Allosteric modulation of rat brain nitric oxide synthase by the pterin-site enzyme inhibitor 4-aminotetrahydrobiopterin

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We investigated the functional and allosteric effects of the 4-amino analogue of tetrahydrobiopterin, (6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine (4-amino-H_4biopterin) on pteridine-free rat neuronal nitric oxide synthase. In the presence of added (6*R*)-5,6,7,8-tetrahydro-L-erythrobiopterin (H_4biopterin; 10 μM), 4-amino-H_4biopterin completely inhibited the conversion of both L-arginine and N^G-hydroxy-L-arginine with half-maximally effective concentrations of 1.1 ± 0.09 and 1.3 ± 0.09 μM, respectively. Inhibition was reversible, as shown by a time-dependent restoration of citrulline formation upon dilution of the inhibitor-treated enzyme ($t_{1/2}$ = 3.0 min). Binding of 4-amino-H_4biopterin led to a complete conversion of the haem from low-spin to high-spin state, and to the formation of stable homodimers which partially survived electrophoresis under denaturating conditions. These results show that oxidation of both L-arginine and N^G-hydroxy-L-arginine is pteridine-dependent, and that the allosteric effects of H_4biopterin do not fully explain the essential role of the pteridine cofactor in nitric oxide biosynthesis.

INTRODUCTION

Nitric oxide is formed from the guanidino group of L-arginine by nitric oxide synthases (NOSs) (EC 1.14.13.39) [1,2]. The neuronal NOS (type I) (nNOS), and the endothelial isoform, are constitutively expressed and require micromolar free Ca^{2+} for activity, whereas the isoform first described in murine macrophages is cytokine-inducible and Ca^{2+}-independent. The enzymes consist of a catalytic oxygenase domain, containing a prosthetic haem group, and a flavin-containing reductase domain, shuttling reducing equivalents from the co-substrate NADPH to the haem.

Unlike other cytochrome *P*-450s, all NOS isoforms require (6*R*)-5,6,7,8-tetrahydro-L-erythrobiopterin (H_4biopterin) as a co-factor, but the function of the pteridine in nitric oxide (NO) biosynthesis is not well understood [3]. It may play a specific role as a reactant in the enzymic hydroxylation of L-arginine to N^G-hydroxy-L-arginine, and/or the subsequent oxidation of the hydroxylated intermediate to NO and L-citrulline [4–7]. However, the allosteric effects of H_4biopterin binding, resulting in profound changes in protein conformation, are more obvious and have been studied thoroughly in the past. Presence of the pteridine cofactor was shown to be essential for dimerization of inducible NOS [8] and for formation of stable nNOS dimers [9]. Since NOS dimerization is dependent critically on the presence of haem [8,10,11], the effect of H_4biopterin on subunit assembly may be a consequence of the low-spin-to-high-spin conversion that has been observed upon titration of pteridine-free NOS with H_4biopterin [12–14].

On the basis of the close correlation in the time course of haem spin conversion and enzyme activation [14], it is conceivable that the interaction with the haem and/or stabilization of protein dimers are crucial events that might fully explain the pterin dependence of NOS. To test this hypothesis, we investigated whether enzyme inhibition by the pterin-site NOS inhibitor (6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine (4-amino-H_4biopterin; also known as 4-amino-4-desoxy-H_4biopterin) [15,16] is due to the inability of the antagonist to produce the allosteric effects observed with the natural cofactor H_4biopterin.

MATERIALS AND METHODS

Materials

Rat nNOS containing less than 0.1 equivalent of H_4biopterin was obtained from recombinant baculovirus-infected Sf9 cells as described [14,17,18]. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was from Amersham, purchased through MedPro (Vienna, Austria). N^G-Hydroxy-L-[2,3,4,5-^{3}H]arginine was prepared enzymically from radioactively labelled L-arginine, as described [19]. The EMT-6 cell line was kindly provided by Drs. Jean-Pierre Tenu and Michel Lepoivre (University of Paris, France). Pteridines were from Dr. B. Schircks Laboratories, (Jona, Switzerland), and unlabelled N^G-hydroxy-L-arginine from ALEXIS, Läufelfingen, Switzerland. Other chemicals were from Sigma.

Determination of enzyme activity

NOS activity was determined as the formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine [20]. Incubations were for 10 min at 37 °C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.2 μg of nNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (approx. 60000 c.p.m.), 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, nNOS (0.1 μM; 0.16 mg/ml) was preincubated at 4 °C for 10 min in the presence of 1 mM L-arginine with or without 10 μM 4-amino-H_4biopterin, followed by 10-fold dilution of the samples and determination of L-citrulline formation at 37 °C for 1–30 min with 0.1 mM H_4biopterin. To obtain the apparent dissociation rate constant K_d, the data were fitted for t = 0.1–30 min according

Abbreviations used: (n)NOS, (neuronal) nitric oxide synthase (type 1); NO, nitric oxide; H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-erythrobiopterin [also known as (6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pterin]; 4-amino-H_4biopterin, (6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1,2-dihydroxypropyl)pteridine; IC_{50}, concentration producing half-maximal inhibition.

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to $(p_I/p_0)*100 = \{[1+\exp(-K_d*t)-1]/(K_d*t)\}*c$ with p_I and p_0 denoting product formation at time t in the presence and absence of the inhibitor, respectively, and the maximal relative activity $(p_I/p_0)*100$ at $t = \infty(c)$ set at 70 % to account for the residual presence of the inhibitor in the final assay mixture.

Reconstitution of pteridine-free nNOS with 4-amino-H_4biopterin

Aliquots of 0.20 ml containing approx. 0.25 mg of nNOS were incubated with 0.2 mM 4-amino-H_4biopterin in the presence of 1 mM L-arginine at ambient temperature for 30 min and then subjected to gel-filtration chromatography, as described [10], followed by the determination of 4-amino-H_4biopterin, protein and enzyme activity. 4-Amino-H_4biopterin was determined by acidic oxidation and HPLC using the method described for the analysis of NOS-bound H_4biopterin [21]. Calibration curves were established with 50–500 nM authentic 4-amino-H_4biopterin.

PAGE

Formation of stable nNOS dimers was analysed by low-temperature SDS/PAGE as described [9]. The protein was incubated for 5 min at 37 °C in 50 μl of 50 mM triethanolamine/HCl buffer (pH 7.4) in the absence or presence of H_4biopterin or 4-amino-H_4biopterin (0.2 mM each) and L-arginine (1 mM) as indicated. Incubations were terminated by the addition of 50 μl of chilled Laemmli buffer [22] containing 0.125 M Tris/HCl (pH 6.8), 4 % (w/v) SDS, 20 % (w/v) glycerol and 0.02 % (w/v) Bromophenol Blue. For complete denaturation, the protein was heated to 95 °C for 5 min in Laemmli buffer. Samples containing approx. 6 μg of protein were subjected to SDS/PAGE for 90 min at 120 V on discontinuous 6 % SDS slab gels (70 × 80 × 1 mm). Gels and buffers, prepared according to Laemmli [22], were equilibrated at 4 °C and the buffer tank was cooled during electrophoresis in an ice bath. Gels were stained for protein detection with Coomassie Blue and densitometrically analysed using the VDS 800 video system and H1D Software of Hirschmann (Hirschmann, Taufkirchen, Germany).

Optical measurements

Absorbance spectra were recorded at ambient temperature with a Hewlett–Packard 8452A Diode Array Spectrophotometer. nNOS stock solutions were diluted with 50 mM triethanolamine/HCl buffer (pH 7.0) to approx. 4.7 μM and incubated for up to 2 h in the presence of H_4biopterin or 4-amino-H_4biopterin (10 μM each). The kinetics of the low-spin-to-high-spin transition were measured as the change in the peak-to-trough absorbance differences at 394 and 424 nm.

RESULTS AND DISCUSSION

Pteridine-free nNOS incubated with 10 μM exogenous H_4biopterin catalysed formation of L-citrulline from L-arginine and N^G-hydroxy-L-arginine with specific activities of 0.43 ± 0.026 and 0.52 ± 0.012 μmol mg^{-1} per min, respectively (means ± S.E., $n = 3$]. 4-Amino-H_4biopterin inhibited the oxidation of both substrates with similar potency [$IC_{50} = 1.1 \pm 0.09$ μM (arginine) and 1.3 ± 0.09 μM (N^G-hydroxy-L-arginine); mean ± S.E.M., $n = 3$] (Figure 1A). Whereas the presence of 10 μM 4-amino-H_4biopterin almost completely inhibited the pteridine-free enzyme (see Figure 1), the inhibitor reduced the activity of conventional nNOS preparations containing 0.4–0.5 equivalents of endogenous H_4biopterin per subunit only by 40–50 % [15], supporting our previous suggestion that H_4biopterin is not readily displaced from its NOS-binding site by exogenous ligands [23]. The restoration of activity upon dilution of the enzyme that had been pretreated with 4-amino-H_4biopterin (Figure 1B) showed that the effect of 4-amino-H_4biopterin was reversible with a $t_{\frac{1}{2}}$ of about 3.0 min at 37 °C. The fact that the enzyme activity did not recover completely from inhibition (approx. 65 % of the controls) is probably explained by the presence of residual 4-amino-H_4biopterin (1 μM) in the final assay mixtures. The calculated K_d(app) of 0.23 ± 0.031 min^{-1} indicates that dissociation of the 4-amino derivative may be faster than that of H_4biopterin [14,23], with the high affinity of the inhibitor resulting mainly from its rapid association to the protein.

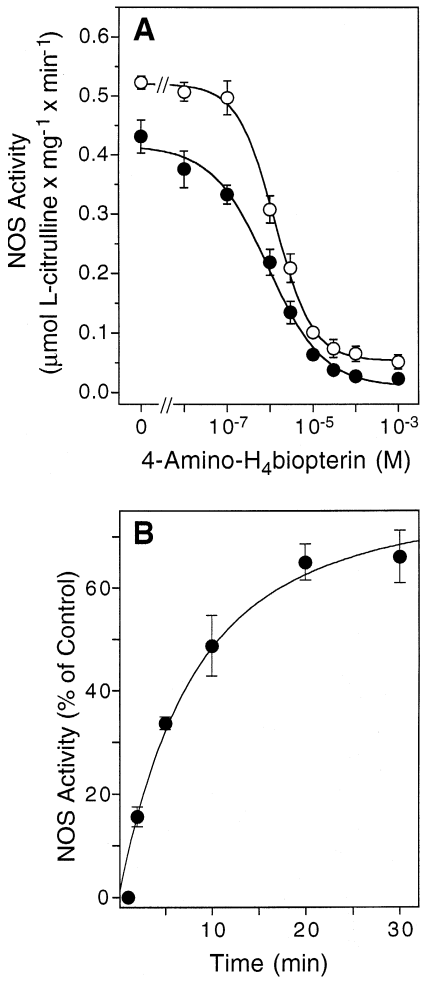

Figure 1 Reversible inhibition of pteridine-free nNOS by 4-amino-H_4biopterin

(**A**): The concentration-dependent inhibition by 4-amino-H_4biopterin of L-citrulline formation from L-arginine and N^G-hydroxy-L-arginine is shown. Incubations were for 10 min at 37 °C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 0.2 μg of nNOS, 0.1 mM L-[2,3,4,5-^{3}H]arginine (approx. 60000 c.p.m.; ●) or N^G-hydroxy-L-[2,3,4,5-^{3}H]arginine (approx. 20000 c.p.m; ○), 10 μM H_4biopterin, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.2 mM CHAPS and 4-amino-H_4biopterin, as indicated. Data were fitted according to the Hill equation (means ± S.E.M., $n = 3$). (**B**): The reversibility of inhibition is shown. nNOS (0.1 μM) was preincubated at 4 °C for 10 min in the presence of 1 mM L-arginine with or without 10 μM 4-amino-H_4biopterin, followed by 10-fold dilution of the samples and determination of L-citrulline formation at 37 °C for the indicated periods of time. The data expressed, as percentages of controls preincubated in the absence of 4-amino-H_4biopterin, were fitted as described in the Materials and methods section (means ± S.E.M., $n = 3$).

The tightness of binding of 4-amino-H_4biopterin to nNOS was studied by preincubation of the pteridine-free enzyme with the inhibitor, followed by gel-filtration chromatography at ambient

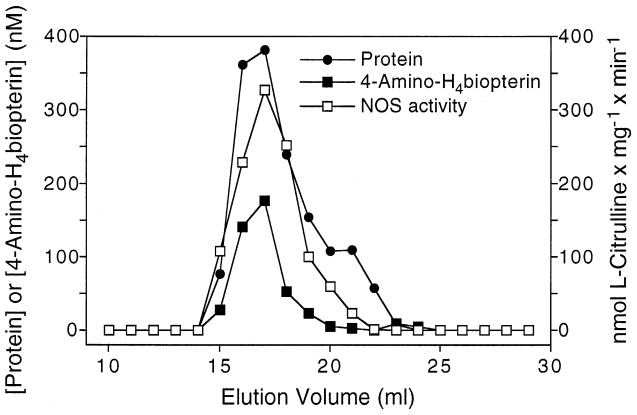

Figure 2 Reconstitution of nNOS with 4-amino-H_4biopterin

Aliquots of 0.20 ml containing approx. 0.25 mg of nNOS were incubated with 0.2 mM 4-amino-H_4biopterin in the presence of 1 mM L-arginine at ambient temperature for 30 min and then subjected to gel-filtration chromatography on a Superose 6 column (HR 10/30, Pharmacia Biotech, Vienna, Austria), followed by analysis of the eluate for protein (●), 4-amino-H_4biopterin (■) and L-citrulline formation (□), as described in the Materials and methods section. The chromatogram shown is representative of four separate experiments.

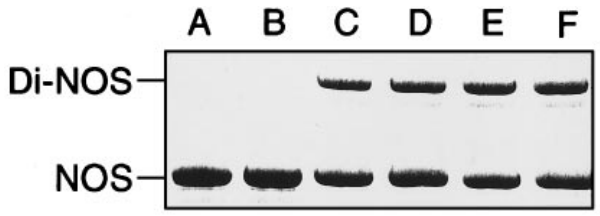

Figure 3 Stabilization of nNOS dimers by H_4biopterin and 4-amino-H_4biopterin

Purified nNOS (1.5 μM) was preincubated at 37 °C for 5 min in a 50 mM triethanolamine/HCl buffer (pH 7.0) with H_4biopterin or 4-amino-H_4biopterin (0.2 mM each) in the absence or presence of L-arginine (1 mM), followed by low-temperature SDS/PAGE analysis on 6% slab gels and protein staining with Coomassie Blue. From left to right, lanes shown are as follows: lane A, boiled; lane B, unboiled; lane C, H_4biopterin; lane D, 4-amino-H_4biopterin; lane E, H_4biopterin plus L-arginine; and lane F, 4-amino-H_4biopterin plus L-arginine. NOS and Di-NOS refer to nNOS monomers and dimers with apparent molecular masses of approximately 160 kDa and 320 kDa, respectively. The gel shown is representative of three separate experiments.

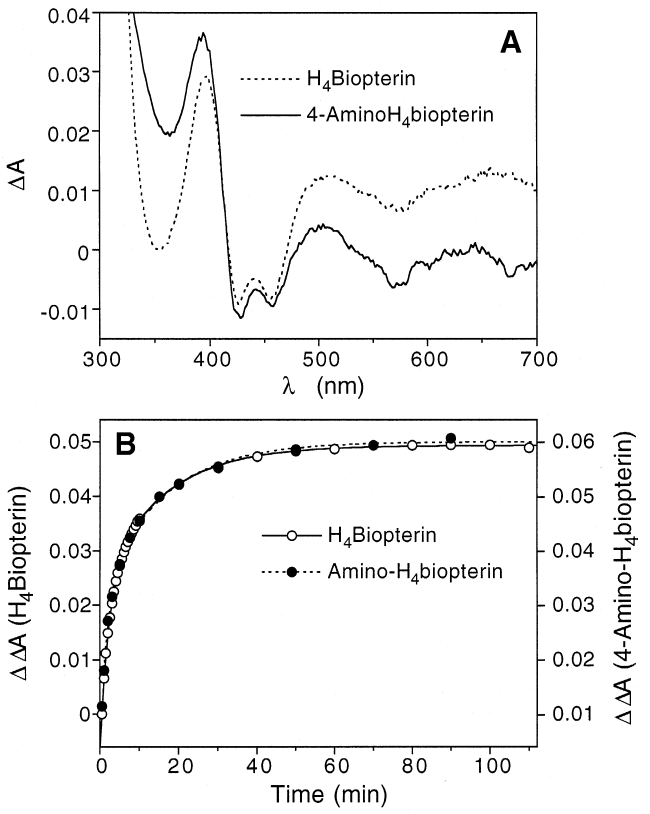

Figure 4 Spectral perturbations caused by H_4biopterin and 4-amino-H_4biopterin

Purified nNOS (2.5 μM) was preincubated in 50 mM triethanolamine/HCl buffer (pH 7.4) for the indicated periods of time at ambient temperature with pteridines as indicated. (**A**): Absorbance difference spectra of nNOS preincubated for 10 min with 10 μM H_4biopterin (dotted line) or 10 μM 4-amino-H_4biopterin (solid line). The absorbance spectrum of control incubations in buffer alone was subtracted from the spectra obtained with pteridine-treated preparations. (**B**): The kinetics of low-spin-to-high-spin conversion induced by H_4biopterin (○) or 4-amino-H_4biopterin (●) at ambient temperature was measured as the change of the peak-to-trough absorbance difference between 392 and 424 nm, and plotted as a function of the incubation time by fitting according to the sum of two first-order reactions (for kinetic details, see [14]). One representative experiment is shown out of three performed.

temperature and analysis of the eluted protein for the bound 4-amino compound. As shown in Figure 2, the pretreated protein eluted as a single peak with a hydrodynamic radius of 8.1 nm corresponding to dimeric nNOS [10]. The peak fraction contained 0.43 ± 0.03 equivalents of 4-amino-H_4biopterin, indicating that the inhibitor binds to the high-affinity pteridine site of nNOS similarly to the native cofactor [10]. NOS activity, assayed in each fraction as the formation of L-citrulline in the presence of 10 μM exogenous H_4biopterin, was about 80% (0.33 μmol mg^{-1} per min) of controls preincubated with buffer instead of 4-amino-H_4biopterin before gel-filtration chromatography. These results further corroborate that binding of 4-amino-H_4biopterin to nNOS is reversible.

We have shown previously that presence of H_4biopterin results in formation of highly stable nNOS dimers resistant to SDS/PAGE [9]. To study whether this remarkable effect on dimer stability explains the pterin requirement of NO synthesis, the effect of 4-amino-H_4biopterin was investigated in low-temperature SDS/PAGE experiments. As shown in Figure 3, the protein migrated mainly as the 160 kDa monomer when it had been either boiled or incubated at 37 °C without additions before electrophoresis (lanes A and B, respectively), whereas preincubation in the presence of H_4biopterin (lane C) or 4-amino-H_4biopterin (lane D) led to the appearance of an approx. 320 kDa band. Preincubation in the additional presence of L-arginine slightly enhanced the effects of both pteridines (lanes E and F). Thus the low-temperature SDS/PAGE experiments clearly demonstrated that H_4biopterin and its inhibitory 4-amino analogue both stabilize nNOS dimers, suggesting that this effect of H_4biopterin is not sufficent to boost NO synthesis from L-arginine.

It is well documented that H_4biopterin shifts the haem in pteridine-free NOS from low-spin to high-spin state [12–14]. The high-spin state of the haem is essential for the enzymic function of NOS and other cytochrome *P*-450s [14,24,25], suggesting that the allosteric interaction of the pteridine cofactor with the haem

might be the factor associated with H_4biopterin that leads to NOS activation. However, Figure 4(A) shows that H_4biopterin and 4-amino-H_4biopterin both led to spectral perturbations that are typical for low-spin-to-high-spin transitions of cytochrome *P*-450s. As described previously [14], spin conversion of nNOS occurred in a biphasic manner and the rates were independent of the pteridine concentrations (results not shown). From kinetic data (see Figure 4B for a representative experiment), we have calculated first-order rate constants of 0.45 ± 0.02 min^{-1} ($t_{1/2} =$ 1.54 min) and 0.58 ± 0.07 min^{-1} ($t_{1/2} = 1.20$ min) ($n = 3$ each) for the fast ($\leqslant 10$ min) transitions caused by H_4biopterin and the 4-amino compound, respectively. These data agree well with the haem-spin equilibrium of nNOS reported previously [14].

The present study shows that binding of the potent pterin-site inhibitor 4-amino-H_4biopterin to nNOS leads to allosteric changes of the protein which are indistinguishable from the effects seen with the active cofactor H_4biopterin. Reversible binding of 4-amino-H_4biopterin resulted in stabilization of protein dimers and caused a conversion of the prosthetic haem group from low spin to high spin. Nevertheless, the 4-amino compound did not support NOS activity but, instead, potently inhibited the stimulation of enzyme by H_4biopterin, suggesting that the described allosteric effects of the pteridine cofactor do not fully explain its role in NOS catalysis. With an amino functional group in position C-4 of the pteridine ring, 4-amino-H_4biopterin resembles methotrexate, an established inhibitor of dihydrofolate and dihydropteridine reductases. In fact, 4-amino-H_4biopterin turned out to be an even more potent inhibitor of dihydropteridine reductase than methotrexate [15]. Thus if such an intrinsic reductase activity of NOS was essentially involved in L-arginine oxidation, 4-amino-H_4biopterin may inhibit NO synthesis through interference with pteridine redox-cycling. Although it cannot be excluded that the pterin dependence of NOS is explained by other, as yet unrecognized, effects of H_4biopterin, the results in here further limit the mechanistic possibilities for H_4biopterin-supported NOS catalysis that need further investigation in the future.

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