Tetrahydrobiopterin Inhibits Monomerization and Is Consumed during Catalysis in Neuronal NO Synthase*

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Andreas Reif, Lothar G. Fröhlich, Peter Kotsonis, Armin Frey, Heike M. Bömmel, David A. Wink, Wolfgang Pfeiderer, and Harald H. H. W. Schmidt

From the Department of Pharmacology and Toxicology, Julius-Maximilians-University, Würzburg, 97078 Germany, the National Cancer Institute, Bethesda, Maryland 20892, and the Faculty of Chemistry, University of Konstanz, Konstanz, 78434 Germany

The biosynthesis of nitric oxide (NO) is catalyzed by homodimeric NO synthases (NOS). For unknown reasons, all NOS co-purify with substoichiometric amounts of (6R)-5,6,7,8-tetrahydrobiopterin (H\(_4\)Bip) and require additional H\(_4\)Bip for maximal activity. We examined the effects of H\(_4\)Bip and pterin-derived inhibitors (anti-pterins) on purified neuronal NOS-I quaternary structure and H\(_4\)Bip content. During l-arginine turnover, NOS-I dimers time dependently dissociated into inactive monomers, paralleled by a loss of enzyme-associated pterin. Dimer dissociation was inhibited when saturating levels of H\(_4\)Bip were added during catalysis. Similar results were obtained with pterin-free NOS-I expressed in Escherichia coli. This stabilizing effect of H\(_4\)Bip was mimicked by the anti-pterin 2-amino-4,6-dioxo-3,4,5,6,8,8a,9,10-octahydro-oxazolo[1,2f]-pteridine (PHS-32), which also displaced NOS-associated H\(_4\)Bip in a competitive manner. Surprisingly, H\(_4\)Bip not only dissociated from NOS during catalysis, but was only partially recovered in the solute (50.0 ± 16.5% of control at 20 min). NOS-associated H\(_4\)Bip appeared to react with a NOS catalysis product to a derivative distinct from dihydrobiopterin or biopterin. Under identical conditions, reagent H\(_4\)Bip was chemically stable and fully recovered (95.5 ± 3.4% of control). A similar loss of both reagent and enzyme-bound H\(_4\)Bip and dimer content was observed by NO generated from spermine NONOate. In conclusion, we propose a role for H\(_4\)Bip as a dimer-stabilizing factor of neuronal NOS during catalysis, possibly by interfering with enzyme destabilizing products.

Nitric oxide (NO), a widespread signaling molecule with important physiological as well as pathophysiological functions (1–3), is synthesized from l-arginine at the expense of NADPH and O\(_2\) by a family of NO synthases (NOS; EC 1.14.13.39) comprised of three isoforms (NOS I –III or nNOS, iNOS, and eNOS, respectively; Ref. 4). The native, active enzyme is homodimeric (5) and each subunit contains both a reductase and an oxygenase domain (6). The reductase domain contains binding sites for NADPH, FAD, and FMN and is homologous to the NADPH-dependent cytochrome P450 reductase (7, 8). Binding of calmodulin to NOS promotes electron flow from the reductase to the oxygenase domain (9). In NOS-I and III, this occurs in response to elevated free intracellular calcium concentrations (10), while NOS-II binds calmodulin with high affinity even at the free calcium concentration of resting cells and, therefore, is primarily regulated at the expression level (9, 11). In the NOS oxygenase domain, which shares spectroscopic but not structural properties with other cytochrome P450-type enzymes (12), a prosthetic heme group (7, 13) forms the catalytic center together with the binding site for l-arginine. A major difference of all NOS isoforms to other P450 enzymes is their additional requirement for tetrahydrobiopterin (H\(_4\)Bip) for maximal activity (14–16). H\(_4\)Bip is known to bind in the oxygenase domain close to the heme and l-arginine, although how H\(_4\)Bip participates in catalysis is unclear. H\(_4\)Bip was first discovered as a cofactor of aromatic amino acid hydroxylases (17) where it acts as the main oxygen acceptor and reacts stoichiometrically to quinoid H\(_2\)Bip. In NOS, this function is fulfilled by the prosthetic heme group (18, 19) and any attempts to show redox cycling of H\(_4\)Bip have yielded negative results (20–22) making such a role for H\(_4\)Bip in NOS catalysis unlikely.

Recent studies on the crystal structure of NOS have yielded controversial results; unlike the initial report by Crane et al. (23), H\(_4\)Bip per se does not seem to induce a large conformational shift in the NOS oxygenase domain (24). However, H\(_4\)Bip has been reported to stabilize enzyme structure, possibly by keeping essential thiol groups in a reduced state (25, 26). Moreover, H\(_4\)Bip exerts multiple allosteric effects on NOS by lowering the K\(_d\) for the substrate l-arginine (22, 29), facilitating coupled electron transfer between the reductase and oxygenase domains (20, 30), and promoting subunit assembly of NOS-II (31) but not NOS-I (32, 33). Furthermore, H\(_4\)Bip stabilizes NOS dimers in the presence of protein denaturants such as calmodulin; CHAPS; 3-[3-cholamidopropyl]dimethylammoniomio]-2-hydroxy-1-propanesulfonate; FPLC, fast performance liquid chromatography; H\(_4\)Bip, (6R)-5,6,7,8-tetrahydrobiopterin; H\(_2\)Bip, (6R)-5,6-dihydro-1-biopthorin; H\(_{4}\)Bip, (6R)-5,6,7,8-tetrahydro-1-biopthorin; HPLC, high performance liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; NOS, nitric oxide synthase; PHS, anti-pterin code number; PIN, protein inhibitor of nNOS; RNS, reactive nitrogen species; ROS, reactive oxygen species; TEA, triethanolamine hydrochloride; PAGE, polyacrylamide gel electrophoresis.
as 5 mM urea (34) or 4% SDS (35). However, the relevance of the above findings in the physiological regulation of NOS is unclear given that the dependence on H4Bip, at least after several rounds of l-arginine turnover, is absolute.

Herein, we investigate possible structural, non-catalytic roles of H4Bip for NOS, describe the monomerization of NOS-I during l-arginine turnover, and demonstrate an important role of H4Bip in inhibiting this process. Additionally, we characterized the separated dimers and monomers. Finally, in order to gain further insight into NOS and pterin interactions, we analyzed the effects of anti-pterins on NOS quaternary structure and measured enzyme-associated and total pterin during catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—l-(12,3,4,5-3H)Arginine HCl (>2.15 TBq mmol⁻¹ and the ECL Western blotting Kit were purchased from Amersham (Braunschweig, Germany); (6R)-5,6,7,8-tetrahydro-l-biotin from Dr. Schircks Laboratories (Jona, Switzerland); NADP from AppliChem (Darmstadt, Germany); FAD and GSH from Roche Molecular Biochemicals (Mannheim, Germany); FMN, from Fluka (Buchs, Switzerland); horse heart cytochrome c from Sigma (Deisenhofen, Germany); spermine from Fluka (Buchs, Switzerland); Da-cutter (Mallipol, Freiburg, Germany); NOS-I (in the indicated amount) was expressed in a baculovirus/Sf9 cell system, purified by 2'-5'-ADP-Sepharose affinity chromatography. Native enzyme had a specific activity up to 227 nmol of citrulline mg⁻¹ min⁻¹. Recombinant human NOS-I was expressed in a baculovirus/Sf9 cell system, purified by 2'-5'-ADP-Sepharose affinity chromatography and subsequent calmodulin-Sepharose affinity chromatography, and had a specific activity of up to 379 nmol of citrulline mg⁻¹ min⁻¹. Pterin-free NOS-I was expressed in *Escherichia coli*, purified to a specific activity of 443 nmol mg⁻¹ min⁻¹ as previously published (33) and kindly provided by Dr. Roman (University of Texas, San Antonio, TX). All other chemicals were of the highest purity available and obtained from either Sigma or Merck AG. Water was deionized to 18 MΩ cm (Milli-Q; Millipore, Eschborn, Germany) and, for some experiments, deionized with argon.

**NOS Incubation**—To determine NOS activity and dimer stability, NOS-I (in the indicated amount) was incubated at 37 °C for up to 30 min in a total assay volume of 100 μl containing 50 mM triethanolamine (TEA) buffer (pH 7.2), 25 μM l-arginine, 1 mM NADPH, 5 μM FAD, 10 μM FMN, 0.5 μM CaM, 1 mM CaCl₂, and TEA buffer (50 mM, pH 7.0) in a final volume of 100 μl CHAPS. In the presence or absence of 2 μM H4Bip, reduced cytochrome c reduction was calculated using an extinction coefficient of 21 μm⁻¹ cm⁻¹ (39). Blank values were determined in the absence of NOS.

**Protein Determination and Immunoblot**—Protein concentrations were determined spectrophotometrically in a microplate assay according to the method of Bradford (40) using bovine serum albumin as a standard. NOS-I was immunodetected by Western blot using a peroxidase-enhanced chemiluminescence kit (ECL Western blotting kit; Amersham Pharmacia Biotech, Freiburg, Germany) as previously published (37). Incubations were carried out in a total volume of 300 μl containing 50 mM MOPS (pH 7.0), 1 mM NADPH, 1 mM CaCl₂, and TEA buffer (50 mM, pH 7.0) in a final volume of 100 μl CHAPS for 25 min at 37 °C. Reduced cytochrome c was measured at 550 nm in a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA). The rate of cytochrome c reduction was calculated using an extinction coefficient of 21 μm⁻¹ cm⁻¹ (39). Blank values were determined in the absence of NOS.

**Pterin Determination**—Pterin concentrations were determined spectrophotometrically in a microplate assay according to the method of Bradford (40) using bovine serum albumin as a standard. NOS-I was immunodetected by Western blot using a peroxidase-enhanced chemiluminescence kit (ECL Western blotting kit; Amersham Pharmacia Biotech) and NOS-I antibody as described (41).

**Statistical Analysis**—Data are expressed as mean ± S.E. Statistical analysis was performed using Student’s *t* test for unequal data. *p* values < 0.05 were considered statistically significant.

**RESULTS**

**NOS-I Monomerizes during l-Arginine Turnover**—In order to investigate possible structural changes of NOS-I during catalysis, we measured the dimer and monomer content of recombinant human NOS-I (17 μg) after 0 and 15 min of incubation in the absence of H4Bip. Samples were then analyzed by size exclusion chromatography as described under “Experimental Procedures.” Without incubation (*t* = 0 min), the enzyme eluted as a single NOS-I immunoreactive protein peak at 12.6 ± 0.05 ml (Fig. 1A, solid line), equivalent to a Stokes radius of 7.89 ± 0.03 nm, which is in the published range for NOS-I dimers (5, 42). Importantly, after 15 min of l-arginine turnover, NOS-I immunoreactive protein eluted in two peaks (Fig. 1A, broken line): the first coincided with the same elution volume as enzyme without any period of catalysis, the second, at 14.09 ± 0.06 ml, correlating to a Stokes radius of 6.07 ± 0.06 nm, which is in the range of that published for NOS-I monomers (32).

**Only NOS-I Dimers Are Active and Contain H4Bip**—To characterize NOS dimers and monomers formed during catalysis,
Tetrahydrobiopterin in NO Synthase

Fig. 1. NOS-I dissociates during l-arginine turnover into inactive and pterin-free monomers. A, recombinant NOS-I (17 μg) was incubated for 0 (solid line) and 15 min (broken line) in the absence of H₄Bip as described under “Experimental Procedures.” Typical chromatograms are shown and representative of n = 3–7 individual experiments. “D” indicates the dimer and “M” the monomer peak. B, NOS activity in the FPLC eluent fractions was determined as described under “Experimental Procedures” and is reported as absolute citrulline formation. C, citrulline formation and cytochrome c reductase. Only fractions containing dimeric NOS-I were found to convert L-arginine to L-citrulline. Addition of H₄Bip to the L-citrulline assay mixture markedly enhanced NOS-I activity.

In contrast, samples containing only monomeric NOS-I, pterin was not detected (fractions 9 to 12; detection limit was 0.1 to 0.2 pmol pterin per FPLC fraction). The plot represents the mean ± S.E. of three individual experiments, each performed in triplicate.

we determined the pterin content and enzyme activities (citrulline formation and cytochrome c reductase). In these experiments, the eluent from size exclusion chromatography was fractionated (300 μl). Fractions containing dimeric NOS-I formed L-citrulline (Fig. 1B, open bars) and this enzyme could be stimulated by 10-fold with the addition of 2 μM H₄Bip (total fraction 5, 5.96 ± 0.23 nmol/15 min; Fig. 1B, closed bars), similar to enzyme that was not preincubated. In contrast, fractions containing monomeric enzyme did not convert L-arginine both in the absence and presence of H₄Bip.

To examine whether enzyme activities of NOS-I dimers and monomers and their H₄Bip content were correlated, NOS-associated pterin was determined (Fig. 1C). While dimeric NOS contained 6.13 ± 0.39 pmol of H₄Bip in the peak fraction (fraction 5; Fig. 1C), the pterin content in the monomer-containing fractions (9–12) was below the detection limit. These data suggested that only NOS dimers contained enzyme-associated H₄Bip, as previously reported for rat NOS-I (5).

Since NOS monomers obtained by other procedures usually retain their reductase activity (13), we measured the reduction of cytochrome c in monomers generated under our conditions. Indeed, in contrast to L-citrulline formation, both the dimer and monomer peak fractions (5 and 10, respectively) were able to reduce cytochrome c (8.58 ± 0.28 and 8.6 ± 0.08 nmol of reduced cytochrome c ml⁻¹ min⁻¹, respectively), confirming that the ability of NOS-I to reduce electron acceptors neither requires dimeric enzyme (43) nor the presence of enzyme-associated H₄Bip.

NOS Monomerization Is Time-dependent in Correlation to Loss of Activity—To determine the kinetics of NOS-I monomerization during catalysis, NOS-I was incubated for different time points (0–30 min; Fig. 2A, open circles) and then analyzed for dimer content and rate of citrulline formation. Monomerization of NOS-I was found to occur mainly during the first 10 min of l-arginine turnover. Similar results were obtained by low temperature SDS-PAGE under the same incubation conditions, although some monomeric NOS-I was observed also at t = 0 min (not shown). This was probably due to the presence of SDS in this method of analysis. Under the same conditions, the rate of citrulline formation also rapidly declined and then ceased, suggesting that dimer loss and loss in activity may correlate (Fig. 2B, open circles).

H₄Bip Inhibits NOS Monomerization in Correlation with Activity—To examine the effect of H₄Bip on the time course of NOS-I monomerization, recombinant human NOS-I was incubated as above but in the presence of 2 μM H₄Bip before size exclusion chromatography. At all time points, H₄Bip increased the dimer content (Fig. 2A, closed circles). Similar results were obtained by low temperature SDS-PAGE analysis (not shown). Moreover, in kinetic experiments under the same conditions, the loss of citrulline formation was also greatly inhibited (Fig. 2B, closed circles). The use of higher H₄Bip concentrations to examine whether this would further stabilize the enzyme activity beyond 5 min is complicated, given that high H₄Bip concentrations inhibit NOS-I activity (22). Eventually, NOS-I activity can be abolished with super-saturating H₄Bip concentrations. Thus H₄Bip was added repetitively in lower concentrations in order to retain full catalytic activity as much as possible (see below). The mechanism underlying the inhibitory effect of H₄Bip and a possible antagonism with l-arginine (24) is under investigation. In conclusion, stabilization of the dimers and changes in activity appeared to correlate, although additional mechanisms of inactivation and stabilization of NOS may be involved. Nevertheless, the increase of the dimer/monomer ratio represents a direct, hydrodynamic measurement of an enzyme-stabilizing mechanism of H₄Bip during NOS catalysis.

Pterin-free NOS-I Also Monomerizes in an H₄Bip-inhibitable

M. Pantke and H. H. H. W. Schmidt, unpublished observation.
FIG. 2. NOS-I monomerizes in a time-dependent manner during catalysis. A, before size exclusion chromatography, recombinant NOS-I (17 µg) was incubated for different time points in the absence (open circles) or presence (closed circles) of 2 µM H₄Bip as described under "Experimental Procedures." NOS-I monomerizes in a time-dependent manner and the addition of H₄Bip markedly reduced this, most prominently during the first 15 min. At later time points, mixed effects (i.e. formation of both reactive nitrogen and oxygen species) take place (see Footnote 3) which may explain the destabilization of the dimer even in the presence of H₄Bip. Each symbol represents the mean ± S.E. of n = 3–7 individual experiments. B, recombinant human NOS-I (0.6 µg) was incubated for different time points in the absence (open circles) or presence (closed circles) of 2 µM H₄Bip. Thereafter, NOS activity was determined as described under "Experimental Procedures." In the presence of H₄Bip, the rapid loss of L-citrulline formation was inhibited. C, pterin-free recombinant rat NOS-I (11 µg) was incubated for 15 min in the absence (dashed line) and presence (solid line) of 100 µM H₂Bip and subsequently analyzed by size exclusion chromatography as described under "Experimental Procedures." The addition of H₂Bip to the reaction mixture markedly inhibited the formation of NOS-I monomers, indicating again a dimer stabilizing function for H₂Bip. Each chromatogram is representative of three individual experiments yielding similar results.

Manner—As NOS-I expressed in a baculovirus/Sf9 expression system contains substoichiometric amounts of endogenously bound H₄Bip (63% in the preparation used in this study), we were also interested in analyzing pterin-free NOS-I, i.e. expressed in E. coli (33). Monomerization of pterin-free NOS-I was examined by incubating 11 µg of enzyme for 15 min under catalytic conditions (i.e. all cofactors and 0.75 mM L-arginine) in the absence or presence of 100 µM H₂Bip. Thereafter size exclusion chromatography was performed as described above. E. coli-derived NOS-I that had not been incubated was entirely dimeric. Upon 15 min of L-arginine turnover, a prominent monomer peak appeared (Fig. 2C, dashed line), suggesting that pterin-free NOS monomerizes and inactivates after a few rounds of catalysis similar to Sf9 cell-derived NOS-I. When H₂Bip was included in the incubation mixture, monomerization was markedly reduced (Fig. 2C, solid line) suggesting again a dimer stabilizing role of H₂Bip also for pterin-free NOS-I.

Effect of Anti-pterins on the NOS-I Structure, Pterin Content, and Activity—To examine whether anti-pterins mimic the stabilizing effect of H₂Bip on NOS, monomerization of NOS-I during catalysis was investigated in the absence or presence of the anti-pterins PHS-32 and 4-aminotetrahydrobiopterin (PHS-203). These compounds have recently been shown to selectively bind to the H₂Bip-binding site of NOS, displace enzyme-bound H₂Bip, and inhibit H₂Bip-stimulated NOS activity (22, 44). In the presence of PHS-32, monomerization was inhibited at t = 15 min (a typical chromatogram is given in Fig. 3A, solid line), and the H₂Bip content in the dimer fractions was greatly reduced (Fig. 3B). These latter data are consistent with our previous observation that PHS-32, which has a simi-
lar K_p as H_4Bip,^3 displaces enzyme-associated H_4Bip almost completely (22) and, thus, readily occupies the occupied high affinity (45) pterin-binding site of dimeric NOS-I. This interaction was reversible since the addition of 2 μM H_4Bip after size exclusion chromatography in the subsequent citrulline assay restored NOS activity to 86 ± 2% of control. Interestingly, both anti-pterins (at 100 μM) increased dimer stability during l-arginine turnover analogous to H_4Bip (at 2 μM; Fig. 4, closed bars). PHS-203 was even more effective than the natural cofactor. However, in contrast to H_4Bip, which stimulates NOS activity, the anti-pterins had no effect on NOS activity in the absence of H_4Bip (Fig. 4, open bars). Thus dimer stabilization alone is not sufficient to maintain maximal activity and cannot fully explain the mechanism of action of H_4Bip.

Resubstitution of NOS with H_4Bip Enhances Subsequent Activity—To examine whether such hypothetically dimeric and inactive enzyme was due to the source of NOS from a recombinant expression system, we performed kinetic studies with native NOS-I purified from porcine cerebellum. NOS activity of this preparation was found to cease after only 3 min of L-arginine turnover (Fig. 6A). Interestingly, the earlier H_4Bip was added to the incubation mixture, the more pronounced was the effect on subsequent citrulline formation (Fig. 5B). These data suggest that there is a population of NOS-I which is reversibly inactivated during l-arginine turnover and can be reactivated upon addition of H_4Bip. Consistent with our data using recombinant human NOS-I, the anti-pterin PHS-32 (in the absence of H_4Bip) had no effect on the initial velocity of catalysis under basal conditions and only marginally affected the subsequent loss of porcine NOS-I activity (Fig. 5A). However, in order to maintain maximal NOS activity for more than 15 min, H_4Bip had to be added repetitively every 15 min (Fig. 5C). This suggested that during l-arginine turnover, which generated about 5 μM reactive nitrogen oxide (RNS) species, initially saturating levels of H_4Bip (2 μM) progressively decreased to suboptimal levels. As mentioned above, higher concentrations of H_4Bip (>100 μM) could not be tested.

Loss of NOS-associated H_4Bip during Catalysis—To further analyze the observed loss of H_4Bip during l-arginine turnover, we examined chemical interactions between H_4Bip and NOS-derived reactive species. While a role for reactive oxygen species (ROS) has already been suggested,^3 we here focused on the possible involvement of RNS. These results were compared with the non-enzymatic decomposition of reagent H_4Bip (in the absence of NOS). Under NOS assay conditions, reagent H_4Bip was found to be surprisingly stable (Fig. 6A, open circles). In contrast, during the first minutes of l-arginine turnover, NOS-associated H_4Bip not only rapidly dissociated from the enzyme...
into solution but the total recovery of H$_4$Bip, i.e. enzyme-associated plus dissociated, was increasingly incomplete and decreased to a much greater extent (Fig. 6B) than expected from the chemical stability of H$_4$Bip (see Fig. 6A). The observed dissociation of H$_4$Bip from NOS-I appeared to coincide with the loss in dimer (see Fig. 2A). It is unlikely that this loss in total H$_4$Bip was due to autodestruction because neither concomitant dihydrobiopterin nor bioprotein generation, which would have been expected in the case of oxidative breakdown, was observed. Rather, these data suggested that activated NOS plays a direct role in the decomposition of enzyme-associated H$_4$Bip to a product distinct from H$_2$Bip and bioprotein.

**NO and H$_4$Bip Interactions**—To examine whether the apparent loss of total pterin during l-arginine turnover could be explained in part by a reaction of H$_4$Bip with NO, we measured the recovery of reagent H$_4$Bip when incubated with NO in amounts that were similar to those which could be formed during l-arginine turnover. Indeed, in the presence of NO, H$_4$Bip decreased to 59 ± 4.5% of control (Fig. 6A, closed circles). Similarly, when reagent H$_4$Bip was incubated in the same manner with NO$^-$ derived from Angeli’s salt (a model compound for alternative arginine-derived products from NOS; Ref. 37), the H$_4$Bip loss was even more pronounced (Fig. 6A, closed squares). To further confirm our hypothesis, we electrochemically monitored the release of NO from the NO donor compound spermine NONOate (37). When a steady state NO signal was reached, H$_4$Bip was added (Fig. 6C, arrow at t = 0 min) upon which the NO signal was abolished. With respect to the mechanism of interference between NO and H$_4$Bip, the formation of superoxide during H$_4$Bip autoxidation and the subsequent diffusion-limited reaction of NO with superoxide to peroxynitrite (46) has been suggested. However, recently published EPR data arguing against superoxide formation from the mechanism of interference between NO and H$_4$Bip (47) make this mechanism unlikely. Thus, the mechanism of interaction remains open and may in fact involve a direct interaction between NO and H$_4$Bip.

**Exogenous NO Decreases Enzyme-associated H$_4$Bip and NOS Dimer Content**—To test our hypothesis that, in addition to ROS, RNS could also contribute to NOS dimer destabilization, the effects of reagent NO, generated by the NO donor compound spermine NONOate, on both NOS-associated H$_4$Bip and dimer content were examined. Recombinant human NOS-I (17 µg, that would generate a flux of 64 µM NO min$^{-1}$ under catalytic conditions) was exposed for 2 min to 10 mM spermine NONOate (generating, under our conditions, a flux of approximately 91 µM NO min$^{-1}$) under non-catalytic conditions (i.e. in the absence of NADPH and l-arginine) and assayed for pterin content (see “Experimental Procedures”). In control experiments, NOS-I was incubated under identical conditions but in the absence of spermine NONOate. In the presence of spermine NONOate, the amount of enzyme-associated H$_4$Bip decreased to 58 ± 10% of control (Fig. 7, open bars). A further 10 ± 3% of total H$_4$Bip dissociated from NOS-I and were recovered in the solute (Fig. 7, hatched bar). Thus the total recovery of H$_4$Bip (enzyme-associate and free) was lowered to 68% of control. This suggests that NO rapidly diffuses in the catalytic center of NOS-I and then reacts with bound H$_4$Bip to a yet unknown derivative, which thereafter dissociates from the enzyme.

Additionally, the effects of reagent NO on the quaternary structure of NOS-I were investigated. Similar to the dimer loss that was induced in NOS-I during l-arginine turnover (21 ± 2% after 2.5 min; see Fig. 2A), exposure to reagent NO also significantly decreased the dimer content of quiescent NOS-I by 16 ± 3% (after 2 min; Fig. 7, closed bar). Almost identical results were observed when the effects of NO on pterin-free rat NOS-I (11 µg) were studied. Here, the dimer content was lowered by 12 ± 3%. Collectively, these data suggest that pterin loss and destabilization of the quaternary structure are mechanistically linked.

**DISCUSSION**

The finding that dimeric NOS-I time dependently monomerizes during catalysis (Scheme I) is novel and may be important in the regulation of NO, RNS, and ROS synthesis. To date, purified native NOS-I has only been observed in its dimeric state (5, 35) and monomerization was shown to occur in the presence of a 10-kDa protein inhibitor of neuronal NOS, PIN (48), although recent evidence questions this finding (49). NOS also monomerizes in the presence of protein denaturants. For example, treatment of NOS-II with 2 M urea causes unfolding of the NOS reductase domain and flavin release; a higher concentration of urea (5 M) is required to cause subunit dissociation (50, 51). Moreover, SDS induces NOS-I monomerization associated with a dramatic increase in α-helical structure (35). In contrast to these studies, the present analysis using native NOS describes the appearance and detection of inactive, monomeric enzyme during l-arginine turnover in the absence of PIN and protein denaturants. In agreement with our findings, Stuehr’s (31) laboratory found that only dimeric NOS-II is active with respect to l-citrulline formation.

Monomerization and inactivation of NOS-I was paralleled by a loss of enzyme-associated H$_4$Bip. While the addition of H$_4$Bip largely inhibited monomerization, also of pterin-free NOS-I, we were not able to achieve reactivation of monomerized NOS-I. In contrast, NOS-II monomers generated by exposure to 5 M urea reassemble upon preincubation with l-arginine and H$_4$Bip to form active enzyme (51). Thus, the underlying mechanism by which H$_4$Bip stabilizes the NOS-I dimer complex is unclear and appears to be isomer-specific. H$_4$Bip may act as a molecular clip to prevent NOS-I subunit dissociation, as Crane et al. (23) recently suggested that H$_4$Bip might induce a large conformational change upon binding to the NOS-II oxygenase domain. However, recent structural data argue against such a mechanism as the H$_4$Bip-free and containing structures of the NOS-III oxygenase domain show no major differences (24). Thus, H$_4$Bip may alternatively prevent uncoupled catalysis and the formation of ROS (30) which otherwise destabilize NOS-I dimers. Nevertheless, the present findings are consistent with the stabilizing effect of H$_4$Bip on SDS-denatured NOS-I (35) and therefore provide a means to examine structural interactions between H$_4$Bip and native NOS-I during l-arginine turnover.

It is still controversial whether NOS-II (13, 31, 51, 52) and NOS-III (53–57) require H$_4$Bip for the initial subunit assembly. However, this can be excluded for NOS-I (32, 33, 58). In contrast, our data suggest that the intracellular pterin content may regulate NOS-I dimer dissociation during catalysis after the initial, H$_4$Bip-independent subunit assembly during protein synthesis.

The stabilizing effect of H$_4$Bip was mimicked by anti-pterins. Given that these compounds are effective inhibitors of NOS, this hints to a partial dissociation of dimer stability and activity. We therefore suggest that there are distinct conformations of the NOS-I dimer (Scheme I, modified from Ref. 13): active (green) and inactive (red) with respect to l-arginine turnover. As we have previously shown, NOS is able to convert l-arginine even in the absence of added H$_4$Bip. This basal active enzyme was termed NOS* (Ref. 22; see Scheme I). Interestingly, this enzyme is not affected by the type of anti-pterins used in the present study despite the fact that these compounds effectively displace enzyme-associated pterin. However, NOS* rapidly inactivates during catalysis, possibly due to autoinactivation by NOS-derived NO or ROS. The resulting species, NOS* (III in
during catalysis. Alicate. Blicate. Clicate. Dicate. Eicate. Ficate. Gicate. Hicate. Iicate. Jicate. Kicate. A. Tetrahydrobiopterin in NO Synthase

FIG. 6. Chemical stability and loss of NOS-associated pterin during catalysis. A, chemical decomposition of H$_4$Bip. H$_4$Bip (2 µM) was incubated in the absence of NOS but otherwise under NOS assay conditions (i.e., 50 µM l-arginine, 1 mM NADPH, 5 µM FAD, 10 µM FMN, 500 mM CaM, 1 mM CaCl$_2$, 250 µM CHAPSO) either in the absence (open circles) or presence of 100 µM NO (closed circles) or 150 µM NO$^-$ (squares). NO$^-$ was applied by using Angeli’s salt; the source of NO was a NO-saturated solution. At the time points indicated, total pterin was measured as described. NO as well as NO$^-$ reduced total pterin recovery in a time-dependent manner whereas H$_4$Bip remained stable in the control experiments. At all incubation time points, neither H$_2$Bip nor biopterin could be detected. During catalysis, enzyme-bound H$_4$Bip was rapidly lost due to chemical decomposition of H$_4$Bip. H$_4$Bip (2 µM) or presence of 100 µM NO min$^{-1}$ which is similar to the NO flux that would be generated during l-arginine turnover by the amount of NOS-I used (64 µM NO min$^{-1}$). Therefore, the samples were analyzed for pterin content and dimer content as described under “Experimental Procedures.” Open bars indicate the amount of enzyme-associated H$_4$Bip, hatched bars H$_4$Bip in the solute, and closed bars the loss of NOS-I dimers expressed in % of control. The addition of spermine NONOate markedly reduced enzyme-bound pterin; furthermore, NOS-I dimers were decreased indicating a corresponding destabilization of the enzyme’s quaternary structure. Values represent mean ± S.E. of four independent experiments, each performed in triplicate.

B. Total recovery of NOS-associated pterin was lowered during l-arginine turnover. Native porcine NOS-I (17 µg) was incubated for 2 min at 37 °C in a total volume of 100 µl of 50 mM TEA buffer (pH 7.4) containing 1 µM CaM, 1 mM CaCl$_2$, and in the absence (control) or presence (NO) of 10 mM spermine NONOate. The NO flux rate, under these conditions, was approximately 91 µM NO min$^{-1}$ which is similar to the NO flux that would be generated during l-arginine turnover by the amount of NOS-I used (64 µM NO min$^{-1}$). Therefore, the samples were analyzed for pterin content and dimer content as described under “Experimental Procedures.” Open bars indicate the amount of enzyme-associated H$_4$Bip, hatched bars H$_4$Bip in the solute, and closed bars the loss of NOS-I dimers expressed in % of control. The addition of spermine NONOate markedly reduced enzyme-bound pterin; furthermore, NOS-I dimers were decreased indicating a corresponding destabilization of the enzyme’s quaternary structure. Values represent mean ± S.E. of three individual experiments. The asterisk indicates a significant difference from control value (p < 0.05).

Scheme I, is at first reversibly inactive and can be re-activated by adding exogenous H$_4$Bip to obtain a maximally active state of NOS-I. Otherwise, III converts to an irreversibly inactive dimer (IV), which subsequently monomerizes (V). Even the time point of H$_4$Bip addition to basally active enzyme during l-arginine turnover appears to be important in determining subsequent enzyme activity. The later the addition took place, the lower was the subsequently recoverable activity suggesting that increasing amounts of III had converted to irreversibly inactive NOS species, i.e. IV or V.

As depicted in Scheme I, it may also be obtained from II by adding exogenous H$_4$Bip, resulting not only in an increase in $V_{max}$ but also in stability. At present, we attribute this stabilizing effect of H$_4$Bip to its capability to scavenge autoinhibitory products from NOS, i.e. ROS$^3$ and RNS. The mechanism of this inactivation of NOS remains to be established; however, since NOS contains essential protein thiols within the catalytic center (12, 25), thiol oxidation is one candidate target. During enzyme isolation, unbound pterin is removed so that the pterin content of NOS-I dimers is rapidly scavenged by H$_4$Bip. The NO signal then immediately ceased correlating well with the disappearance of H$_4$Bip upon exposure to NO$^-$ (A).
when compared between different studies. Anti-pterins compete with exogenous H4Bip to prevent the formation of I. However, they do not affect L-arginine turnover by II and apparently inhibit the monomerization, but not the inactivation process. Thus, in the presence of anti-pterins NOS-I is locked in stage II-IV.

During L-arginine turnover, we observed a progressive loss of enzyme-associated pterin as detected by our HPLC method. Moreover, reagent as well as enzyme-bound H4Bip was incompletely recovered when incubated with NO or NO2. Furthermore, reagent NO was scavenged when incubated with H4Bip. This raises the possibility that NOS-associated H4Bip reacts with ROS/RNS catalysis products in a similar manner, which may therefore account for at least some of the loss of H4Bip during catalysis. Further spectroscopic studies aimed at the identification of the product of H4Bip with RNS/ROS and the H4Bip metabolite generated in NOS during L-arginine turnover are presently under way.

The present findings may have important implications for the regulation of NOS-I depending on intracellular H4Bip concentrations. In situations where both NO and H4Bip synthesis are high, e.g., stimulated macrophages (59), NOS is likely to be replenished with H4Bip. However, at H4Bip levels of resting cells, constitutive NOS may become H4Bip deficient, especially if turnover is occurring in neighboring NOS molecules. Interestingly, H4Bip has been reported to prevent NOS from an autoinactivation process by NO (27) as well as causing oxidation of NO to ONOO- in the presence of superoxide (46). Monomerization of NOS-I then could contribute to the prevention of harmful peroxynitrite production, which occurs at subsaturating H4Bip levels (60).

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