Contrasting effects of N^5-substituted tetrahydrobiopterin derivatives on phenylalanine hydroxylase, dihydropteridine reductase and nitric oxide synthase

Ernst R. WERNER*[1], Hans-Jörg HABISCH†, Antonius C. F. GORREN†, Kurt SCHMIDT†, Laura CANEVARI‡, Gabriele WERNER-FELMAYER* and Bernd MAYER†

*Institute for Medical Chemistry and Biochemistry of the University of Innsbruck, Fritz-Pregl-Str. 3, A-6020 Innsbruck, Austria, †Institute for Pharmacology and Toxicology, University of Graz, Universitätsplatz 2, A-8010 Graz, Austria, and ‡Department of Biochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, U.K.

Tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-L-biopterin, H_4 biopterin] is one of several cofactors of nitric oxide synthases (EC 1.14.13.39). Here we compared the action of N^5-substituted derivatives on recombinant rat neuronal nitric oxide synthase with their effects on dihydropteridine reductase (EC 1.6.99.7) and phenylalanine hydroxylase (EC 1.14.16.1), the well-studied classical H_4biopterin-dependent reactions. H_4biopterin substituted at N^5 with methyl, hydroxymethyl, formyl and acetyl groups were used. Substitution at N^5 occurs at a position critical to the redox cycle of the cofactor in phenylalanine hydroxylase/dihydropteridine reductase. We also included $N^{2'}$-methyl H_4biopterin, a derivative substituted at a position not directly involved in redox cycling, as a control. As compared with N^5-methyl H_4biopterin, N^5-formyl H_4biopterin bound with twice the capacity but stimulated nitric oxide synthase to a lesser extent. Depending on the substituent used, N^5-substituted derivatives were redox-active: N^5-methyl- and N^5-hydroxylmethyl H_4biopterin, but not N^5-formyl- and N^5-acetyl H_4biopterin, reduced 2,6-dichlorophenol indophenol. N^5-Substituted H_4biopterin derivatives were not oxidized to products serving as substrates for dihydropteridine reductase and, depending on the substituent, were competitive inhibitors of phenylalanine hydroxylase: N^5-methyl- and N^5-hydroxymethyl H_4biopterin inhibited phenylalanine hydroxylase, whereas N^5-formyl- and N^5-acetyl H_4biopterin had no effect. Our data demonstrate differences in the mechanism of stimulation of phenylalanine hydroxylase and nitric oxide synthase by H_4biopterin. They are compatible with a novel, non-classical, redox-active contribution of H_4biopterin to the catalysis of the nitric oxide synthase reaction.

Key words: biopterin, inhibition, reaction mechanism, redox cycling, stimulation.

INTRODUCTION

The role of tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-L-biopterin, H_4biopterin] in nitric oxide synthase (NOS; EC 1.14.13.39) has attracted attention due to its differences to the well-known function of this pteridine in the phenylalanine hydroxylase reaction [1], and due to the prospects of using this knowledge for inhibitor design. In contrast to phenylalanine hydroxylase (EC 1.14.16.1), NOS is stimulated by much lower concentrations of the cofactor and exhibits a much higher selectivity for the (6*R*) 6-(L-erythro-1,2-dihydroxypropyl) side chain [2,3]. In common with phenylalanine hydroxylase, only tetrahydro- but no dihydroderivatives of pteridines stimulate the reaction [4,5]. In addition to increasing NOS activity, H_4biopterin promotes dimerization of the enzyme [6–8], stimulates the shift of the haem iron from low spin to high spin [9,10], and increases the affinity of the enzyme for the substrate L-arginine [4]. However, these allosteric effects alone are insufficient to explain the role of H_4biopterin in the NOS reaction, since the 4-amino analogue exerts all of these allosteric effects without stimulating the enzyme's activity [10,11]. Experimental results hint at a redox-active role of H_4biopterin in the NOS reaction: the decay of the ferrous oxygen complex of the neuronal NOS (nNOS) oxygenase domain is accelerated by H_4biopterin [12]. Low-temperature UV spectra of nNOS suggest a reductive activation of the ferrous oxygen complex by H_4biopterin [13].

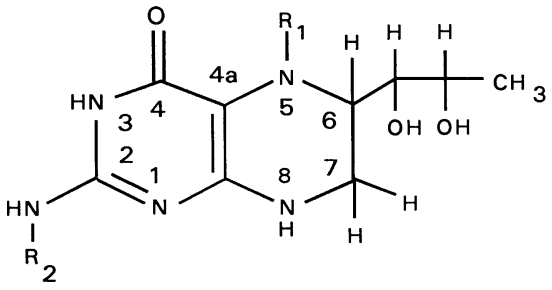

Figure 1 Structural formula of H_4biopterin derivatives used in this study

N^5-substituted derivatives, R_1 = methyl, hydroxymethyl, formyl or acetyl, and R_2 = H. $N^{2'}$-Methyl H_4biopterin, R_1 = H and R_2 = methyl. H_4biopterin ($R_1 = R_2$ = H) leaves the phenylalanine hydroxylase reaction as 4*a*-hydroxy derivative. This 4*a*-hydroxy group together with the hydrogen atom (R_1) at N^5 is then cleaved off as water to yield the quinonoid 6,7-[8H]dihydrobiopterin, which is the substrate of the dihydropteridine reductase reaction [1].

Abbreviations used: H_4biopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; DCPIP, 2,6-dichlorophenol indophenol; NOS, nitric oxide synthase; nNOS, neuronal NOS.

[1] To whom correspondence should be addressed (e-mail ernst.r.werner@uibk.ac.at).

We have shown recently that the N^5-methyl derivative of H_4biopterin stimulates nNOS, although it does not react with molecular oxygen [14]. Here we compare the action of a series of N^5-substituted H_4biopterin derivatives (Figure 1) on phenylalanine hydroxylase, dihydropteridine reductase (EC 1.6.99.7) and nNOS. Substitution at N^5 occurs at a position critical to the redox cycle of the cofactor in phenylalanine hydroxylase/dihydropteridine reductase. As a control, we included $N^{2'}$-methyl H_4biopterin (Figure 1), a derivative substituted at a position not directly involved in redox cycling. We show that N^5-substituted derivatives do not support the dihydropteridine reductase reaction and are inhibitors of phenylalanine hydroxylase. The nNOS stimulatory potential of N^5-substituted H_4biopterin derivatives correlates with their reductive potential, but not with their affinity to nNOS. Our data are compatible with a novel, non-classical, redox-active role of the pterin cofactor in the NOS reaction.

EXPERIMENTAL

Materials

All pteridine derivatives were obtained from Dr Schircks Laboratories, Jona, Switzerland. H_4biopterin content of the novel N^5- and $N^{2'}$-substituted H_4biopterin derivatives was checked by HPLC with electrochemical detection as described in [15]. All N^5-derivatives and the $N^{2'}$-methyl derivative had no detectable H_4biopterin ($< 0.0033\,\%$), except for N^5-hydroxymethyl H_4biopterin, which contained $1.1\,\%$ H_4biopterin. [3′-^{3}H]-H_4biopterin (14 Ci/mmol) was enzymically prepared from [8,5′-^{3}H]GTP as detailed elsewhere [16]. L-[2,3,4,5-^{3}H]Arginine (57 Ci/mmol) was from Amersham Pharmacia Biotech (Uppsala, Sweden). H_4biopterin-free rat nNOS was prepared from baculovirus-infected insect cells treated with 2,4-diamino-6-hydroxypyrimidine as described in [17]. Sheep liver dihydropteridine reductase (100 units/mg) and rat liver phenylalanine hydroxylase (0.069 units/mg) were obtained from Sigma (St.Louis, MO, U.S.A.). All other chemicals were from Sigma or Merck (Darmstadt, Germany).

Determination of phenylalanine hydroxylase activity

Phenylalanine hydroxylase was incubated under conditions modified from [18] and the amount of tyrosine formed was determined by HPLC with fluorescence detection. Briefly, phenylalanine hydroxylase (60 μg/ml) was preincubated with 200 μM phenylalanine for 10 min at 37 °C, and the reaction was started by addition of a mixture of H_4biopterin derivative (10 μM–1 mM) and 10^4 units/ml catalase (Serva, Heidelberg, Germany). After a further 20 min at 37 °C, the reaction was stopped by addition of 10 μl of 1 M HCl. Then, 10 μl were injected on to an RP-18 column (125 × 4 mm, Lichrosphere, 5 μm particle size; Merck) and eluted with 75 mM KH_2PO_4 buffer containing $20\,\%$ (v/v) acetonitrile, $10\,\%$ (v/v) methanol, 0.44 g/l SDS and 1.5 μM EDTA, pH 3.1 (adjusted with 1 M trichloroacetic acid) at a flow rate of 0.8 ml/min. Tyrosine was detected by fluorescence (excitation 285 nm, emission 325 nm) with a detection limit of 2.5 pmol.

Assay for dihydropteridine reductase

Two assays were used. (i) A method based on oxidation of the H_4biopterin derivative by peroxidase/hydrogen peroxide and monitoring of the consumed NADH was updated from [19]. In 200 μl of 50 mM Tris/HCl, pH 7.2, the following components were incubated at 37 °C for 5 min: hydrogen peroxide ($0.003\,\%$), peroxidase (1 unit, Sigma), H_4biopterin derivative (1–35 μM), NADH (150 μM) and dihydropteridine reductase (2 μg/ml). The reaction was started by addition of dihydropteridine reductase and the consumption of NADH was monitored by UV absorption at 340 nm in a microplate reader (Anthos 2001, Anthos, Salzburg, Austria). (ii) The second method used to determine dihydropteridine reductase monitors the consumption of 2,6-dichlorophenol indophenol (DCPIP) [3], which is used to oxidize the tetrahydropterin to the 'quinonoid' 6,7-[8H]dihydrobiopterin. In a total volume of 200 μl, 2 μg/ml dihydropteridine reductase are incubated with 0.1–3.4 μM H_4biopterin in the presence of 150 μM NADH and 150 μM DCPIP for 5 min at 37 °C. The reaction is started by addition of DCPIP, and the consumption of the dye is quantified by measurement of the absorption at 620 nm in a microplate reader (Anthos).

Measurement of NOS activity

NOS activity was determined by quantification of [^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine as described in [20]. Incubations were performed in 0.1 ml of 50 mM triethanolamine/HCl, pH 7.4, containing 0.1–0.2 μg of purified, recombinant H_4biopterin-free nNOS [17], 0.1 mM [2,3,4,5-^{3}H]L-arginine (≈ 60000 c.p.m.), 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.2 mM CHAPS and 10^{-8}–10^{-3} M H_4 biopterin derivative. In some experiments, 5 μM haemoglobin was added to scavenge NO to inactivate feedback inhibition, since NO rather than peroxynitrite is the product of the NOS reaction with N^5-methyl H_4biopterin [14].

Binding of H_4biopterin derivatives to nNOS

Purified, H_4biopterin-free rat nNOS was incubated for 10 min at 37 °C with 10 nM [3′-^{3}H]H_4biopterin (14 nCi), 0.1 mM L-arginine and 10^{-8}–10^{-3} M H_4biopterin derivative in 0.1 ml of triethanolamine/HCl buffer, pH 7.4, followed by rapid vacuum filtration using the MultiScreen Assay System from Millipore. Determination of the radioactivity retained on the filters by liquid scintillation counting was performed as in [3].

Reduction of DCPIP by H_4biopterin derivatives

At room temperature in volumes of 200 μl, H_4biopterin derivatives (150 μM) or water were mixed with DCPIP (150 μM) at the following pH values in the following buffer systems (100 mM each): pH 7.2 (K_2HPO_4/KH_2PO_4), pH 6.0 (K_2HPO_4/KH_2PO_4); pH 4.5 (acetic acid/sodium acetate) and pH 3.0 (KH_2PO_4/H_3PO_4). Within 2 min, the absorbance at 620 nm (pH 7.2 and 6.0) and at 540 nm (pH 4.5 and 3.0) was measured with a microplate reader, and the absorbance of the wells containing no H_4biopterin derivative was set to $100\,\%$. Stopped-flow experiments to analyse the kinetics of reduction of DCPIP by the H_4biopterin derivatives were performed with a Biosequential SX-17 MV ASVD apparatus (Applied Photophysics, Leatherhead, U.K.). Solutions of pteridines and DCPIP, 100 μl each, were mixed at 25 °C and the decrease of UV absorption at 600 nm was monitored up to 30 s. The final reaction mixture consisted of 0.2 M K_2HPO_4/HCl buffer (pH 6.0), 150 μM H_4biopterin derivative and 25 μM DCPIP. Six independent experiments yielded the means ± S.E.M. mentioned in the Results section. All values shown are means ± S.E.M. of three independent experiments unless indicated otherwise.

RESULTS

Depending on the nature of the N^5-substituent, N^5-substituted H_4biopterin derivatives are still capable of donating electrons. This is exemplified by the equilibrium concentrations of DCPIP in reaction mixtures with the H_4biopterin derivatives (Figure 2). Whereas H_4biopterin and its $N^{2'}$-methyl derivative reduced DCPIP at all investigated pH values to 100%, N^5-substituted derivatives showed declining reductive potential in the order of the N^5-substituent hydrogen > methyl > hydroxymethyl. N^5-formyl and N^5-acetyl derivatives were not capable of reducing DCPIP in the investigated pH range (Figure 2). A similar ranking was observed by studying the kinetics of the reaction of DCPIP with the H_4biopterin derivatives: Second-order rate constants declined from $170 \pm 4\ mM^{-1} \cdot s^{-1}$ for the N^5-substituent hydrogen, to $1.3 \pm 0.3\ mM^{-1} \cdot s^{-1}$ for the N^5-methyl, and $6.0 \pm 0.3\ mM^{-1} \cdot s^{-1}$ for the N^5-hydroxymethyl derivative. N^5-formyl and N^5-acetyl H_4biopterin did not react at all. The $N^{2'}$-methyl derivative, in contrast, reduced DCPIP with a second-order rate constant of $200 \pm 7\ mM^{-1} \cdot s^{-1}$, which is as fast as the parent compound H_4biopterin.

Substitution of H_4biopterin at $N^{2'}$ with a methyl group did not affect the ability of the compound to serve as substrate for the dihydropteridine reductase reaction (Figure 3). N^5-substitution, however, completely blocked the ability of the compounds to serve as substrates for dihydropteridine reductase regardless of the nature of the N^5-substituent [Figure 3, assay (i)]. When given at a 10-fold excess (35 μM) together with H_4biopterin (3.5 μM), none of the N^5-substituted H_4biopterin derivatives inhibited the dihydropteridine reductase reaction [assay (ii), not shown].

When the derivatives were tested for their ability to serve as substrates for the phenylalanine hydroxylase reaction, only $N^{2'}$-methyl H_4biopterin could act as cofactor for the enzyme instead of H_4biopterin (Figure 4). Whereas the K_m of $N^{2'}$-methyl H_4biopterin ($25.2 \pm 7.4\ \mu$M) was comparable with that of H_4biopterin ($17.4 \pm 1.2\ \mu$M), only 24.6 ± 2.8% of the V_{max} of H_4biopterin was achieved. When given together with H_4biopterin, N^5-formyl H_4biopterin as well as the N^5-acetyl derivative had no effect on phenylalanine hydroxylase activity. N^5-methyl H_4biopterin ($K_i = 23.1 \pm 5.4\ \mu$M) and N^5-hydroxymethyl H_4biopterin ($K_i = 237 \pm 70\ \mu$M), in contrast, inhibited phenylalanine hydroxylase activity competitively with H_4biopterin.

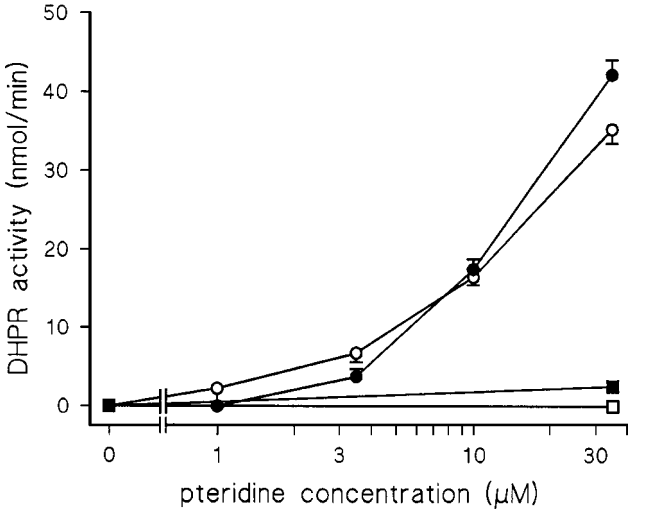

Figure 3 Ability of H_4biopterin derivatives to provide substrates to the dihydropteridine reductase reaction

Pteridines were used in the indicated concentrations in the assay for dihydropteridine reductase, which used hydrogen peroxide/peroxidase to oxidize the pterins and monitor the consumption of NADH at 340 nm (assay i, see the Experimental section). Results show means ± S.D. of four parallel incubations. ○, H_4biopterin; ●, $N^{2'}$-methyl H_4biopterin; ■, N^5-hydroxymethyl H_4biopterin; □ (= no stimulation), N^5-methyl H_4biopterin, N^5-formyl H_4biopterin and N^5-acetyl H_4biopterin.

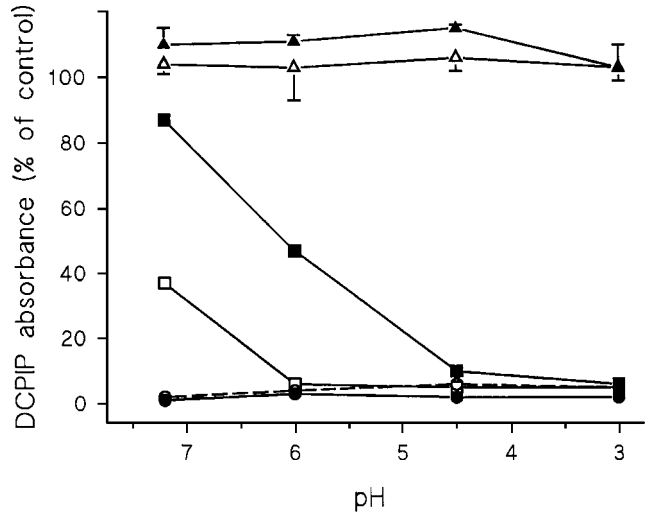

Figure 2 Reduction of DCPIP by H_4biopterin derivatives as a function of pH

Pteridine derivatives (final concentration 150 μM) were mixed with DCPIP (final concentration 150 μM) at room temperature, and the absorbance determined at 620 and 540 nm within 2 min. The control was the absorption of DCPIP mixed with water instead of H_4biopterin-derivative-containing solution. Values shown are means ± S.D. of 8 determinations. ○, H_4biopterin; ●, $N^{2'}$-methyl H_4biopterin; □, N^5-methyl H_4biopterin; ■, N^5-hydroxymethyl H_4biopterin; △, N^5-formyl H_4biopterin; ▲, N^5-acetyl H_4biopterin.

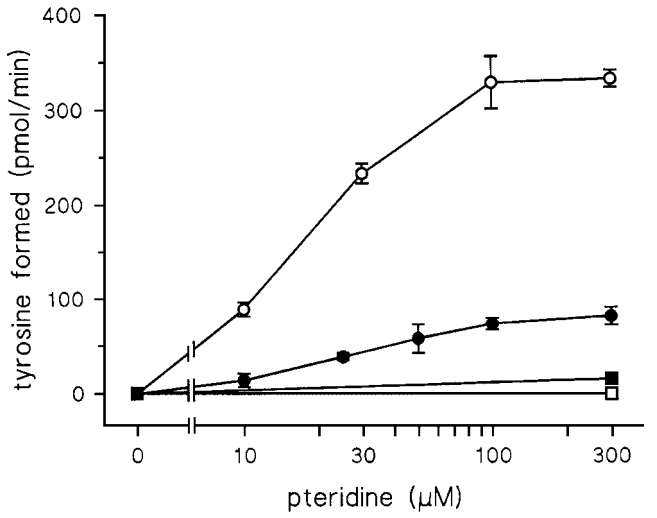

Figure 4 Stimulation of phenylalanine hydroxylase by H_4biopterin derivatives

Phenylalanine hydroxylase was incubated with the indicated concentrations of H_4biopterin derivatives and the formation of tyrosine from phenylalanine measured by HPLC with fluorescence detection. Values shown are means ± S.D. of three parallel incubations. ○, H_4biopterin; ●, $N^{2'}$-methyl H_4biopterin; ■, N^5-hydroxymethyl H_4biopterin; □ (= no stimulation), N^5-methyl H_4biopterin, N^5-formyl H_4biopterin and N^5-acetyl H_4biopterin.

Table 1 Binding and stimulatory characteristics of H_4biopterin derivatives on recombinant, H_4biopterin-free rat nNOS

H_4biopterin-free, recombinant nNOS was prepared as described in [17]. Binding of H_4biopterin derivatives to the enzyme was assayed by replacement of enzyme-bound [3′-^{3}H]H_4biopterin according to [3]. NOS activities were determined by measurement of [2,3,4,5-^{3}H]citrulline formed from L-[2,3,4,5-^{3}H]arginine in the presence of haemoglobin to avoid feedback inhibition by nitric oxide, which is the product of the NOS reaction in the presence of N^5-methyl H_4biopterin [14]. Values are means ± S.E.M. of three experiments. $\%V_{max}$ shows V_{max} as a percentage of that of the parent compound, H_4biopterin (= 100%).

Derivative	Binding to nNOS (K_d or K_i, μM)	Stimulation of nNOS (EC_{50}, μM)	Stimulation of nNOS ($\%V_{max}$)
H_4biopterin	0.26 ± 0.01	0.041 ± 0.015	100 ± 13
$N^{2'}$-Methyl H_4biopterin	15 ± 6	48 ± 14	62 ± 10
N^5-Methyl H_4biopterin	5.6 ± 1.2	7.0 ± 1.8	108 ± 15
N^5-Formyl H_4biopterin	2.5 ± 0.2	3.2 ± 1.7	47 ± 10
N^5-Acetyl H_4biopterin	130 ± 55	None	None

Table 1 compares binding and stimulation of H_4biopterin-free rat nNOS by H_4biopterin and its derivatives studied in this work. Substitution at $N^{2'}$ with a methyl group greatly reduced the affinity of H_4biopterin to the enzyme, and stimulation required a concentration of the pterin derivative three orders of magnitude higher. Substitution at N^5 with acetyl led to a complete loss in stimulation of nNOS, and binding was very poor (Table 1). Substitution at N^5 with methyl yielded a compound that could bind to nNOS and lead to full stimulation of the enzyme, albeit with a 170-fold higher EC_{50} compared with the parent compound. This is consistent with the more than two orders of magnitude lower affinity for the N^5-methyl derivative (Table 1). N^5-formyl H_4biopterin exhibits twice the affinity to nNOS as N^5-methyl H_4biopterin. Consistent with this, only about half of the concentration of N^5-formyl H_4biopterin as compared with N^5-methyl H_4biopterin is required for half-maximal stimulation of nNOS. The V_{max} reached with N^5-formyl H_4biopterin, however, is less than 50% when compared with N^5-methyl H_4biopterin or H_4biopterin (Table 1). N^5-Hydroxymethyl H_4biopterin bound with a K_i of 1.5 ± 0.7 μM, and stimulated the enzyme with an EC_{50} of 6.3 ± 1.9 μM to 84 ± 19% of the V_{max} observed with H_4biopterin. Since the 1.1% H_4biopterin contained in this preparation could contribute strongly to these observed values, they were omitted from the Table and not considered for further interpretation.

DISCUSSION

The role of H_4biopterin in the NOS reaction has fascinated researchers since its detection about 10 years ago [2,21,22]. A straightforward approach has been to assume that the first step of the NOS reaction, the hydroxylation of L-arginine, depends on H_4biopterin by a mechanism comparable with the phenylalanine hydroxylase reaction [23]. Several pieces of experimental evidence have made it more and more difficult to maintain this hypothesis. H_4biopterin was not required in stoichiometric amounts compared with product formation [24,25], and recycling of H_4biopterin was not observed [25]. In addition, the second step of the NOS reaction, the conversion of N^G-hydroxy-L-arginine to citrulline and NO, was found to require H_4biopterin [26] and is inhibited by the 4-amino analogue of the pterin cofactor [11]. Further, as shown in the present study, N^5-methyl H_4biopterin stimulates the NOS reaction, even though it is an inhibitor of the phenylalanine hydroxylase reaction and cannot be oxidized to products serving as substrate for the dihydropteridine reductase reaction. This demonstrates that the mechanisms of stimulation of phenylalanine hydroxylase and NOS by H_4biopterin may be fundamentally different. A comparison of data on NOS stimulation and binding of N^5-substituted H_4biopterin derivatives reveals a striking discrepancy between binding and stimulatory capacity: N^5-formyl H_4biopterin binds with twice the affinity compared with N^5-methyl H_4biopterin. Nevertheless, only N^5-methyl H_4biopterin yields a V_{max} comparable with H_4biopterin. The V_{max} obtained with N^5-formyl H_4biopterin is less than half of the V_{max} of the former two compounds. An explanation for this difference could be the different redox potentials of the two H_4biopterin derivatives, N^5methyl and N^5-formyl H_4biopterin. Whereas cyclic voltammograms of the two compounds have a similar shape, the reductive potential of the N^5-formyl derivative is weaker by 0.53 V (A. C. F. Gorren, A. Kungl, K. Schmidt, E. R. Werner and B. Mayer, unpublished work). The N^5-formyl derivative, therefore, can no longer react with DCPIP. If a reductive potential is relevant for NOS catalysis by the pterin, this difference might explain the lower V_{max} for the N^5-formyl derivative. This interpretation is compatible with previous suggestions [13,27,28] that H_4biopterin may stimulate the NOS reaction by donating electrons in an unconventional way, thus coupling NADPH oxidation to NO and citrulline formation [29]. Detection of a trihydrobiopterin radical in the inducible-NOS oxygenase domain [30] impressively supports these suggestions.

The behaviour of $N^{2'}$-methyl H_4biopterin is difficult to interpret. Although the redox properties of this derivative are indistinguishable from the H_4biopterin parent compound, the V_{max} reached for stimulation of NOS is only 60% of V_{max} reached with H_4biopterin. The rather poor affinity of this compound to NOS may prevent saturation from being reached. Alternatively, the $N^{2'}$-methyl substitution may interfere with a potential interaction of the pterin with the haem in NOS. Crystal structures of NOS oxygenase domains containing pterin [28,31,32] suggest N^3 as a potential candidate for mediating an electronic interaction. Methyl at $N^{2'}$ might interfere with this interaction. Remarkably, calculated electronic structures of tetrahydropteridines [33] show that the two stimulators of NOS, H_4biopterin and its N^5-methyl derivative, share comparable potentials around N^3. In contrast, the 4-amino analogue of H_4biopterin, which inhibits NOS, has a totally different electronic structure at N^3.

The nature of the oxidation products of N^5-substituted H_4biopterin derivatives are not known thus far. As we show here, these oxidation products cannot serve as substrates in the dihydropteridine reductase reaction. Since H_4biopterin is not consumed in the NOS reaction in stoichiometric amounts [24,25], NOS must also have a means of restoring the cofactor with the electron(s) it has donated to the reaction, if the assumption of single electron donation by the pterin [13,27,28] is correct. Our results suggest clearly that this potential recycling in NOS may occur by a mechanism different to the dihydropteridine reductase reaction.

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