA (right panel). Reactions contained either no added pterin and DTT (0 μM) or 10 μM 4-amino-H_4B with 100 μM DTT. Some reactions (labeled 2× NOS) contained twice as much 4-amino-H_4B-FLiNOS (final concentration 320 nM, or 2 μg).

of 4-amino-H_4B-FLiNOS, indicating that although the amount of product formed in the arginine reaction is small, this is indeed an enzyme-catalyzed reaction.

(C) Inorganic Product. The inorganic product of these reactions was quantified as NO_2^-/NO_3^-. All of the FLiNOS samples formed detectable quantities of NO_2^-/NO_3^- with either arginine or NHA as the substrate (data not shown). The amount of NO_2^-/NO_3^- observed in each case is in good agreement with the amount of amino acid formed in these reactions, consistent with the expected 1:1 stoichiometry of inorganic to amino acid products. The presence of NO_2^-/NO_3^- does not distinguish between $^\bullet$NO and NO^- as the inorganic product since both of these molecules can decompose to NO_2^- and NO_3^- in aerobic solution. However, since neither 4-amino-H_4B- nor pterin-free FLiNOS forms $^\bullet$NO as measured by the oxyhemoglobin assay, the inorganic product in these reactions is probably NO^-.

The ability of the tetrahydrobiopterin analogues to support various aspects of catalysis by HDiNOS and FLiNOS is summarized in Table 1, to facilitate comparison of their activity with that of the native H_4B cofactor.

DISCUSSION

Full-length enzyme (FLiNOS) and the heme domain (HDiNOS) can be reconstituted with H_4B or with tetrahydrobiopterin analogues (Figures 1 and 2). A complete conversion of the heme to the high-spin state was observed with both proteins in the presence of arginine, suggesting complete occupancy of the pterin-binding site with either H_4B or the analogues. When reconstitution was carried out in the absence of substrate, spectral changes representing ~85% high-spin heme were achieved with H_4B or 4-amino-H_4B, whereas the ability of FLiNOS or HDiNOS to bind 5-methyl-H_4B was compromised (~45 and 0% high-spin heme, respectively). These observations illustrate the cooperative nature of the binding of substrate and the pterin cofactor, which is well established for the native H_4B cofactor (*30*−*32*), and demonstrate the decreased binding affinity of

Table 1: Summary of H_4B and Tetrahydrobiopterin Analogue Activities[a]

	H_4B	5-methyl-H_4B	4-amino-H_4B	pterin-free
HDiNOS[b]				
pterin stoichiometry	0.83 ± 0.18 ($n = 6$)	0.38 ± 0.19 ($n = 6$)	0.79 ± 0.15 ($n = 6$)	none detected[c]
pterin radical formation				
$H_3B^\bullet$ stoichiometry	~0.8[d]	~0.6	0	0[d]
$H_3B^\bullet$ $k_{formation}$ (s^{-1})	~15−20[d]	~30	n/a	n/a
$H_3B^\bullet$ k_{decay} (s^{-1})	~0.12[d]	~0.0003	n/a	n/a
arginine hydroxylation				
NHA stoichiometry	0.78 ± 0.11 ($n = 4$)[d]	0.47 ($n = 1$)	0 ($n = 1$)	0 ($n = 1$)[d]
NHA oxidation				
product identity	citrulline, CN-orn	citrulline, CN-orn, unknown	citrulline, CN-orn, unknown	citrulline, CN-orn, unknown
% citrulline	86.5 ± 1.5 ($n = 4$)	44.1 ± 2.9 ($n = 4$)	46.1 ± 7.9 ($n = 4$)	20.9 ± 9.3 ($n = 4$)
product stoichiometry				
RFQ-EPR samples[e]	0.63 ($n = 1$)[d]	0.55 ($n = 1$)	0.65 ($n = 1$)	n/a
dithionite reactions[f]	1.12 ± 0.05 ($n = 4$)	1.04 ± 0.04 ($n = 4$)	1.07 ± 0.27 ($n = 4$)	1.01 ± 0.26 ($n = 4$)
•NO formation	yes	yes	no	no
FLiNOS[g]				
oxyhemoglobin assays[h]				
arginine (%)	100	33.7 ± 1.5 ($n = 3$)	0.1 ± 0.1 ($n = 2$)	0.4 ($n = 1$)
NHA (%)	100	38.0 ± 7.1 ($n = 3$)	0.8 ± 0.6 ($n = 2$)	0.4 ($n = 1$)
endpoint assays[i]				
arginine				
amino acid products	citrulline, NHA	citrulline, NHA	citrulline	none detected
relative activity (%)	100	53.5 ± 8.5 ($n = 3$)	1.6 ± 0.7 ($n = 3$)	0 ($n = 3$)
NHA				
amino acid products	citrulline (CN-orn)	citrulline, CN-orn	citrulline, CN-orn	citrulline, CN-orn
% citrulline	99.9 ± 0.2 ($n = 4$)	98.6 ± 0.6 ($n = 4$)	84.3 ± 10.3 ($n = 4$)	62.8 ± 1.9 ($n = 3$)
relative activity (%)	100	48.4 ± 7.5 ($n = 4$)	25.5 ± 5.9 ($n = 4$)	19.9 ± 5.6 ($n = 3$)

[a] The ability of H_4B and tetrahydrobiopterin analogues to support various aspects of NOS catalysis was examined, as detailed in Experimental Procedures. [b] All HDiNOS samples were reconstituted with the indicated pterin, then desalted to remove any unbound pterin; no excess pterin was present in any of these experiments. [c] Data from ref *19*. [d] Data from ref *17*. [e] Samples were titrated with dithionite until the heme was completely reduced (1−2.5 electrons per HDiNOS). [f] Samples were reduced with a slight excess of dithionite to maximize product formation (4 electrons per HDiNOS). [g] FLiNOS samples were reconstituted with the indicated pterin then assayed in the presence of additional pterin. Activities for each assay are reported relative to that of H_4B-FLiNOS with the indicated substrate. [h] Assays contained 1 mM substrate and 8 μM appropriate pterin; the rate reported is the initial rate of •NO formation at 37 °C. [i] Assays contained 0.5 mM substrate and 10 μM appropriate pterin; the activity reported is the relative amount of product formed in 2 h of reaction at 25 °C.

NOS for 5-methyl-H_4B, also reflected in the low stoichiometry of pterin binding in the 5-methyl-H_4B-HDiNOS reconstituted samples. Despite the limitations associated with the low pterin content of the 5-methyl samples (discussed below), we examined the ability of 5-methyl-H_4B- and 4-amino-H_4B-HDiNOS to form a pterin radical and to catalyze product formation from both arginine and NHA. We also compared the ability of these tetrahydrobiopterin analogues to support catalysis in full-length iNOS.

The behavior of 5-methyl-H_4B-HDiNOS in RFQ-EPR experiments is similar to that of H_4B-HDiNOS previously characterized. In the presence of arginine, a radical with a $g = 2.0$ signal accumulates significantly with either protein, whereas very little radical is formed with NHA as the substrate (Figure 3). The spectra obtained with both proteins are similar in overall shape and width, although there are small differences in the hyperfine structure of the signal (Figure 4), providing additional support that the signal is attributable to a pterin radical. The presence of a methyl group at the N5 position of the pterin ring substitutes three β-hydrogens for the one α-hydrogen of H_4B; the resulting differences in the hyperfine structure indicate strong hyperfine coupling to the *N*5-methyl hydrogens. Two recent papers have reported the formation of a radical of 5-methyl-H_4B bound to heme domains of eNOS (*38*) and iNOS (*41*). Simulations of the observed EPR signal show hyperfine coupling to the three equivalent *N*5-methyl hydrogens, with hyperfine coupling constants in the range of 10 G. These observations are consistent with significant spin density in the pterin radical being localized to the N5 nitrogen, as observed in EPR studies of pterin radical cations in solution (*55*, *56*), as well as in previous studies of $H_3B^\bullet$ where EPR spectra of HDiNOS reconstituted with N5-^{14}N- and N5-^{15}N-substituted H_4B were compared (*17*). Simulations of the $H_3B^\bullet$ signal have led to conflicting assignments of the protonation state of the pterin radical (*38*, *39*, *41*). For simplicity, we represent the pterin radical as $H_3B^\bullet$ or 5-methyl-$H_3B^\bullet$, although the relevant species may be neutral or cationic.

The kinetics of formation and decay of the two radicals were compared (Table 1). Although the rate of radical formation is similar in both cases, the rate of decay of 5-methyl-$H_3B^\bullet$ is much slower (~400-fold) than that of $H_3B^\bullet$. The radical of 5-methyl-H_4B is likely stabilized by electron donating effects of the methyl substituent; however, the dramatic stabilization observed is unexpected (50% of the signal remaining at $t = 40$ min). Methylation at the N5 position of H_4B and other tetrahydropterins makes them resistant to autoxidation (ref *46* and references therein) and disfavors the formation of the 6,7-quinonoid dihydropterin form that is the kinetically preferred product of pterin oxidation. A recent report on the electrochemical properties of 5-methyl-H_4B indicates that this analogue participates in one-electron redox cycling (between tetrahydrobiopterin and trihydrobiopterin radical), but no electrochemically reversible two-electron transitions (to dihydrobiopterin) were observed (*57*). In the RFQ-EPR experiments with HDiNOS, the reducing equivalents come from sodium dithionite; additions of dithionite were carefully titrated to ensure complete

reduction of HDiNOS without excess reducing equivalents present. Under these conditions, the pterin radical once formed must decay via oxidation to the dihydro-oxidation state. The electrochemical data suggest that this is an unfavorable reaction for 5-methyl-$H_3B^\bullet$ and likely account for the exceptional stability of this radical. Consistent with our observations, a slower rate of decay of 5-methyl-$H_3B^\bullet$ as compared to $H_3B^\bullet$ has recently been reported (*41*); however, the magnitude of the stabilization reported was much smaller (~3-fold instead of 400-fold) than what we observe. The stability of 5-methyl-$H_3B^\bullet$ is probably not relevant under conditions of multiple turnover by full-length NOS; if it were, the slow rate of decay of the pterin radical would result in apparent inhibition of NOS activity by this analogue, which is not observed (vide infra and refs *46* and *47*). Instead, NADPH provides the electrons required to reduce the radical via the flavoprotein reductase domain, allowing facile and rapid regeneration of the tetrahydro- form of 5-methyl-H_4B.

In contrast to the results obtained with H_4B- and 5-methyl-H_4B-HDiNOS, no radical signal was observed with 4-amino-H_4B-HDiNOS at any time point with either substrate (Figure 3). The inability of 4-amino-H_4B to undergo one-electron oxidation when bound to HDiNOS is consistent with reports that this analogue does not support NOS catalysis, and in fact, inhibits H_4B-dependent activity (*43*−*45*). However, it is not clear why this analogue does not form a radical species under these conditions. Two potential explanations for this observation can be eliminated. (1) The lack of pterin radical formation is not due to poor binding affinity of 4-amino-H_4B to HDiNOS. The stoichiometry of 4-amino-H_4B binding is comparable to that of H_4B in these samples, and the affinity of NOS for 4-amino-H_4B is reported to be even higher than that for H_4B (*43*). Furthermore, despite the low stoichiometry of 5-methyl-H_4B binding, a radical is observed in that case. (2) The inactivity of 4-amino-H_4B is not explained by electrochemical data. The solution one-electron oxidation potentials of H_4B and the two analogues used here have been reported (*57*). The potential of 4-amino-H_4B is ~60 mV higher (more positive) than that of H_4B, a difference probably too small to explain the inactivity of this analogue. Furthermore, the oxidation potential of 5-methyl-H_4B is the highest of the three pterins, ~130 mV higher than that of H_4B and ~70 mV higher than that of 4-amino-H_4B. Clearly, the solution electrochemistry of 4-amino-H_4B alone does not account for our observations. Instead, it appears that the inability of 4-amino-H_4B to form a radical in these experiments reflects unique properties of this analogue when bound to NOS. Structures of the iNOS heme domain with H_4B or 4-amino-H_4B bound reveal that both pterins bind in an essentially identical manner (*24*). The hydrogen-bonding interactions that characterize the H_4B-binding site are preserved with 4-amino-H_4B, including bonds between the heme propionate and the N3 nitrogen and the 2-amino substituent of the pteridine. A key difference between H_4B and the 4-amino analogue is the preferred tautomeric form of these two pterins. With 4-amino-H_4B, the 4-amino tautomer is favored over the 4-imino form that corresponds to the preferred 4-oxo tautomer of H_4B (see Scheme 1). To maintain the same hydrogen bonding with the heme carboxylate as that observed with H_4B, the N3 nitrogen of 4-amino-H_4B would have to be protonated. Protonation of the pterin ring is more favorable for 4-amino-H_4B than H_4B since the substitution of an amino group at the 4 position raises the pterin pK_a by ~1.1 pH units in solution (6.7 for 4-amino-H_4B vs 5.6 for H_4B; ref *24*). This difference in the protonation state of 4-amino-H_4B and its implications for the redox properties and reactivity of the pterin and heme cofactors has been extensively discussed (*24*). A net positive charge on 4-amino-H_4B when bound to NOS would likely stabilize the tetrahydro- form, increasing its oxidation potential and possibly precluding the formation of the radical species.

To address the relevance of the pterin radical to NOS turnover, we examined the ability of these tetrahydrobiopterin analogues to support catalysis by HDiNOS and FLiNOS. Product formation by HDiNOS in the RFQ-EPR experiments and under similar, single-turnover conditions shows that the activity of the heme domain reconstituted with 5-methyl-H_4B is similar to that when H_4B is bound (Table 1). Like H_4B, 5-methyl-H_4B supports the conversion of arginine to NHA and catalyzes the formation of $^\bullet$NO from NHA. However, the amino acid products of NHA oxidation by 5-methyl-H_4B-reconstituted HDiNOS include CN-orn, a product characteristic of the pterin-free reaction previously described for FLiNOS (*25*) and HDiNOS (*19*). The interpretation of these results is complicated by the low binding affinity of NOS for the 5-methyl analogue. HDiNOS samples reconstituted with 5-methyl-H_4B contain contaminating pterin-free HDiNOS; in fact, the stoichiometry of bound 5-methyl-H_4B (0.38 ± 0.19) indicates that there is more pterin-free than 5-methyl-H_4B-bound HDiNOS in these samples. Since pterin-free HDiNOS also catalyzes the oxidation of NHA, the identity of the amino acid product of NHA oxidation by 5-methyl-H_4B-bound HDiNOS is obscured in these experiments. Additional experiments with full-length enzyme were carried out to elucidate the intrinsic reactivity of 5-methyl-H_4B. The activity of 5-methyl-H_4B-FLiNOS was assessed by several methods (Figures 6 and 7). Activity is observed with both arginine and NHA in the oxyhemoglobin assay (Figure 6A), which measures the rate of $^\bullet$NO formation, and the rate is somewhat faster with NHA. Endpoint amino acid analysis shows that citrulline is the product of the 5-methyl-H_4B-FLiNOS reaction with either substrate (Figure 7A), with a small amount of NHA observed in reactions where arginine is the substrate. The activity profile of 5-methyl-H_4B-FLiNOS is qualitatively similar to that of H_4B-iNOS, although the rate of product formation and the amount of product are decreased with 5-methyl-H_4B-FLiNOS. In part, this decrease is due to the poor binding affinity of FLiNOS for this analogue, as evidenced by the effect of added pterin in the assays. Whereas the inclusion of 8 μM H_4B in the oxyhemoglobin assay results in a slight increase (10−20%) in the activity of H_4B-FLiNOS (data not shown), the activity of 5-methyl-H_4B-FLiNOS is nearly completely dependent on the addition of pterin to the assay, with 8 μM 5-methyl-H_4B causing a 10-fold increase in the rate of $^\bullet$NO formation (Figure 6B). The activity of 5-methyl-H_4B-FLiNOS in endpoint assays with arginine (Figure 7B) and NHA (Figure 7C) is also dependent on the concentration of added pterin, although the effect is much less pronounced in this case (~25% increase in total amino acid product from arginine and ~20% from NHA in the presence of 100 μM 5-methyl-H_4B). A direct comparison of these results is

difficult, however, since •NO formation is measured as an initial rate (t <2 min), and the amino acid products are quantified at long reaction times (t = 2 h). Although some CN-orn is observed in the reaction of 5-methyl-H_4B-FLiNOS with NHA, higher concentrations of added 5-methyl-H_4B in the assay decrease the fraction of CN-orn formed to essentially 0% of the total product, suggesting that CN-orn formation in these samples is catalyzed by the fraction of the protein that does not have bound pterin. Additional support for this conclusion comes from reactions of 5-methyl-H_4B-FLiNOS where arginine is the substrate (Figure 7B). In these assays, the only amino acid products detected are citrulline and NHA (no CN-orn) even in the absence of added pterin. The likely explanation for this result is that NHA in these samples can only be formed at the active site of FLiNOS that has bound pterin since pterin-free FLiNOS does not hydroxylate arginine, and the subsequent oxidation of NHA in this case (to citrulline with no CN-orn) represents only the activity of the pterin-bound fraction of the 5-methyl-H_4B-FLiNOS preparation. These data together suggest that the reactivity of 5-methyl-H_4B-FLiNOS is identical to that of H_4B-FLiNOS with regard to the identity of the products formed in both steps of the NOS reaction: arginine is hydroxylated to NHA, and NHA is oxidized exclusively to citrulline and •NO when the protein is saturated with this pterin analogue.

In contrast, the activity of HDiNOS and FLiNOS when 4-amino-H_4B is bound resembles that of pterin-free NOS (Table 1). In single-turnover experiments with the heme domain, the 4-amino analogue does not catalyze the hydroxylation of arginine. NHA is oxidized by 4-amino-H_4B-HDiNOS, but the reaction leads to a mixture of amino acid products as previously described for pterin-free HDiNOS. The relative fraction of citrulline formed in the 4-amino-H_4B-HDiNOS reaction is substantially higher (~50% of total amino acid product) than that observed for pterin-free HDiNOS (<25% citrulline), suggesting that there are some differences in NOS reactivity that result from binding of the 4-amino analogue. Despite these differences, the most notable feature of NHA oxidation by 4-amino-H_4B-HDiNOS is that no •NO is detected in this reaction. As previously discussed for the reaction of pterin-free FLiNOS (*25*) or pterin-free HDiNOS (*19*), the product of the 4-amino-H_4B-HDiNOS reaction is probably NO^-, a product that is one-electron reduced as compared to •NO. All of the observations with 4-amino-H_4B-HDiNOS are paralleled in reactions of the full-length enzyme with bound 4-amino-H_4B. By the oxyhemoglobin assay, 4-amino-H_4B-FLiNOS is essentially inactive irrespective of substrate (Figure 6A), indicating that no •NO is formed. A very small amount of amino acid product (<2% that observed with H_4B-FLiNOS) is observed at long reaction times with arginine as the substrate (Figure 7A); this reaction is enzyme-catalyzed since the activity is dependent on the concentration of 4-amino-H_4B-FLiNOS in the assay (Figure 7D). The oxidation of NHA by 4-amino-H_4B-FLiNOS leads to a mixture of citrulline and CN-orn, although the fraction of CN-orn is substantially less than that observed with pterin-free FLiNOS (~10 vs 35% in this particular experiment). Although 4-amino-H_4B-HDiNOS and 4-amino-H_4B-FLiNOS have some catalytic activity, it resembles that of pterin-free NOS: the hydroxylation of arginine to NHA is very unfavorable, and the products of NHA oxidation indicate that this reaction is a two-electron oxidation rather than the three-electron oxidation observed with bound H_4B or 5-methyl-H_4B. Since the affinity of iNOS for 4-amino-H_4B is high, these results represent the intrinsic reactivity of the enzyme when this analogue is bound.

A comparison of the RFQ-EPR results with the activity data obtained with HDiNOS and FLiNOS reveals a requirement for the pterin radical in NOS catalysis (Table 1). In the first step of the NOS reaction, the hydroxylation of arginine, there is essentially no reaction in the absence of a redox-active bound pterin that is able to undergo one-electron oxidation. H_4B and 5-methyl-H_4B are able to support the conversion of arginine to NHA; pterin radicals are observed with these two pterins. In contrast, the 4-amino analogue is virtually inactive with respect to NHA formation, and no pterin radical is observed. The tight coupling of radical and product formation is further evidenced by comparing the stoichiometry of the bound pterin radical to that of NHA formed in the RFQ-EPR HDiNOS samples (0.8 radical: 0.8 NHA for H_4B-HDiNOS; 0.6 radical: 0.5 NHA for 5-methyl-H_4B-HDiNOS; and 0 radical: 0 NHA for 4-amino-H_4B- and pterin-free HDiNOS). The pterin cofactor is proposed to provide the electron to reduce the ferrous−dioxygen complex in the hydroxylation of arginine (step 4 in Scheme 2A; refs *17* and *35*). Decay of the ferrous−dioxygen complex is accelerated in the presence of H_4B (*58*, *59*); furthermore, the rate of pterin radical formation and that of decay of the ferrous−dioxygen complex are comparable (*37*, *40*, *41*), suggesting that these represent a single chemical step. Delivery of the second electron to the NOS heme (step 4) is essential for the formation of the proposed oxidant in the hydroxylation of arginine, the high-valent iron−oxo species ($[FeO]^{3+}$; see Scheme 2A) implicated in hydroxylations catalyzed by cytochrome P450. Without this second electron, the enzyme undergoes autoxidation (step 4′), releasing superoxide and regenerating the resting ferric heme without any product formation. The requirement for the pterin as an electron donor is argued to be a kinetic one in preventing this uncoupled dissociation of superoxide (*26*); in iNOS mutants (Trp457Phe and Trp457Ala) where the rate of electron transfer from the pterin is decreased, there is a corresponding decrease in the stoichiometry of NHA formed (*40*, *41*). In the absence of pterin, the importance of this kinetic effect is apparently sufficient to either completely prevent the reaction with arginine (pterin-free FLiNOS or HDiNOS; this paper and refs *19* and *25*) or result in a highly uncoupled reaction where 32 electron equiv are required to form 1 equiv of citrulline (and NO^-) from arginine (pterin-free nNOS; ref *26*). The ability of 4-amino-H_4B-FLiNOS to support some arginine hydroxylation (<2% of H_4B-FLiNOS activity) may be attributable to indirect effects of pterin binding on the reactivity of the heme, allowing reduction of the ferrous−dioxygen complex directly by the flavins of the reductase domain rather than by the pterin cofactor. Alternatively, an undetectable amount of 4-amino-H_3B• may form and account for the small amount of product observed here. In either case, the hydroxylation of arginine is clearly very unfavorable in the presence of this analogue. The role of H_4B in electron transfer to the ferrous−dioxygen complex is reminiscent of that of cytochrome b_5 in some P450 reactions (*60*, *61*). The requirement for the pterin in NOS is much more specific, however, since the addition of this

second electron directly from the reductase domain is apparently too slow to support catalysis.

The dependence of the second step of the NOS reaction, the oxidation of NHA, on the formation of a pterin radical is less clear. All of the FLiNOS and HDiNOS samples, including pterin-free FLiNOS and HDiNOS, are able to catalyze this reaction, although the products of NHA oxidation depend on the identity of the bound pterin (Table 1). The distribution of amino acid products appears to be influenced both by the direct involvement of the pterin cofactor in catalysis and by indirect effects of the pterin on the NOS structure and active site. The redox properties of the bound pterin, however, are essential to the formation of •NO as the inorganic product. Only H_4B and 5-methyl-H_4B, the two pterins that form radicals in the arginine reaction, support •NO formation in the NHA reaction. These results suggest that the most important role of H_4B in the oxidation of NHA is also in electron transfer to the ferrous−dioxygen complex (step 4 in Scheme 2B). In this reaction, as described above for the arginine reaction, dissociation of superoxide (step 4′) can also occur if the second electron is not delivered to the heme at the proper time. In contrast to arginine, however, NHA can react with superoxide (*62*−*66*), resulting in oxidized products. Our experiments do not distinguish between free superoxide (constrained to the active site or in solution) and a heme−superoxide species (the ferrous−dioxygen complex, which can also be described as a ferric−superoxide complex; see Scheme 2) as the oxidant in this reaction; however, it is clear that chemistry can take place even without the addition of the second electron. This reaction differs from the normal catalytic pathway most notably in that it represents a two-electron oxidation rather than the three-electron oxidation observed with bound H_4B or 5-methyl-H_4B, compelling evidence for a one-electron redox role of the pterin in NHA oxidation. Differences in the amino acid products between pterin-free and 4-amino-H_4B-FLiNOS/HDiNOS likely reflect the effects of pterin binding on the structure of the NOS active site (one possibility is that the presence or absence of bound pterin dictates whether NHA reacts with a heme-bound superoxide or superoxide in solution; the steric constraints of the substrate binding site may lead to more normal products than a solution reaction). Although a pterin radical is presumed to form in step 4 of the NHA reaction, it does not accumulate to a significant extent when NHA is the substrate. To account for this observation and for the ultimate formation of •NO (rather than NO^-) from nucleophilic attack of the ferric−peroxide species on NHA (step 5 in Scheme 2B), the pterin cofactor is proposed to act as an electron acceptor in this step, becoming re-reduced to the tetrahydro- form (*19*, *26*, *36*, *67*, *68*). Tight coupling of steps 4 and 5 would preclude the accumulation of a pterin radical in NHA oxidation. Taken together, our results implicate a pterin radical in catalysis with both arginine and NHA as substrates; furthermore, they suggest that H_4B has the same role—transfer of the second electron—in both steps of the NOS reaction. The additional involvement of the pterin as an electron acceptor in NHA oxidation ensures that •NO rather than NO^- is the ultimate product of the NOS reaction.

In conclusion, the ability of tetrahydrobiopterins to support product formation by NOS and to undergo one-electron oxidation when bound to NOS has been compared in these studies. Our data presented here imply a strong correlation between catalysis and formation of an enzyme-bound pterin radical. The correct products of the NOS reaction—hydroxylation of arginine to NHA and subsequent oxidation of NHA to citrulline and •NO—are only obtained when a redox-active pterin capable of forming a pterin radical is bound to HDiNOS or FLiNOS. Although the inability of 4-amino-H_4B to be oxidized to the radical species is surprising based on its binding affinity and solution electrochemistry, this result explains the lack of activity observed with this analogue. The involvement of H_4B in electron transfer in NOS, evidenced by the formation of a pterin radical as an intermediate in the reaction, is an integral part of NOS catalysis and would appear to be the most critical function of this cofactor.

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REFERENCES

1. Marletta, M. A., Hurshman, A. R., and Rusche, K. M. (1998) Catalysis by nitric oxide synthase, *Curr. Opin. Chem. Biol. 2*, 656−663.
2. Stuehr, D. J., and Ghosh, S. (2000) Enzymology of nitric oxide synthases, *Handbook Exp. Pharmacol. 143*, 33−70.
3. Alderton, W. K., Cooper, C. E., and Knowles, R. G. (2001) Nitric oxide synthases: structure, function, and inhibition, *Biochem. J. 357*, 593−615.
4. Stuehr, D. J., Kwon, N. S., Nathan, C. F., Griffith, O. W., Feldman, P. L., and Wiseman, J. (1991) N^{ω}-hydroxy-L-arginine is an intermediate in the biosynthesis of nitric oxide from L-arginine, *J. Biol. Chem. 266*, 6259−6263.
5. Pufahl, R. A., Nanjappan, P. G., Woodard, R. W., and Marletta, M. A. (1992) Mechanistic probes of N-hydroxylation of L-arginine by the inducible nitric oxide synthase from murine macrophages, *Biochemistry 31*, 6822−6828.
6. White, K. A., and Marletta, M. A. (1992) Nitric oxide synthase is a cytochrome P-450-type hemoprotein, *Biochemistry 31*, 6627−6631.
7. Stuehr, D. J., and Ikeda-Saito, M. (1992) Spectral characterization of brain and macrophage nitric oxide synthases. Cytochrome P-450-like hemeproteins that contain a flavin semiquinone radical, *J. Biol. Chem. 267*, 20547−20550.
8. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. (1992) Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide, *Proc. Natl. Acad. Sci. U.S.A. 89*, 11141−11145.
9. Hevel, J. M., White, K. A., and Marletta, M. A. (1991) Purification of the inducible murine macrophage nitric oxide synthase. Identification as a flavoprotein, *J. Biol. Chem. 266*, 22789−22791.
10. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., and Nathan, C. F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein, *Proc. Natl. Acad. Sci. U.S.A. 88*, 7773−7777.
11. Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G., and Böhme, E. (1991) Brain nitric oxide synthase is a biopterin- and flavin-containing multi-functional oxido-reductase, *FEBS Lett. 288*, 187−191.

12. Hevel, J. M., and Marletta, M. A. (1992) Macrophage nitric oxide synthase: relationship between enzyme-bound tetrahydrobiopterin and synthase activity, *Biochemistry 31*, 7160−7165.
13. Schmidt, H. H. H. W., Smith, R. M., Nakane, M., and Murad, F. (1992) Ca^{2+}/calmodulin-dependent NO synthase type I: a biopterin-oflavoprotein with Ca^{2+}/calmodulin-independent diaphorase and reductase activities, *Biochemistry 31*, 3243−3255.
14. Sheta, E. A., McMillan, K., and Masters, B. S. (1994) Evidence for a bidomain structure of constitutive cerebellar nitric oxide synthase, *J. Biol. Chem. 269*, 15147−15153.
15. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) Structure of nitric oxide synthase oxygenase dimer with pterin and substrate, *Science 279*, 2121−2126.
16. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S., and Poulos, T. L. (1998) Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center, *Cell 95*, 939−950.
17. Hurshman, A. R., Krebs, C., Edmondson, D. E., Huynh, B. H., and Marletta, M. A. (1999) Formation of a pterin radical in the reaction of the heme domain of inducible nitric oxide synthase with oxygen, *Biochemistry 38*, 15689−15696.
18. Boggs, S., Huang, L., and Stuehr, D. J. (2000) Formation and reactions of the heme−dioxygen intermediate in the first and second steps of nitric oxide synthesis as studied by stopped-flow spectroscopy under single-turnover conditions, *Biochemistry 39*, 2332−2339.
19. Hurshman, A. R., and Marletta, M. A. (2002) Reactions catalyzed by the heme domain of inducible nitric oxide synthase: Evidence for the involvement of tetrahydrobiopterin in electron transfer, *Biochemistry 41*, 3439−3456.
20. Pufahl, R. A., and Marletta, M. A. (1993) Oxidation of N^G-hydroxy-L-arginine by nitric oxide synthase: Evidence for the involvement of the heme in catalysis, *Biochem. Biophys. Res. Commun. 193*, 963−970.
21. Pufahl, R. A., Wishnok, J. S., and Marletta, M. A. (1995) Hydrogen peroxide-supported oxidation of N^G-hydroxy-L-arginine by nitric oxide synthase, *Biochemistry 34*, 1930−1941.
22. Clague, M. J., Wishnok, J. S., and Marletta, M. A. (1997) Formation of N^δ-cyanoornithine from N^G-hydroxy-L-arginine and hydrogen peroxide by neuronal nitric oxide synthase: implications for mechanism, *Biochemistry 36*, 14465−14473.
23. Tierney, D. L., Huang, H., Martasek, P., Masters, B. S., Silverman, R. B., and Hoffman, B. M. (1999) ENDOR spectroscopic evidence for the position and structure of N^G-hydroxy-L-arginine bound to holo-neuronal nitric oxide synthase, *Biochemistry 38*, 3704−3710.
24. Crane, B. R., Arvai, A. S., Ghosh, S., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (2000) Structures of the N^ω-hydroxy-L-arginine complex of inducible nitric oxide synthase oxygenase dimer with active and inactive pterins, *Biochemistry 39*, 4608−4621.
25. Rusche, K. M., Spiering, M. M., and Marletta, M. A. (1998) Reactions catalyzed by tetrahydrobiopterin-free nitric oxide synthase, *Biochemistry 37*, 15503−15512.
26. Adak, S., Wang, Q., and Stuehr, D. J. (2000) Arginine conversion to nitroxide by tetrahydrobiopterin-free neuronal nitric-oxide synthase. Implications for mechanism, *J. Biol. Chem. 275*, 33554−33561.
27. Baek, K. J., Thiel, B. A., Lucas, S., and Stuehr, D. J. (1993) Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme, *J. Biol. Chem. 268*, 21120−21129.
28. Abu-Soud, H. M., Loftus, M., and Stuehr, D. J. (1995) Subunit dissociation and unfolding of macrophage NO synthase: relationship between enzyme structure, prosthetic group binding, and catalytic function, *Biochemistry 34*, 11167−11175.
29. Klatt, P., Schmidt, K., Lehner, D., Glatter, O., Bachinger, H. P., and Mayer, B. (1995) Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer, *EMBO J. 14*, 3687−3695.
30. Klatt, P., Schmid, M., Leopold, E., Schmidt, K., Werner, E. R., and Mayer, B. (1994) The pteridine binding site of brain nitric oxide synthase. Tetrahydrobiopterin binding kinetics, specificity, and allosteric interaction with the substrate domain, *J. Biol. Chem. 269*, 13861−13866.
31. White, K. A. (1994) Studies of the heme environment and electron flow in the inducible murine macrophage nitric oxide synthase, Ph.D. Thesis, The University of Michigan, Ann Arbor, MI.
32. Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) Endothelial nitric-oxide synthase. Expression in *Escherichia coli*, spectroscopic characterization, and role of tetrahydrobiopterin in dimer formation, *J. Biol. Chem. 271*, 11462−11467.
33. Presta, A., Weber-Main, A. M., Stankovich, M. T., and Stuehr, D. J. (1998) Comparative effects of substrates and pterin cofactor on the heme midpoint potential in inducible and neuronal nitric oxide synthases, *J. Am. Chem. Soc. 120*, 9460−9465.
34. Rusche, K. M., and Marletta, M. A. (2001) Reconstitution of pterin-free inducible nitric-oxide synthase, *J. Biol. Chem. 276*, 421−427.
35. Bec, N., Gorren, A. C., Voelker, C., Mayer, B., and Lange, R. (1998) Reaction of neuronal nitric-oxide synthase with oxygen at low temperature. Evidence for reductive activation of the oxy-ferrous complex by tetrahydrobiopterin, *J. Biol. Chem. 273*, 13502−13508.
36. Bec, N., Gorren, A. C., Mayer, B., Schmidt, P. P., Andersson, K. K., and Lange, R. (2000) The role of tetrahydrobiopterin in the activation of oxygen by nitric-oxide synthase, *J. Inorg. Biochem. 81*, 207−211.
37. Wei, C. C., Wang, Z. Q., Wang, Q., Meade, A. L., Hemann, C., Hille, R., and Stuehr, D. J. (2001) Rapid kinetic studies link tetrahydrobiopterin radical formation to heme−dioxy reduction and arginine hydroxylation in inducible nitric-oxide synthase, *J. Biol. Chem. 276*, 315−319.
38. Schmidt, P. P., Lange, R., Gorren, A. C., Werner, E. R., Mayer, B., and Andersson, K. K. (2001) Formation of a protonated trihydrobiopterin radical cation in the first reaction cycle of neuronal and endothelial nitric oxide synthase detected by electron paramagnetic resonance spectroscopy, *J. Biol. Inorg. Chem. 6*, 151−158.
39. Du, M., Yeh, H. C., Berka, V., Wang, L. H., and Tsai, A. L. (2003) Redox properties of human endothelial nitric-oxide synthase oxygenase and reductase domains purified from yeast expression system, *J. Biol. Chem. 278*, 6002−6011.
40. Wang, Z. Q., Wei, C. C., Ghosh, S., Meade, A. L., Hemann, C., Hille, R., and Stuehr, D. J. (2001) A conserved tryptophan in nitric oxide synthase regulates heme−dioxy reduction by tetrahydrobiopterin, *Biochemistry 40*, 12819−12825.
41. Wei, C. C., Wang, Z. Q., Arvai, A. S., Hemann, C., Hille, R., Getzoff, E. D., and Stuehr, D. J. (2003) Structure of tetrahydrobiopterin tunes its electron transfer to the heme−dioxy intermediate in nitric oxide synthase, *Biochemistry 42*, 1969−1977.
42. Presta, A., Siddhanta, U., Wu, C., Sennequier, N., Huang, L., Abu-Soud, H. M., Erzurum, S., and Stuehr, D. J. (1998) Comparative functioning of dihydro- and tetrahydropterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase, *Biochemistry 37*, 298−310.
43. Werner, E. R., Pitters, E., Schmidt, K., Wachter, H., Werner-Felmayer, G., and Mayer, B. (1996) Identification of the 4-amino analogue of tetrahydrobiopterin as a dihydropteridine reductase inhibitor and a potent pteridine antagonist of rat neuronal nitric oxide synthase, *Biochem. J. 320*, 193−196.
44. Pfeiffer, S., Gorren, A. C., Pitters, E., Schmidt, K., Werner, E. R., and Mayer, B. (1997) Allosteric modulation of rat brain nitric oxide synthase by the pterin-site enzyme inhibitor 4-aminotetrahydrobiopterin, *Biochem. J. 328*, 349−352.
45. Mayer, B., Wu, C., Gorren, A. C., Pfeiffer, S., Schmidt, K., Clark, P., Stuehr, D. J., and Werner, E. R. (1997) Tetrahydrobiopterin binding to macrophage inducible nitric oxide synthase: heme spin shift and dimer stabilization by the potent pterin antagonist 4-amino-tetrahydrobiopterin, *Biochemistry 36*, 8422−8427.
46. Riethmuller, C., Gorren, A. C., Pitters, E., Hemmens, B., Habisch, H. J., Heales, S. J., Schmidt, K., Werner, E. R., and Mayer, B. (1999) Activation of neuronal nitric-oxide synthase by the 5-methyl analog of tetrahydrobiopterin. Functional evidence against reductive oxygen activation by the pterin cofactor, *J. Biol. Chem. 274*, 16047−16051.
47. Werner, E. R., Habisch, H. J., Gorren, A. C., Schmidt, K., Canevari, L., Werner-Felmayer, G., and Mayer, B. (2000) Contrasting effects of N5-substituted tetrahydrobiopterin derivatives on phenylalanine hydroxylase, dihydropteridine reductase, and nitric oxide synthase, *Biochem. J. 348*, 579−583.
48. Battino, R. (1981) *IUPAC Solubility Data Series*, Permagon Press, Oxford, UK.
49. Hevel, J. M., and Marletta, M. A. (1994) Nitric-oxide synthase assays, *Methods Enzymol. 233*, 250−258.

50. Rusche, K. M. (1998) Studies of the nitric oxide synthase catalytic and structural requirement for 6(*R*)-tetrahydro-L-biopterin, Ph.D. Thesis, The University of Michigan, Ann Arbor, MI.
51. White, R. E., and Coon, M. J. (1982) Heme ligand replacement reactions of cytochrome P-450. Characterization of the bonding atom of the axial ligand trans to thiolate as oxygen, *J. Biol. Chem. 257*, 3073−3083.
52. Ghosh, D. K., Abu-Soud, H. M., and Stuehr, D. J. (1996) Domains of macrophage NO synthase have divergent roles in forming and stabilizing the active dimeric enzyme, *Biochemistry 35*, 1444−1449.
53. Gorren, A. C., List, B. M., Schrammel, A., Pitters, E., Hemmens, B., Werner, E. R., Schmidt, K., and Mayer, B. (1996) Tetrahydrobiopterin-free neuronal nitric oxide synthase: Evidence for two identical highly anticooperative pteridine binding sites, *Biochemistry 35*, 16735−16745.
54. Gorren, A. C., Schrammel, A., Schmidt, K., and Mayer, B. (1997) Thiols and neuronal nitric oxide synthase: Complex formation, competitive inhibition, and enzyme stabilization, *Biochemistry 36*, 4360−4366.
55. Ehrenberg, A., Hemmerich, P., Müller, F., Okada, T., and Viscontini, M. (1967) Über Pterinchemie. 18. Monohydro- und Trihydropterin-Radikale, *Helv. Chim. Acta 50*, 411−416.
56. Bobst, A. (1968) Über Pterinchemie. 24. Charakterisierung des 5,6,7,8-Tetrahydropterin-(THP)-Radikals in saurer Lösung, *Helv. Chim. Acta 51*, 607−613.
57. Gorren, A. C., Kungl, A. J., Schmidt, K., Werner, E. R., and Mayer, B. (2001) Electrochemistry of pterin cofactors and inhibitors of nitric oxide synthase, *Nitric Oxide 5*, 176−186.
58. Abu-Soud, H. M., Gachhui, R., Raushel, F. M., and Stuehr, D. J. (1997) The ferrous-dioxy complex of neuronal nitric oxide synthase. Divergent effects of L-arginine and tetrahydrobiopterin on its stability, *J. Biol. Chem. 272*, 17349−17353.
59. Sato, H., Sagami, I., Daff, S., and Shimizu, T. (1998) Autoxidation rates of neuronal nitric oxide synthase: Effects of the substrates, inhibitors, and modulators, *Biochem. Biophys. Res. Commun. 253*, 845−849.
60. Bonfils, C., Balny, C., and Maurel, P. (1981) Direct evidence for electron transfer from ferrous cytochrome b_5 to the oxyferrous intermediate of liver microsomal cytochrome P-450 LM_2, *J. Biol. Chem. 256*, 9457−9465.
61. Pompon, D., and Coon, M. J. (1984) On the mechanism of action of cytochrome P-450. Oxidation and reduction of the ferrous dioxygen complex of liver microsomal cytochrome P-450 by cytochrome b_5, *J. Biol. Chem. 259*, 15377−15385.
62. Sennequier, N., Boucher, J. L., Battioni, P., and Mansuy, D. (1995) Superoxide anion efficiently performs the oxidative cleavage of C═NOH bonds of amidoximes and *N*-hydroxyguanidines with formation of nitrogen-oxides, *Tetrahedron Lett. 36*, 6059−6062.
63. Mansuy, D., Boucher, J. L., and Clement, B. (1995) On the mechanism of nitric oxide formation upon oxidative cleavage of C═N(OH) bonds by NO synthases and cytochromes P450, *Biochimie 77*, 661−667.
64. Jousserandot, A., Boucher, J. L., Henry, Y., Niklaus, B., Clement, B., and Mansuy, D. (1998) Microsomal cytochrome-P450-dependent oxidation of *N*-hydroxyguanidines, amidoximes, and ketoximes: mechanism of the oxidative cleavage of their C═N(OH) bond with formation of nitrogen oxides, *Biochemistry 37*, 17179−17191.
65. Moali, C., Boucher, J. L., Renodon-Corniere, A., Stuehr, D. J., and Mansuy, D. (2001) Oxidations of N^{ω}-hydroxyarginine analogues and various *N*-hydroxyguanidines by NO synthase II: key role of tetrahydrobiopterin in the reaction mechanism and substrate selectivity, *Chem. Res. Toxicol. 14*, 202−210.
66. Mansuy, D., and Boucher, J. L. (2002) Oxidation of *N*-hydroxyguanidines by cytochromes P450 and NO synthases and formation of nitric oxide, *Drug Metab. Rev. 34*, 593−606.
67. Gorren, A. C., Bec, N., Schrammel, A., Werner, E. R., Lange, R., and Mayer, B. (2000) Low-temperature optical absorption spectra suggest a redox role for tetrahydrobiopterin in both steps of nitric oxide synthase catalysis, *Biochemistry 39*, 11763−11770.
68. Wang, Z. Q., Wei, C. C., and Stuehr, D. J. (2002) A conserved tryptophan 457 modulates the kinetics and extent of *N*-hydroxy-L-arginine oxidation by inducible nitric-oxide synthase, *J. Biol. Chem. 277*, 12830−12837.

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