Inducible nitric-oxide synthase (NOS) was expressed and purified in the absence of 6(R)-tetrahydro-L-biop-terin (H$_4$B). Pterin-free NOS exhibits a Soret band (416–420 nm) characteristic of predominantly low spin heme and does not catalyze the formation of nitric oxide (NO) (Rusche, K. M., Spiering, M. M., and Marletta, M. A. (1998) Biochemistry 37, 15503–15512). Reconstitution of pterin-free NOS with H$_4$B was monitored by a shift in the Soret band to 396–400 nm, the recovery of NO-forming activity, and the measurement of H$_4$B bound to the enzyme. As assessed by these properties, H$_4$B binding was not rapid and required the presence of a reduced thiol. Spectral changes and recovery of activity were incomplete in the absence of reduced thiol. Full reconstitution of holoenzyme activity and stoichiometric H$_4$B binding was achieved in the presence of 5 mM reduced glutathione (GSH). Preincubation with GSH before the addition of H$_4$B decreased, whereas lower concentrations of GSH extended, the time required for reconstitution. Six protected cysteine residues in pterin-free NOS were identified by labeling of NOS with cysteine-directed reagents before and after reduction with GSH. Heme and metal content of pterin-free and H$_4$B-reconstituted NOS were also measured and were found to be independent of H$_4$B content. Additionally, pterin-free NOS was reconstituted with 6-methylpterin analogs, including redox-stable deazapterins. Reconstitution with the redox-stable pterin analogs was neither time- nor thiol-dependent. Apparent binding constants were determined for the 6-methyl- (50 μM) and 6-ethoxymethyl (200 μM) deazap-terins. The redox-stable pterin analogs appear to bind to NOS in a different manner than H$_4$B.

Nitr-oxide synthase (NOS, EC 1.14.13.39) has been char-acterized in three isoforms: a membrane-associated, constitutive enzyme from the vascular endothelium; a soluble, constitutive enzyme from neuronal cells; and an inducible enzyme best characterized from murine macrophages (1). All of the isoforms catalyze the formation of nitric oxide (NO) and citrul-line from l-arginine (2–4). The reaction requires NADPH and O$_2$ and proceeds via the intermediate N$^\text{6}$-hydroxy-L-arginine (NHA) (5, 6). NOS is homodimeric and binds an equivalent of each of FAD, FMN (7–9), and protoporphyrin IX heme (10–12) per subunit. Additionally, each NOS subunit binds 1 equiv-alent of tetrahydrobiopterin (H$_4$B), which is required for full activity (9, 13, 14). Calmodulin binds to the constitutive isoforms reversibly in response to the intracellular calcium concentration (15, 16) and nearly irreversibly in an apparently Ca$^{2+}$-independent manner to the inducible isoform (4, 17).

The respective roles of the heme and H$_4$B cofactors in the NOS mechanism are not yet clear. The first step of the reaction, the hydroxylation of arginine, appears to require the involvement of the heme due to the inhibition of the reaction by CO (10). However, hydrogen peroxide and iodosobenzene fail to support this reaction (18), and pterin-free NOS does not cata-lyze arginine hydroxylation in the presence of either hydrogen peroxide or NADPH (19). Thus, H$_4$B appears to play a role in the first step of the reaction. H$_4$B has been proposed to participate in electron transfer in NOS. Bec et al. (20) infer a role for H$_4$B in the reduction of the ferrous dioxygen complex of the heme by one electron to form the heme-derived oxidant, which would result in a pterin radical. Raman et al. (21) subsequently suggested, based on structural evidence, that the H$_4$B-binding site could stabilize a pterin radical cation (21). Direct evidence of a pterin radical, which is proposed to be formed in the arginine reaction, has been obtained by rapid freeze-quench electron paramagnetic resonance studies of the iNOS heme domain (22). Evidence to support heme catalysis in the oxida-tion of NHA to citrulline and NO consists of inhibition of the reaction by CO (23) and studies of peroxide-dependent catalysis (18, 24). Additionally, a direct role for the heme in the second step of the reaction is supported by the fact that oxidation of NHA is catalyzed by pterin-free NOS (19). However, the reaction of NHA catalyzed by pterin-free NOS yields different products than the reaction catalyzed by H$_4$B-bound NOS. It is not clear what the role of H$_4$B may be in the oxidation of NHA. It is apparent, though, that H$_4$B performs one or more crucial functions in the mechanism of NOS.

Structural roles for H$_4$B have also been proposed based on the observed effects of H$_4$B on substrate affinity for NOS (25, 26), enzyme oligomeric structure (27–29), heme spin state equi-librium (30–35), and heme midpoint potential (36). The NOS crystal structures with H$_4$B bound have provided some clues as to the cause of these effects (21, 37, 38). H$_4$B binds near the heme, the nitrogens of the pyrimidine ring interacting with a heme propionate group. H$_4$B also interacts with residues from the symmetry-related subunit, forming a link between the subunits of the dimer. In addition, the structures revealed a metal-binding site at the dimer interface in which a zinc atom is coordinated to four cysteine residues, two from each monomer (21, 38).

Expression and purification of pterin-free NOS has enabled studies examining the role of the reduced pterin cofactor. The ability to reconstitute this enzyme with H$_4$B and recover all physical and catalytic properties complements previous studies.
of pterin-free NOS reactivity (19). These studies support a role for reduced thiol in the reconstitution of H_B as also observed by Sono et al. (39). We have studied the process and requirements of pterin reconstitution of the full-length iNOS in the absence of substrate utilizing H_B as well as the 6-methyl analogs, including redox-stable deazapterins (Fig. 1). Reconstitution was assessed by spectroscopic changes, quantitation of bound H_B, and recovery of NO-forming activity.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods—**Escherichia coli JM109 competent cells were purchased from Promega. Ampicillin, chloramphenicol, and isopropyl-β-D-thiogalactopyranoside were purchased from Roche Molecular Biochemicals. NHA was obtained from Alexis Corp. 2′,5′-ADP Sepharose 4B, the S200 16/60 gel filtration column, and HiTrapp™ desalting columns were purchased from Amersham Pharmacia Biotech. DEAE Bio-Gel A, Coomassie Blue R-250, and Bradford protein dye reagent were purchased from Bio-Rad. Reaction vials and silicone/Teflon septa were purchased from Pierce. Centrifugal filtration units (Ultrafree-15, Biomax-50K NMMWL membrane) were purchased from Millipore. H_B and 6(R,S)-methyltetrahydropterin (MPH) were purchased from Dr. B. Schircks Laboratory (Jona, Switzerland). The tri-peptide γ-L-Glu-L-Ser-Gly (GSH) was a generous gift of Dr. Richard Armstrong, Vanderbilt University. All other reagents were purchased from Sigma.

Stock solutions of H_B or MPH were prepared in 100 mM Hepes (pH 7.4) either in an anaerobic chamber (Coy Laboratory Products, Inc.) or in buffer containing 100 mM dithiothreitol (DTT). H_B concentrations were determined by dilution into 50 mM Hepes (pH 8), and the absorbance was recorded at 287 nm (ε = 5780 M⁻¹ cm⁻¹) (40). MPH concentrations were determined by dilution into 0.1 M HCl, and the absorbance was recorded at 265 nm (ε = 14, 380 M⁻¹ cm⁻¹) (41). 6(R,S)-Methyl-5-deazapteridinopterin (DZPH) was synthesized as described previously (13, 42) and used as the monofluoroureacetal salt. DZPH (4–6 mg) was dissolved in 0.5 ml of 0.1 M NaOH and diluted to 4 ml with 200 mM Hepes (pH 7.4). The pH was adjusted to 7.4 with HCl, the concentration was determined by dilution into 100 mM Hepes (pH 8), and the absorbance was recorded at 279 nm (ε = 15, 500 M⁻¹ cm⁻¹) (42). 6(R,S)-Ethoxymethyl-5-deazapteridinopterin (EtOMeDZPH) was a generous gift from Dr. Edward C. Taylor (Princeton University) and was prepared similarly to DZPH.

**Purification of iNOS—**Expression and purification of pterin-free NOS were as described previously (19) with the following modifications. Supernatant derived from the cell pellets of 6 liters of culture was prepared by desalting on a HiTrap P-25 superfine) equilibrated with metal-free buffer. The buffer, 20 mM Hepes (pH 7.4) was equilibrated to 15 °C before and after initiation with NHA with or without GSH. Aliquots (4 μg of NOS in 5 ml) were removed from the reaction vial with a gas-tight syringe and used to initiate aerobic oxyhemoglobin assays. Assays were carried out with arginine at 15 °C, and additional H_B (or thiol) was not added. Therefore, H_B in the absence of substrate was not removed by the enzyme derivative from the enzyme incubation. Similarly, the GSH concentration in the assays reflected the 100-fold dilution of the enzyme aliquot. Zero time points were obtained by assaying pterin-free NOS with these same concentrations of H_B with or without GSH (0.5 and 50 μM, respectively). For longer incubations in the absence of reduced thiol, samples were prepared in multiple reaction vials and assayed at various time points, as repeated piercing of the silicone/Teflon septum resulted in anaerobic acidification.

**Reconstitution of Pterin-free NOS with MPH—**Pterin-free NOS was reconstituted with MPH (50 μM) in the presence of 680 μM DTT (or 2.5 mM ascorbate) at 25 °C. Spectra were recorded at various times on a Cary 3E UV-visible spectrophotometer (Varian) with a Neslab RTE-111 circulating water bath. Aliquots (5 ml) were withdrawn from the cuvette at various times and assayed at 37 °C for NO formation from arginine by the oxyhemoglobin assay.

**Reconstitution of Pterin-free NOS with H_B—**Pterin-free NOS (5 μM) was reconstituted with H_B (50 μM) in the presence of 680 μM DTT (or 2.5 mM ascorbate) at 25 °C. Spectra were recorded at various times on a Cary 3E UV-visible spectrophotometer (Varian) with a Neslab RTE-111 circulating water bath. Aliquots (5 ml) were withdrawn from the cuvette at various times and assayed at 37 °C for NO formation from arginine by the oxyhemoglobin assay.

**Reconstitution of Pterin-free NOS with Deazapterin—**Increasing concentrations of DZPH or EtOMeDZPH were added to pterin-free NOS at 25 °C. Spectra were recorded initially and after each addition. An apparent binding constant was calculated from non-linear analysis of the spectral difference data. In addition, an apparent binding constant for arginine binding to DZPH-bound NOS was determined by the spectral changes occurring with increasing substrate concentration (an absorbance increase at 395 and a decrease around 416 nm). NOS in the presence of DZPH (530 μM) was assayed (25 and 37 °C) with NHA as the substrate by an HPLC assay of amino acid products as described above.

**Metal Analysis and Heme Content—**Samples for analysis of zinc content were prepared by desalting on a HiTrapp column (Sephadex G-25 superfine) equilibrated with metal-free buffer. The buffer, 20 mM Hepes (pH 7.4), was prepared by passage over a Chelex-100 column (Bio-Rad) and filtered through 0.2 μm filters. H_B and MPH-reconstituted NOS samples were incubated with 50 μM H_B and 5 mM GSH for 3 h at 15–20 °C before desaturation. Pterin-free NOS samples were desalted as isolated. Inducively coupled plasma/MS (Plasmaspec III, Leeman labs, Hudson, NH) analysis of samples (3–4 μl, by the Bradford protein assay) was carried out by Dr. Ted J. Huston (Dept. of Geological Sciences, University of Michigan, Ann Arbor). In the buffer controls, zinc was either not
detected or was <2% of the concentration of metal in the protein samples. The heme content of the same samples was determined by HPLC analysis with myoglobin standards using a Beckman System Gold HPLC and a previously described method (43). The mobile phase consisted of 0.1% aqueous trifluoroacetic acid, and samples were eluted with a 0–75% linear gradient over 20 min of 0.1% trifluoroacetic acid in acetonitrile.

Labeling of Cysteine Residues in NOS—Pterin-free NOS in 0.5 mM Tris and 5 mM guanidine HCl (pH 7) was reacted with a 10-fold molar excess (23 mol of cysteine/mol of NOS) of N-ethylmaleimide for 75 min at 37 °C. The reaction was quenched with a 10-fold molar excess (over protein thiol plus N-ethylmaleimide) of 10 mM DTT and incubated at 37 °C for 1 h, then diluted into 0.5 mM Tris and 5 mM guanidine HCl (pH 8) with a 10-fold molar excess (over total thiol concentration) of iodoacetamide. After 1 h at 37 °C, the reaction was quenched with a 10-fold molar excess of β-mercaptoethanol. The concentration of guanidine HCl was decreased by dilution and concentration with an Ultrafiltrate-15 (Biomax-30 kDa NMWL, Millipore), and the resulting sample was gel-purified (Novex 3–8% Tris acetate gels). Bands corresponding to NOS from multiple lanes were excised, washed with 50% acetonitrile, and submitted to the Harvard Microchemistry Facility (Harvard University) for tryptic digestion and peptide sequencing. Sequence analysis was performed by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer. The MS/MS spectra were then correlated with the known iNOS sequence using the algorithm Sequest, developed at the University of Washington (44) and programs developed at the Harvard facility (45).

Pterin Content—The pterin content of NOS samples was determined by HPLC analysis with a Nova-Pak C18 column (3.9 × 150 mm, 4 μm; Waters) equipped with an Alltech C18-LF guard column (5 μm; Alltech) using a Hewlett Packard 1090 Series II HPLC with a diode array detector. The mobile phase consisted of 50 mM sodium acetate, 5 mM citric acid, and 0.1% aqueous trifluoroacetic acid, and samples were eluted with a 0–75% linear gradient over 3 min and a 1-min wash with 100% methanol (0.5 ml/min). Detection wavelengths were 297 (λmax for H4B) and 280 nm (λmax for 7, 8-H2B). Under these conditions, H4B elutes at 4 min, and 7,8-dihydro-7,8-bioterpin (7, 8-H2B) elutes at 4.8 min. NOS samples were concentrated to ~40 μM with centrifugal filtration units (Ultrafilter-0.5, Biomax-10K NMWL membrane, from Millipore). H4B-reconstituted NOS was prepared by incubation with 50 μM H4B and 0.7 mM DTT for 3 h at 4 °C. Samples (50–75 μl) were prepared for analysis by desalting with Micro Bio-Spin P-30 columns (Bio-Rad) equilibrated with 10 mM DTT and 100 mM HEPES (pH 7.4). Control experiments were carried out to ensure the efficiency of the desalting procedure. H4B and DTT (50 μM and 0.7 mM, respectively) in 50–75 μl aliquots were desalted with the Micro Bio-Spin columns and analyzed for H4B. H4B or 7,8-dihydro-7,8-bioterpin was not detected in these experiments, evidence that the method of desalting was adequate. Guanidine HCl was added to desalted protein samples for a final concentration of 0.67 M. Samples and standards contained 10 mM DTT and were prepared immediately before analysis. 7,8-Dihydro-7,8-bioterpin was not detected in the standards, and only a small amount (~5% of H4B measured) was detected in the NO-forming activity (37 °C) from 0.50 (all time points, including the zero time point, measured with 10 μM H4B, 0.14 mM DTT) to 1.25 μmol/min/mg (Fig. 2B), equivalent to observed holoenzyme activity.

The complications arising from the use of DTT or β-mercaptoethanol (namely, binding to the pterin-free NOS heme) were circumvented by the use of GSH. The spectrum (or activity) of pterin-free NOS was not affected by the addition of up to 5 mM GSH. However, the addition of H4B with 5 mM GSH effected a shift of the Soret from 418 to 398 nm (Fig. 3A). The conversion from low to high spin was direct (three isosbestic points; 410, 465, and 510 nm) and not accompanied by the increase in specific activity (37 °C) from 0.50 (all time points, including the zero time point, measured with 10 μM H4B, 0.14 mM DTT) to 1.25 μmol/min/mg (Fig. 2B), equivalent to observed holoenzyme activity.

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Reconstitution of Pterin-free Nitric-oxide Synthase

in the absorbance changes similar to that observed when H$_4$B and GSH are added simultaneously (Fig. 4B).

The thiol dependence of H$_4$B reconstitution was examined by several methods. First, the presence of 2.5 mM ascorbate did not facilitate reconstitution as observed with GSH (data not shown). Second, 5 mM compared with 1 mM GSH in the reconstitution with 50 μM H$_4$B resulted in more rapid spectral shifts (15 °C, Fig. 5A). In addition, the oxygen-substituted GSH analog, GOH, with 50 μM H$_4$B did not facilitate NOS spectral shifts over those with H$_4$B alone. Third, the effect of an anaerobic preincubation of pterin-free NOS with GSH before initiation with H$_4$B, _versus_ simultaneous addition of H$_4$B and GSH on the H$_4$B-dependent NOS spectral shift (15 °C) was examined (Fig. 5B). Preincubation of pterin-free NOS with GSH resulted in a more rapid shift as compared with simultaneous addition of the same GSH concentration.

**Reconstitution of Pterin-free NOS with MPH$_4$—MPH$_4$ (Fig. 1) reconstitution of pterin-free NOS required considerably higher concentrations of this pterin. MPH$_4$ at 30 μM did not effect any change in the pterin-free NOS spectrum. Reconstitutions were carried out at 25 °C with 500 μM MPH$_4$ in the presence or absence of 5 mM GSH. Similar to H$_4$B, MPH$_4$ in the absence of GSH effected only a small shift of the NOS spectrum. In the presence of GSH, however, reconstitution proceeded rapidly (data not shown). In the anaerobic MPH$_4$ experiment, extended incubation times resulted in the very slow reduction of the flavin cofactors of NOS. This was evidenced by decreases in absorbance between 450 and 500 nm.

**Reconstitution of Pterin-free NOS with Deazapterin—** Increasing concentrations of the redox-stable pterin analog, DZPH$_4$ (Fig. 1), were added to pterin-free NOS. DZPH$_4$ effected a concentration-dependent decrease in absorbance at 418–422 nm and increase in absorbance at 396–399 nm (Fig. 6). The absorbance changes at each concentration occurred rapidly (within the time required for mixing) and did not increase further. These changes did not require the presence of reduced thiol. The apparent spectral binding constant measured for this conversion was 50 ± 13 μM (n = 4). In the presence of 0.2–0.8 mM arginine, the spectral $K_{D_{app}}$ measured for DZPH$_4$ binding dropped to 34 ± 3 μM (n = 6). This increase in pterin affinity in the presence of substrate is similar to, although less than, that reported for H$_4$B affinity, where the $K_D$ for H$_4$B was 250 and 37 mM in the absence and presence of arginine, respectively (25). Arginine binding to DZPH$_4$-bound NOS resulted in a shift of the Soret peak to 395 nm. The spectral binding constant measured for this conversion was 18.8 ± 5.9 μM (n = 2), as compared with 8–13 μM for murine macrophage NOS purified in the presence of H$_4$B (26). EtOMeDZPH$_4$ similarly shifted the NOS Soret band from 419 to 400 nm (data not shown). The $K_{D_{app}}$ determined for this conversion, however, was 200 ± 10 μM,


**FIG. 5.** Effects of increasing GSH concentration and preincubation. A, H4B (50 μM) was added either simultaneously with either 1 mM (triangles) or 5 mM (squares) GSH or with 5 mM GOH (circles). Spectra were recorded over time, and difference spectra were calculated. Changes in absorbance versus time from H4B and GSH (or GOH) addition are plotted. In each experiment the NOS concentration was 1.8 μM. B, the rate of formation of the high spin heme depended not only on the concentration of GSH but also on preincubation with GSH. H4B and GSH (50 μM and 5 mM, respectively) were added either simultaneously to pterin-free NOS (open squares) or after pterin-free NOS was incubated with 5 mM GSH for 1 h on ice (closed circles).

**TABLE I**

| Analyte | Pterin-free NOS | H4B-reconstituted NOS |
|---------|----------------|-----------------------|
| Heme    | 0.74 ± 0.07    | 0.77 ± 0.03           |
| Zinc    | 0.50 ± 0.07    | 0.49 ± 0.04           |
| H4B     | Not detected   | 0.79 ± 0.04           |

Heme quantitation (in duplicate) was carried out on four samples each of pterin-free and H4B-reconstituted NOS.

Copper in all samples was detected at ≤0.01 per NOS monomer (n = 3 for the ICP/MS experiments).

H4B measurements (in duplicate) were carried out on two samples of pterin-free and five samples of H4B-reconstituted NOS.

The lower limit of detection for this method is <0.05 μM, which (with the 30 μM NOS used in this experiment) would correspond to a stoichiometry of <0.003 per NOS monomer.

Pterin-free NOS was incubated with 530 μM DZPH4, and amino acid product formation (citrulline plus N6-cyanoornithine) at 25 °C was measured in the presence and absence of DZPH4. The specific activity of pterin-free NOS in the absence of DZPH4 was 210 ± 40 nmol/min/mg (n = 4). In the presence of DZPH4, the specific activity dropped 48%, to 110 ± 7 nmol/min/mg (n = 2).

Zinc, Heme, and Pterin Content—Pterin-free and H4B-reconstituted NOS were analyzed for zinc, heme, and pterin content. Essentially no difference in the heme or zinc content of pterin-free and H4B-reconstituted NOS was observed (Table I). The heme stoichiometry was determined to be approximately 0.76 per monomer. Zinc was present at 0.5 per NOS monomer, consistent with the crystallographically observed zinc tetra-thiolate center at the dimer interface of the endothelial and inducible NOS isoforms (21, 38). It is interesting to note that the zinc stoichiometry was independent of H4B content. In these experiments 0.5 zinc was reproducibly bound to 1 NOS monomer, implying that pterin-free NOS retains the ability to bind zinc. This conclusion is consistent with the observation by analytical gel filtration that these preparations of pterin-free NOS were mostly dimeric (>90%), with only a small shift in the enzyme elution profile upon H4B reconstitution (data not shown). As expected, H4B was not detected in pterin-free NOS preparations. Incubation of pterin-free NOS with H4B did result in the binding of H4B to NOS, as evidenced by the detection of 0.8 H4B per NOS monomer. The H4B and heme stoichiometries in the same enzyme preparation are equivalent within error.

Cysteine Labeling—Pterin-free NOS cysteine residues were labeled by N-ethylmaleimide before reduction with DTT and by iodoacetamide after reduction. After tryptic digestion of the sample, MS/MS peptide sequencing identified which of the 23 cysteine residues of iNOS were labeled with each reagent. Six cysteine residues did not react with the first reagent (N-ethylmaleimide) and were only labeled by iodoacetamide after reduction. Two of these cysteine residues (Cys-104 and Cys-109) correspond to those reported to be the ligands in the zinc tetra-thiolate center (21, 38). Presumably, bound zinc would protect these cysteine residues until exposure to excess reduced thiol results in removal of the zinc ion. Two other cysteine residues in the heme domain, Cys-284 and Cys-378, as well as two in the reductase domain, Cys-356 and Cys-675, also did not react with the first reagent.

DISCUSSION

Pterin-free NOS exhibits a Soret band (416–420 nm) characteristic of predominantly low spin heme. Upon incubation of NOS with H4B and arginine, the Soret band shifts to 398–400 nm (30,35). We have studied the process and requirements of H4B binding to pterin-free NOS in the absence of substrate. This is important in that L-arginine (or NHA) binding alone...
that may occur upon L-arginine binding or turnover in the inability of ascorbate to substitute for GSH imply that a functions to reduce an NOS moiety, which in the oxidized form, interferes with H4B reconstitution. This proposal is supported reconstitution requires the presence of reduced thiol. Because our experiments were carried out with the full-length enzyme, we were able to correlate the observed spectral changes with full recovery of NO-forming activity (Fig. 3B). We also carried out extensive characterizations of these forms of the enzyme by H4B, heme, and metal quantitation as well as cysteine-labeling experiments. In the absence of reduced thiol, H4B alone effects little change in the pterin-free NOS spectrum and achieves reconstitution of only approximately 50% of full activity (Fig. 4). It is important to remember that pterin-free NOS does not catalyze turnover of arginine in the absence of added H4B (19). For ease of comparison in the activity reconstitution experiments, however, zero time points were designed to control for H4B reconstitution that may occur upon l-arginine binding or turnover in the assay. This was accomplished by assaying pterin-free NOS in the presence of H4B with or without GSH at concentrations that corresponded to those in assays of the timed incubations. These results have indicated that facile and complete H4B reconstitution requires the presence of reduced thiol. Because these experiments were carried out under strictly anaerobic conditions and formation of oxidized pterin in H4B-only studies was not observed in the spectral experiments (<2%), the presence of reduced thiol was not required to maintain a pool of reduced pterin. We considered the possibility that reduced thiol could function to chelate the NOS-bound zinc molecule, which might aid H4B binding by relaxing structural constraints. The zinc stoichiometry of pterin-free NOS incubated with GSH alone, however, was unchanged relative to either pterin-free or H4B-reconstituted NOS (data not shown). Apparently, a thiol functions to reduce an NOS moiety, which in the oxidized form, interferes with H4B reconstitution. This proposal is supported by the dependence of H4B reconstitution on thiol concentration (Fig. 5A), the increased rate of absorbance change observed upon preincubation of pterin-free NOS with GSH (Fig. 5B), and the inability of the oxygen-substituted GSH analog, GOH, to facilitate H4B reconstitution (Fig. 5A). These data as well as the inability of ascorbate to substitute for GSH imply that a protein disulfide must be reduced for facile H4B binding and recovery of activity. To examine this hypothesis, labeling of NOS cysteine residues before and after reduction with DTT was carried out. Of the six cysteine residues observed in the NOS structures (21, 37), a hydrogen bond donor in addition to the absence of the two cysteine residues from the opposing subunit (21, 38) and may be expected to be protected from reaction until dissociation of the zinc atom, induced by high thiol concentrations and/or enzyme denaturation after prolonged incubations at 37 °C. Examination of the NOS heme domain structures (21, 37, 38) or the cytochrome P450 reductase structure (51) does not explain the protection of the remaining two cysteine residues in the heme domain and the two in the reductase domain. It is unlikely but not impossible that some cysteine residues might be inaccessible to the labeling reagent due to structural constraints even though the reactions were carried out in 5 M guanidine HCl. However, based on the results presented in this study it is tempting to speculate that one or more disulfide bonds (intra- or intersubunit) involving these residues are formed during expression and/or purification of NOS in the absence of H4B and reduced thiol. This disulfide bond would then interfere with H4B binding and recovery of activity, perhaps by constraining the structure of the enzyme. The previously observed effects of GSH to stimulate H4B-bound neuronal NOS activity and stabilize activity over long (15–60 min) assays (52) was proposed to be due to reduction of protein thiols, which interact with the pterin-binding site of NOS. Although direct interaction of protein cysteine residues with the pterin-binding site is not observed in the NOS structures (21, 37, 38), the function of GSH in those studies may indeed have been in the reduction of a protein disulfide, which interfered with H4B binding, as supported by the results presented here.

Another interesting result of these studies is the observation that the redox-stable pterin analogs, DZPH4 and EtOMeDZPH4, did not bind to pterin-free NOS in the same fashion as H4B. The absorbance changes effected by the redox-stable analogs were similar to those occurring upon H4B binding. However, the redox-stable analogs bound pterin-free NOS in a concentration-dependent (not time-dependent) manner, without a dependence on the presence of reduced thiol. One difference between the redox-stable analogs and H4B is in the substituent at the C6 position. To control for changes in the C6 substituents, the reconstitution properties of the redox-active MPH4 compared with those of DZPH4 were examined. These compounds both have a 6-methyl group in contrast to the 6-dihydroxypropyl group of H4B. MPH4 has already been shown to reconstitute NOS activity (13) and, as reported here, reconstituted pterin-free NOS in a time- and thiol-dependent manner, similar to H4B. The higher concentrations of MPH4 (as compared with H4B) required for reconstitution are similar to those needed to support NOS catalysis (13). DZPH4, however, is unable to support NOS catalysis (13). Both compounds, MPH4 and DZPH4, are racemic, which may in part explain the observed lowered affinity compared with 6R-H4B (25). However, the 200-fold decrease in the affinity of DZPH4 for NOS may also be due to differences in the pterin side chain. The 6-ethoxymethyl substituent of EtOMeDZPH4 results in an even greater decrease (400-fold as compared with H4B) in affinity. The C6 substituent of the pterin thus appears to be an important determinant of binding affinity; this is supported by the multiple interactions of the biopterin side chain with protein residues observed in the NOS structures (21, 37, 38).

The redox-stable pterin analogs also lack the nitrogen atom at the 5 position, which is present in H4B. The structures of MPH4 and DZPH4 also differ only in the absence of the N5 atom in DZPH4. Therefore, the differences in binding between MPH4 and DZPH4 cannot be attributed to the C6 substituent. DZPH4 lacks a hydrogen bond donor. The mode of DZPH4 binding may be different from that of H4B, particularly in light of the different hydrogen bonding patterns and the difference in reconstitution. Although crystal structures of 7,8-dihydrobiopterin and 4-amino-H4B bound to NOS are indistinguishable from H4B-bound NOS (53), no structure with a 5-deazapterin bound has been determined. In the structure of the iNOS heme domain dimer (37), a hydrogen bond bridges the N5 of H4B (through a water molecule) to Arg-375 of the substrate binding helix, which participates in additional hydrogen bonds. Interestingly, Sono et al. (39) report that the 4-amino-H4B effects a thiol-dependent shift in the nNOS heme domain Soret. Apparently, the thiol-independent effects of the deazapterins are unique to changes at the N5 position. The absence of this hydrogen bond donor in DZPH4 in addition to the absence of the interactions of a 6-dihydroxypropyl side chain may allow
DZPH together to bind in a different orientation compared with biotin terin molecules with a nitrogen at the 5 position. The redox stable pterin analogs most likely bind near the heme, however, because the analogs affect the heme spin-state equilibration, and DZPHH inhibits the pterin-free NOS reaction with NHA and NADPH.

Analysis of the zinc, heme, and H4B content of pterin-free and H4B-reconstituted NOS was carried out to further examine the differences between these two forms of the enzyme. However, the presence or absence of H4B made no difference in the binding of heme and zinc by NOS. NOS bound zinc with a highly reproducible stoichiometry of 0.5 per monomer, entirely consistent with the zinc atom bound at the dimer interface of the NOS heme domain (21, 38). Zinc content was independent of H4B content and not correlated to the heme stoichiometry. H4B content, when reconstituted, and heme content were equivalent within error in these samples. Heme binds to NOS in the absence of H4B, as evidenced by results with the pterin-free samples. From the stoichiometries observed, it appears for these experiments). The former results in an underestimation of the extent of H4B binding, and the latter may be explained by 1) the fact that pterin-free NOS is not completely low spin even in the absence of substrate and pterins and 2) that activity measurements must be carried out in the presence of substrate, which may in itself effect an increase in H4B bound (leading to an increase in the baseline observed for these experiments). The former results in an underestimation of the extent of H4B binding, and the latter may result in an overestimation of H4B binding. In support of these interpretations, the stoichiometry of H4B bound to pterin-free NOS in the absence of reduced thiols was measured by HPLC assay at 0.32/NOS monomer.

In summary, pterin-free NOS can be reconstituted with H4B as evidenced by the electronic absorption spectrum, the recovery of -NO-forming activity, and the measurement of H4B bound to the enzyme. In addition, heme and metal content of NOS are not dependent on the presence of H4B. H4B binding exhibits a dependence on the presence of a reduced thiol. The most likely explanation for this dependence is the requirement for reduction of a protein disulfide bond perhaps involving one or more of the cysteines identified in this study. The reconstitution characteristics of the redox-stable pterin analogs differ from those of H4B, perhaps indicating that these deaza molecules bind to NOS differently than does H4B. H4B does not seem to be required for the structural integrity of NOS, which has also been demonstrated crystallographically (21). The results presented here indicate that binding of H4B to NOS is a complex process. Further studies on the binding of H4B and pterin analogs will aid in the elucidation of the role of H4B in NOS.

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