Tetrahydrobiopterin Binding to Macrophage Inducible Nitric Oxide Synthase: Heme Spin Shift and Dimer Stabilization by the Potent Pterin Antagonist 4-Amino-Tetrahydrobiopterin†

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ABSTRACT: The characteristics of tetrahydrobiopterin (H_4biopterin) binding to pteridine-free recombinant macrophage inducible nitric oxide synthase expressed in *Escherichia coli* were investigated with a special focus given to effects caused by 2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine (4-amino-H_4biopterin), a novel pterin-based inhibitor of nitric oxide synthase. The 4-amino compound completely inhibited enzyme stimulation by 10 μM H_4biopterin with a half-maximally active concentration of 7.2 ± 0.39 μM, whereas H_2biopterin and sepiapterin were much less potent. Binding studies using [^{3}H]H_4biopterin at 4 °C revealed biphasic association of the radioligand according to two first-order reactions with apparent rate constants of 2.2 and 0.05 min^{-1}, each accounting for approximately 50% of total binding. Dissociation of [^{3}H]H_4biopterin occurred with rate constants of 0.005 and 0.0028 min^{-1} in the absence and presence of L-arginine, respectively. Specific binding of 10 nM [^{3}H]H_4biopterin was antagonized by unlabeled H_4biopterin and its 4-amino analog with half-maximal effects at 84 ± 6 and 34 ± 3.2 nM, respectively. Binding of H_4biopterin and 4-amino-H_4biopterin was accompanied by a partial low spin to high spin conversion of the heme that was completed by L-arginine. Similarly, the active cofactor and the inhibitory 4-amino derivative both induced significant formation of stable protein dimers that survived during SDS electrophoresis, suggesting that the allosteric effects caused by H_4biopterin do not explain sufficiently the essential role of the pteridine cofactor in NO biosynthesis.

Nitric oxide is formed by enzymatic oxidation of the guanidino group of L-arginine by different nitric oxide synthases (NOS,[1] EC 1.14.13.39) (Griffith & Stuehr, 1995, Masters et al., 1996, Mayer, 1995). The neuronal (nNOS) and endothelial (eNOS) isoforms are constitutively expressed and require micromolar concentrations of free Ca^{2+} for activity, whereas the isoform first described in murine macrophages (iNOS) is cytokine inducible and Ca^{2+}-independent. Oxidation of L-arginine occurs via reductive activation of molecular oxygen catalyzed by a cytochrome P450-like heme iron localized in the oxygenase domain of the enzyme. The five electron oxidation of L-arginine is accompanied by an eight electron reduction of molecular oxygen with three exogenous electrons shuttled from the cofactor NADPH to heme by an FAD- and FMN-containing cytochrome P450 reductase domain. With this domain structure, NOS appears to be a self-sufficient cytochrome P450, resembling the soluble cytochrome $P450_{(BM-3)}$ from *Bacillus megaterium*, which also contains oxygenase and reductase domains within a single polypeptide (Fulco, 1991). When activated by Ca^{2+}/calmodulin in the presence of low concentrations of L-arginine or H_4biopterin, nNOS exhibits NADPH oxidase activity resulting in formation of superoxide anions and H_2O_2 due to uncoupling of oxygen reduction from L-arginine oxidation (Culcasi et al., 1994; Heinzel et al., 1992; Mayer et al., 1991; Pou et al., 1992). Uncoupling in the absence of L-arginine appears to be a specific feature of nNOS, since neither eNOS (List et al., 1997) nor iNOS (Abu-Soud & Stuehr, 1993; Olken & Marletta, 1993) exhibits considerable NADPH oxidase activity in the absence of a ligand bound to the substrate site.

Unlike other P450s, NOS requires H_4biopterin as a cofactor, but the precise function of the pteridine is not known (Mayer & Werner, 1995). Although H_4biopterin may have a distinct function as a reactant in L-arginine oxidation, its allosteric effects resulting in profound changes in protein conformation are more obvious. Presence of the pteridine

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[1] Abbreviations: NOS, nitric oxide synthase, eNOS, endothelial nitric oxide synthase (type III); iNOS, inducible nitric oxide synthase (type II); nNOS, neuronal nitric oxide synthase (type I); H_4biopterin, 5,6,7,8-tetrahydro-L-*erythro*-biopterin = 5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pterin; H_2biopterin, 5,6,7,8-dihydro-L-*erythro*-biopterin = 5,6,7,8-dihydro-6-(L-*erythro*-1,2-dihydroxypropyl)pterin; 4-amino-H_4biopterin, 2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PAGE, polyacrylamide gel electrophoresis; EC_{50}, concentration producing half-maximal effects; IC_{50}, concentration producing half-maximal inhibition.

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was shown to be essential for dimerization of iNOS monomers (Baek et al., 1993) and for stabilization of nNOS dimers (Klatt et al., 1995). On the basis of the essential role of the prosthetic heme group for dimerization of iNOS (Baek et al., 1993), nNOS (Klatt et al., 1996a), and eNOS (List et al., 1997), the effect of H_4biopterin on subunit assembly may be related to an interaction with the heme as evident by a low spin to high spin transition of the heme that occurs upon pteridine binding to H_4biopterin-free NOS preparations (McMillan & Masters, 1995; Rodriguez-Crespo et al., 1996). It is unknown whether this spin transition of the heme or the stabilization of NOS dimers explain the essential role of H_4biopterin in NOS catalysis. In a previous study, we observed that binding of H_2biopterin to the pteridine site of porcine brain NOS antagonized stimulation of the enzyme by exogenous H_4biopterin, indicating that pteridine binding is necessary but not sufficient to support catalytic activity (Klatt et al., 1994a). This view was further supported by a recent study in which we identified the dihydropteridine reductase inhibitor 4-amino-H_4biopterin as a potent pteridine antagonist of nNOS (Werner et al., 1996). Notwithstanding this evidence supporting a redox-function of H_4biopterin, it cannot be excluded at the present state that enzyme inhibition by 4-amino-H_4biopterin and other inhibitory pteridines results from a lack of these derivatives to induce an appropriate change in the heme environment.

In the present work, we investigated the characteristics of [^{3}H]H_4biopterin binding to dimeric pteridine-free macrophage iNOS expressed in *Escherichia coli* (Wu et al., 1996). The novel compound 4-amino-H_4biopterin was identified as potent iNOS inhibitor that bound with high affinity to the pteridine site of the enzyme. Like the natural cofactor, the inhibitory 4-amino analog partially prevented iNOS subunit dissociation during SDS electrophoresis and caused a low spin to high spin transition of the heme, indicating that the allosteric effects induced by H_4biopterin may not explain the essential role of the pteridine cofactor in NOS catalysis.

MATERIALS AND METHODS

Materials. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was from Amersham, purchased through MedPro (Vienna, Austria). 3′[^{3}H](6*R*)-5,6,7,8-Tetrahydro-L-biopterin (14 Ci/mmol) was prepared from [8,5′-^{3}H]GTP as described (Werner et al., 1994). Mouse macrophage iNOS was expressed in *E. coli* and purified as described (Wu et al., 1996). Pteridines were from Dr. B. Schircks Laboratories, Jona, Switzerland. Other chemicals were from Sigma.

Determination of Enzyme Activity. NOS activity was determined as formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine (Mayer et al., 1994). Incubations were for 2−10 min at 37 °C in 0.1 mL of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.1 μg of iNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (∼80 000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 2.4 mM 2-mercaptoethanol, 0.2 mM CHAPS, and pteridines as indicated. To test for reversibility of inhibition, iNOS (0.1 μM; 0.13 mg/mL) was preincubated at 4 °C for 10 min in the presence of 5 mM L-arginine with or without 50 μM 4-amino-H_4biopterin, followed by 50-fold dilution of the samples and determination of L-citrulline formation at 37 °C for 1−30 min with 0.1 mM H_4biopterin.

H_4Biopterin Binding. All binding experiments (saturation, association, and dissociation) were performed at 4 °C with iNOS that had been preincubated at a concentration of 50 μg of protein/mL in a 50 mM triethanolamine/HCl buffer (pH 7.4) for 30 min at ambient temperature with or without 0.5 mM L-arginine (protein and substrate 5-fold final). For saturation binding, 1 μg of protein was incubated for 45 min at 4 °C with 10 nM [^{3}H]H_4biopterin (∼14 nCi) and increasing concentrations of unlabeled H_4biopterin (10 nM−10 μM) in 0.1 mL of a 50 mM triethanolamine/HCl buffer, pH 7.4, followed by precipitation with polyethylene glycol, vacuum filtration, and determination of bound radioactivity as described (Klatt et al., 1994a,b). For association, the enzyme was incubated for the indicated periods of time under essentially the same conditions but without unlabeled H_4-biopterin. For dissociation, 10 μg of protein was incubated in 1 mL of a 50 mM triethanolamine/HCl buffer (pH 7.4) for 45 min with 10 nM [^{3}H]H_4biopterin, followed by addition of unlabeled H_4biopterin (1 mM final) and processing of 0.1 mL aliquots containing 1 μg of enzyme at the indicated time points. Data were corrected for nonspecific binding in the presence of 1 mM of the unlabeled ligand.

Gel Electrophoresis. To test for dimerization, purified iNOS was analyzed by low temperature SDS−PAGE as described (Klatt et al., 1995). The protein was incubated for 5 min at 37 °C in 50 μL of 50 mM triethanolamine/HCl buffer (pH 7.4) with L-arginine (1 mM) and H_4biopterin or 4-amino-H_4biopterin (0.2 mM each). Incubations were terminated by the addition of 50 μL of chilled Laemmli buffer (Laemmli, 1970) containing 0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, and 0.02% (w/v) bromophenol blue. In some experiments, the protein was boiled for 5 min in Laemmli buffer. Samples containing 8 μg of iNOS were subjected to SDS−PAGE for 60 min at 120 V on discontinuous 8% SDS slab gels (70 × 80 × 1 mm). Gels and buffers, prepared according to Laemmli (Laemmli, 1970), were equilibrated at 4 °C and the buffer tank was cooled during electrophoresis in an ice bath. Gels were stained for protein with Coomassie blue and densitometrically analyzed using the vds 800 video system and H1D-software of Hirschmann (Taufkirchen, Germany).

Determination of H_4biopterin Binding Stoichiometry by Gel Filtration Chromatography. Aliquots of 0.20 mL containing ∼1 mg of purified iNOS were incubated with 0.2 mM H_4biopterin in the presence of 1 mM L-arginine at ambient temperature for 15 min and subjected to gel filtration chromatography as described (Klatt et al., 1996a). Fractions containing dimeric iNOS were pooled and assayed for enzyme activity and H_4biopterin (Klatt et al., 1996b).

Optical Measurements. Absorbance spectra were recorded at ambient temperature with a Hewlett-Packard 8452A Diode Array Spectrophotometer. iNOS was diluted with 50 mM triethanolamine/HCl buffer (pH 7.4) to ∼5 μM from a 50-fold concentrated stock solution and incubated the presence of H_4biopterin, 4-amino-H_4biopterin (10 μM each) or L-arginine (0.2 mM) as indicated. The kinetics of low spin to high spin transition were measured as change in the peak to trough absorbance differences at 392 and 424 nm.

RESULTS

Recombinant iNOS purified from *E. coli* was inactive in the absence of exogenous H_4biopterin. The pteridine stimulated formation of L-citrulline in a concentration-dependent

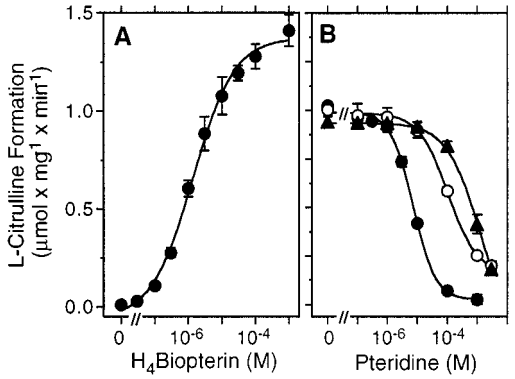

FIGURE 1: Effect of H_4biopterin and 4-amino-H_4biopterin on iNOS activity. Incubations were for 2 min (A) or 10 min (B) at 37 °C in 0.1 mL of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.1 μg of iNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (~80 000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.2 mM CHAPS, and pteridines as indicated. (A) Stimulation by H_4biopterin (3×10^{-8}–10^{-3} M). (B) Effects of 4-amino-H_4biopterin (solid circles), H_2-biopterin (open circles), and sepiapterin (solid triangles) on iNOS activity in the presence of 10 μM H_4biopterin. Data (means ± SE; $n = 3$) were fitted according to the Hill equation.

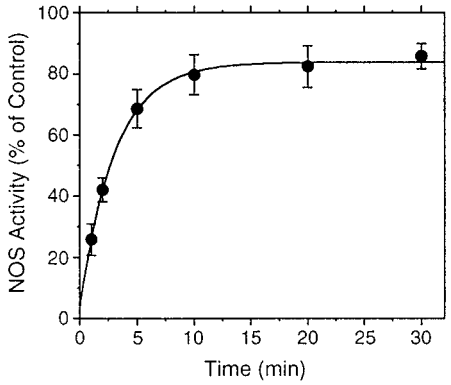

FIGURE 2: Reversibility of iNOS inhibition by 4-amino-H_4biopterin. Purified iNOS (1 μM; 0.13 mg/ml) was preincubated at 4 °C for 10 min in the presence of 5 mM L-arginine with or without 50 μM 4-amino-H_4biopterin, followed by 50-fold dilution of the samples and determination of L-citrulline formation at 37 °C for the indicated periods of time in 0.1 mL volumes in the presence of 0.1 mM H_4-biopterin. Data (means ± SE; $n = 3$) are expressed as percent of enzyme activity determined with iNOS preincubated in the absence of 4-amino-H_4biopterin and were fitted according to first-order kinetics.

manner with an EC_{50} of 1.6 ± 0.43 μM (mean ± SE, $n = 3$) and a maximal effect at about 100 μM (Figure 1A). Incubation for 10 min instead of 2 min resulted in an increase of the EC_{50} to 2.6 ± 0.22 μM (mean ± SE; $n = 3$). H_2-biopterin, 4-amino-H_4biopterin, and sepiapterin were inactive at concentrations of up to 1 mM (data not shown). As shown in Figure 1B, the effect of H_4biopterin was fully antagonized by 4-amino-H_4biopterin, which exhibited an IC_{50} of 7.2 ± 0.39 μM ($n = 3$) in the presence of 10 μM H_4biopterin. H_2-Biopterin and sepiapterin also inhibited iNOS activity but only at much higher concentrations ($IC_{50} \geq 0.1$ mM).

Inhibition by 4-amino-H_4biopterin was reversible, as evident from the comparably rapid regain of enzyme activity that was observed upon dilution of the inhibitor-saturated enzyme in the presence of excess H_4biopterin (0.1 mM). From the data shown in Figure 2, we have calculated a dissociation rate of 0.33 ± 0.02 min^{-1}, corresponding to a half-time of 2.1 min for dissociation of 4-amino-H_4biopterin at 37 °C. Maximal activity of the inhibitor-treated enzyme

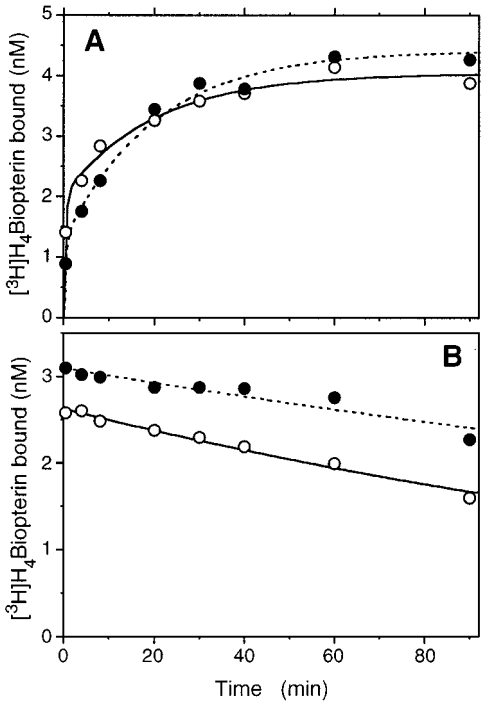

FIGURE 3: Kinetics of [^{3}H]H_4biopterin association (A) and dissociation (B). (A) Association kinetics. Purified iNOS (50 μg/mL) was preincubated for 30 min at ambient temperature in a 50 mM triethanolamine/HCl buffer, pH 7.4, in the absence (open symbols) or presence (filled symbols) of 0.5 mM L-arginine (5-fold final), followed by incubation of 10 μg of protein with 10 nM [^{3}H]H_4-biopterin (~14 nCi) in 1 mL of triethanolamine buffer at 4 °C. At the indicated time points, 0.1 mL aliquots containing 1 μg of protein were removed and assayed for bound [^{3}H]H_4biopterin as described (Klatt et al., 1994a,b). Data (means ± SE; $n = 3$) were fitted according to a function consisting of two first-order reactions. (B) Dissociation kinetics. Purified iNOS (50 μg/mL) was preincubated for 30 min at ambient temperature in a 50 mM triethanolamine/HCl buffer, pH 7.4, in the absence (open symbols) or presence (filled symbols) of 0.5 mM L-arginine (5-fold final), followed by equilibration of the enzyme (10 μg) with 10 nM [^{3}H]H_4biopterin (~14 nCi) in 1 mL of a 50 mM triethanolamine/HCl buffer, pH 7.4, for 45 min at 4 °C and addition of unlabeled H_4biopterin (1 mM final). At the indicated time points, 0.1 mL aliquots were removed for determination of enzyme-bound radioactivity. Data (means ± SE; $n = 3$) were fitted according to first-order kinetics.

was slightly below control values, although the residual 4-amino-H_4biopterin concentration of 1 μM did not produce significant inhibition in normal enzyme assays carried out in the presence of 0.1 mM H_4biopterin (not shown).

Pteridine binding to iNOS was studied using [^{3}H]H_4-biopterin as a radioligand. As shown in Figure 3A, we observed several clear differences in the kinetics of [^{3}H]H_4-biopterin association to iNOS as compared to results obtained earlier with H_4biopterin-free nNOS (Gorren et al., 1996). First, the rate of H_4biopterin binding was considerably higher, which forced us to measure the reaction at 4 °C. Furthermore, the reaction did not follow simple pseudo-first-order kinetics, but, unlike the reaction with nNOS, it neither was zero order. Excellent fits were obtained to a sum of two first-order reactions (50% reacting with a k_{app} of 2.2 min^{-1} and 50% with a k_{app} of 0.05 min^{-1}). Finally, in striking contrast to the nNOS reaction, the rate of H_4biopterin binding did not increase upon incubation with L-arginine. When the association binding kinetics was performed in the absence or presence of L-arginine with 2 μM instead of 10 nM [^{3}H]H_4-biopterin, we observed that binding was complete within the

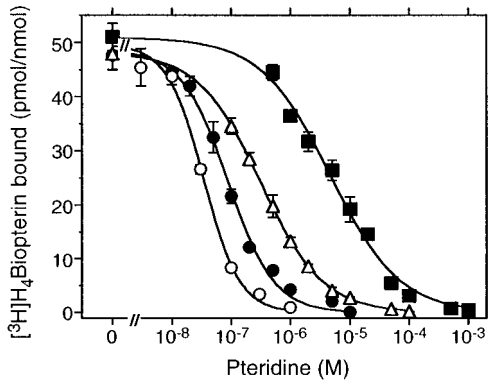

FIGURE 4: Competition of pteridine derivatives for [^{3}H]H$_4$biopterin binding. Purified iNOS (50 μg/mL) was preincubated for 30 min at ambient temperature in a 50 mM triethanolamine/HCl buffer, pH 7.4, in the presence of 0.5 mM L-arginine (5-fold final), followed by incubation of 1 μg of protein with 10 nM [^{3}H]H$_4$biopterin (~14 nCi) in 0.1 mL of a 50 mM triethanolamine/HCl buffer, pH 7.4, for 45 min at 4 °C and determination of enzyme-bound radioligand. Unlabeled H$_4$biopterin (solid circles), 4-amino-H$_4$biopterin (open circles), H$_2$biopterin (triangles), and sepiapterin (squares) were present as indicated. Data (means ± SE; $n = 3$) were fitted according to the Hill equation.

first 30 s of incubation in both conditions, showing that k_{app} of the slow phase was ≥ 1.5 min^{-1}.

Experiments on H$_4$biopterin dissociation kinetics (Figure 3B) showed that the binding was very stable, with only little loss of radiolabel occurring within 1 h after addition of excess unlabeled H$_4$biopterin ($t_{1/2} \approx 2$ h; not shown). Preincubation of iNOS with L-arginine further stabilized H$_4$biopterin binding. Under these conditions, the apparent dissociation rate constant decreased ~2-fold from 0.0050 to 0.0028 min^{-1} ($t_{1/2} \approx 4$ h). For comparison, dissociation of [^{3}H]H$_4$biopterin from H$_4$biopterin-free nNOS was measured at 4 ° C. After preincubation with L-arginine, the dissociation rate constant was 0.013 min^{-1} (mean; $n = 2$).

The complex kinetics of association and the very slow rates of [^{3}H]H$_4$biopterin dissociation made it difficult to obtain reliable K_D or K_I values for binding of pteridine derivatives in competition experiments. Therefore, affinity constants are given as IC$_{50}$ values, i.e., the concentrations of the unlabeled ligands that reduced equilibrium binding of 10 nM [^{3}H]H$_4$-biopterin by 50%. As shown in Figure 4, H$_4$biopterin, 4-amino-H$_4$biopterin, H$_2$biopterin, and sepiapterin antagonized the binding of [^{3}H]H$_4$biopterin to L-arginine-pretreated iNOS with IC$_{50}$ values of 84 ± 7.6, 34 ± 3.2, 260 ± 29, and 4100 ± 84 nM, respectively (mean ± SE, $n = 3$ each). Omission of L-arginine during preincubation led to slightly reduced amounts of the bound radioligand but had no significant effect on the affinity constants (not shown).

Similarly to the radioligand binding experiments, optical studies of pteridine binding to H$_4$biopterin-free iNOS yielded results quite different from those obtained with the neuronal enzyme. Whereas H$_4$biopterin-free nNOS is to a large extent low spin (Gorren et al., 1996, Wang et al., 1995), the different iNOS preparations showed a mixture of spin states that ranged from mostly low spin to mostly high spin. These differences may be explained by a slow ($t_{1/2} \approx 30$ min) high spin to low spin conversion of the enzyme at ambient temperature. Figure 5A shows the spectrum of a preparation containing approximately 60% high spin heme that was shifted toward low spin by H$_4$biopterin or L-arginine. However, H$_4$biopterin alone was not able to fully shift iNOS

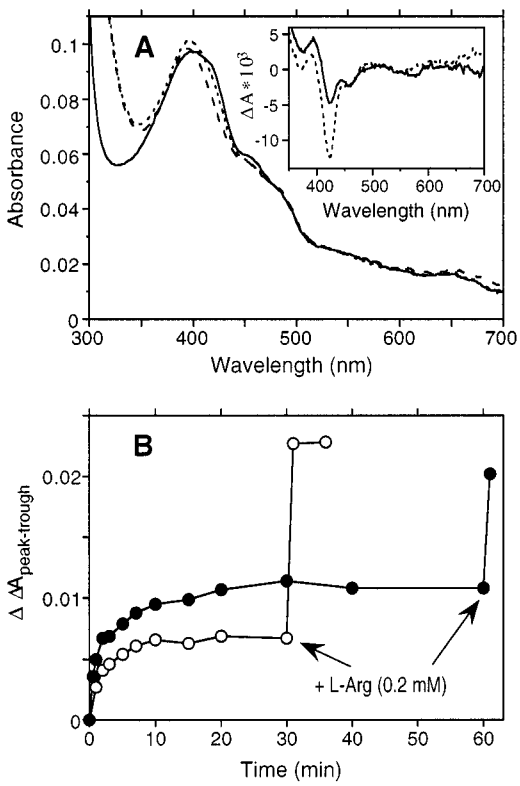

FIGURE 5: Spectral changes induced by H$_4$biopterin and 4-amino-H$_4$biopterin. For measurements of light absorbance, iNOS (2.5 μM) was preincubated in 50 mM triethanolamine/HCl buffer (pH 7.4) for the indicated periods of time (usually 1 h) at ambient temperature with pteridines or L-arginine as indicated. (A) Absorbance spectra of iNOS preincubated in buffer only (solid line), 10 μM H$_4$biopterin (dotted line) or 0.2 mM L-arginine (dashed line). The inset shows the corresponding absorbance difference spectra (H$_4$biopterin, solid line; L-arginine, dashed line). (B) The kinetics of low spin to high spin conversion induced by H$_4$biopterin (solid circles) or 4-amino-H$_4$biopterin (open circles) was measured as change of the peak-to-trough absorbance difference between 392 and 424 nm and plotted as a function of the incubation time. L-Arginine was added at the time points indicated by arrows. Shown is one out of three similar experiments.

to the high spin form as evident from the pronounced absorbance change induced by L-arginine added to the pteridine-equilibrated enzyme (Figure 5B). 4-Amino-H$_4$-biopterin produced spectral changes similar to H$_4$biopterin although the effect was less pronounced. Again, full conversion to high spin was observed upon addition of L-arginine to the 4-amino-H$_4$biopterin-saturated enzyme. These findings contrast observations with nNOS which was fully converted to high spin by H$_4$biopterin as well as the 4-amino analog.[2]

Low temperature SDS−PAGE, which allows to monitor formation of stable nNOS dimers (Klatt et al., 1995), was used to study the effects of pteridines on iNOS dimerization. As shown in Figure 6, iNOS migrated as a single 130 kDa band when it had been either boiled (lane A) or preincubated for 5 min at 37 °C without additions (lane B). Preincubation of the protein with L-arginine (1 mM) and either H$_4$biopterin (0.2 mM; lane C) or 4-amino-H$_4$biopterin (lane D) led to the appearance of an additional band which migrated at ~200 kDa and apparently represented dimeric iNOS (lane C). L-Arginine had no effect when given alone but potentiated

[2] Gorren, A. C. F., and Mayer, B. (unpublished material).

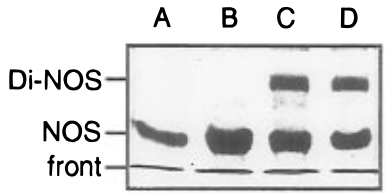

FIGURE 6: Low temperature SDS−PAGE of iNOS. Purified iNOS (8 μg) was preincubated at 37 °C for 5 min in the presence of 1 mM L-arginine and H_4biopterin or 4-amino-H_4biopterin (0.2 mM each), followed by low temperature SDS−PAGE analysis on 8% slab gels, which were stained for protein with Coomassie blue. Lane A, boiled; lane B, unboiled; lane C, H_4biopterin; lane D, 4-amino-H_4biopterin (0.2 mM). NOS and Di-NOS refer to iNOS monomers and dimers with apparent molecular masses of approximately 130 and 200 kDa, respectively. The gel shown is representative of three.

the effect of H_4biopterin (not shown). As estimated by densitometric analysis, dimers accounted for about 30% of total protein. Identical results were obtained when the enzyme was preincubated with 10 μM instead of 0.2 mM H_4biopterin or 4-amino-H_4biopterin (data not shown).

To see whether pteridine binding to iNOS shows anticooperativity as described for the neuronal isoform (Gorren et al., 1996), the enzyme was preincubated with 0.2 mM H_4biopterin for 15 min at ambient temperature in the presence of 1 mM L-arginine followed by gel filtration chromatography for removal of the unbound ligand and determination of H_4biopterin in the pooled column fractions. The pteridine treated enzyme contained 0.28 ± 0.03 equiv of H_4biopterin per subunit ($n = 3$), and omission of H_4biopterin (0.1 mM) from enzyme assays led to a reduction of L-citrulline formation to 35.5 ± 2.0% of controls ($n = 3$). The H_4biopterin-pretreated preparations showed only minor binding of [^{3}H]H_4biopterin (≤10% of pteridine-free controls; $n = 2$), contained 55 ± 5% ($n = 3$) high spin heme, and partially (∼20%) survived LT-PAGE in the presence of SDS without added H_4biopterin ($n = 2$).

DISCUSSION

As reported for other NOS isoforms expressed in *E. coli* (Fossetta et al., 1996, Gerber & Ortiz DeMontellano, 1995, Martasek et al., 1996, Rodriguez-Crespo et al., 1996, Roman et al., 1995), recombinant mouse macrophage iNOS obtained from the bacteria was H_4biopterin-free and required addition of the pteridine cofactor for activity. The H_4biopterin concentration producing half-maximal enzyme stimulation was 1.6 μM and thus significantly higher than that reported for the H_4biopterin-free forms of bovine eNOS (Rodriguez-Crespo et al., 1996), human iNOS (Fossetta et al., 1996), and rat nNOS (Gorren et al., 1996). The apparent decreased potency of H_4biopterin in 10 min assays indicates that the enzyme is unstable at low H_4biopterin concentrations as suggested for eNOS from *E. coli* (Rodriguez-Crespo et al., 1996).

A recent study that we have carried out with pteridine-free nNOS showed that [^{3}H]H_4biopterin bound only to the small fraction of the enzyme containing high spin heme as evident from a slow, zero-order [^{3}H]H_4biopterin association that was governed by the spin equilibrium of the enzyme (Gorren et al., 1996). According to the present data, iNOS behaves differently. Association of the radioligand was at least 100-fold faster, biphasic, clearly non-zero-order, and not stimulated by preincubation with L-arginine. The fast binding of [^{3}H]H_4biopterin and the lack of effect of L-arginine may be explained by the fact that the iNOS preparations were largely high spin even in the absence of bound pterin or substrate. We can only speculate about the reason for the biphasic binding. Since the enzyme is always present in excess over the radioligand, only the more reactive protein species are observed in binding experiments, suggesting that the biphasic kinetics may be due to heterogeneity of the high spin enzyme.

In addition to the very fast association, we observed an about 5-fold slower rate of [^{3}H]H_4biopterin dissociation from iNOS as compared to nNOS. Accordingly, the binding data hint at a very high affinity of the iNOS pteridine site, even though the functional data (Figure 1) show that enzyme stimulation occurred only at relatively high H_4biopterin concentrations. This may be explained by an anticooperativity of the two pteridine binding sites of dimeric iNOS, as suggested by our experiments in which the enzyme was preincubated with excess H_4biopterin and then separated from the unbound ligand by gel filtration chromatography. This procedure resulted in the formation of iNOS dimers containing ∼30% of tightly bound H_4biopterin which was not replaced at significant rates upon addition of the radiolabeled ligand. In contrast to the protein as isolated, H_4biopterin-treated iNOS catalyzed formation of L-citrulline in the absence of exogenous H_4biopterin. As expected from its pteridine content, the enzyme was stimulated approximately 3-fold by added H_4biopterin. With respect to this apparent anticooperativity of pteridine binding, iNOS resembles the neuronal enzyme (Gorren et al., 1996; List et al., 1996), whose high affinity site has an estimated K_D of about 10^{-9} M (Gorren et al., 1997). A similarly low K_D value may be the reason why the high affinity site of iNOS was not apparent in the citrulline assay, which had to be performed at a protein concentration that exceeded this value by 1 order of magnitude (∼10^{-8} M). Our results disagree with the data published by Marletta and colleagues, who reported on a virtually stoichiometrical H_4biopterin content of pteridine-reconstituted iNOS (Hevel & Marletta, 1992) and nNOS (Richards et al., 1996). The reason for this discrepancy is unclear.

Specific binding of [^{3}H]H_4biopterin to iNOS was antagonized by unlabeled H_4biopterin and the pteridine derivatives 4-amino-H_4biopterin, H_2biopterin, and sepiapterin. While the affinities of the 4-amino analog and H_2biopterin were comparable or even higher than that of H_4biopterin, sepiapterin was much less potent, supporting our proposal that the structure of the dihydroxypropyl side chain at C-6 of the pterin ring is crucial for high affinity binding to NOS. As we have recently reported (Werner et al., 1996), presence of an additional hydroxyl group (tetrahydroneopterin) or even epimerization to the D-*threo* derivative (tetrahydrodictyopterin) results in a dramatic loss of binding affinity. Of note, latter derivatives are active NOS cofactors, whereas the deaza analog of H_4biopterin as well as H_2biopterin, which both lack the reducing potential of the tetrahydro compounds, are inactive and inhibit enzyme stimulation by H_4biopterin (Hevel & Marletta, 1992; Klatt et al., 1994a). These previous studies have already indicated that the effect of H_4biopterin may involve redox chemistry, and this conclusion is strongly supported by the present results obtained with 4-amino-H_4biopterin. This compound, which inhibits enzymatic redox cycling of H_4biopterin due to the lack of the oxo group at

C4 (Werner et al., 1996) turned out as a potent and reversible pterin-based NOS inhibitor. At 37 °C, dissociation of 4-amino-H_4biopterin occurred with a rate of 0.33 min^{-1} which is ~120-fold higher than the rate of [^{3}H]H_4biopterin dissociation. Although the radioligand binding studies had to be determined at 4 °C, the large difference in the two off-rates is unlikely to be due to the difference in temperature at which the two assays were carried out but indicates that the 4-amino derivative is less tightly bound than the natural cofactor. In latter case, the potency of the inhibitor to compete with [^{3}H]H_4biopterin binding would be due to a comparably fast rate of association, but radioligand binding studies with radioactive 4-amino-H_4biopterin are needed to decide this issue.

Dimerization of mammalian iNOS was shown to be highly dependent on the presence of H_4biopterin (Baek et al., 1993; Tzeng et al., 1995), whereas the protein obtained from *E. coli* is dimeric even when expressed and purified without L-arginine/H_4biopterin (Wu et al., 1996). Here, we observed that H_4biopterin stabilized the enzyme such that it partially survived SDS electrophoresis in a dimeric state. The effect was less pronounced than with porcine nNOS (Klatt et al., 1995) but similar to that seen with the baculovirus-expressed recombinant rat brain enzyme (Klatt et al., 1996a). The most interesting result of the low-temperature SDS electrophoresis experiments was that the effect of 4-amino-H_4biopterin was indistinguishable from that of H_4biopterin with respect to iNOS dimerization. Together with the spectral data showing that H_4biopterin and the 4-amino derivative both induced a low spin to high spin conversion of the heme, our results indicate that the pterin dependence of NOS is not explained by the changes in protein conformation and point to an as yet unrecognized, highly specific function of H_4biopterin in NOS catalysis that remains to be investigated.

In addition to insights into mechanistic aspects of NOS function, the present results may provide new opportunities for the development of pteridine derivatives as a novel class of therapeutically relevant NOS inhibitors. Considering the pronounced pterin dependence of iNOS during protein expression (Tzeng et al., 1995; Werner-Felmayer et al., 1990), it is conceivable that the inducible enzyme is more sensitive to the 4-amino analog than the constitutively expressed isoforms already containing tightly bound H_4-biopterin. Work is underway in our laboratories to examine the selectivity of 4-amino-H_4biopterin toward different NOS isoforms and other pterin-dependent enzymes.

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