Low-Temperature Optical Absorption Spectra Suggest a Redox Role for Tetrahydrobiopterin in Both Steps of Nitric Oxide Synthase Catalysis†

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ABSTRACT: To investigate the role of tetrahydrobiopterin (BH4) in the catalytic mechanism of nitric oxide synthase (NOS), we analyzed the spectral changes following addition of oxygen to the reduced oxygenase domain of endothelial nitric oxide synthase (NOS) in the presence of different pteridines at −30 °C. In the presence of N^G-hydroxy-L-arginine (NOHLA) and BH4 or 5-methyl-BH4, both of which support NO synthesis, the first observable species were mixtures of high-spin ferric NOS (395 nm), ferric NO-heme (439 nm), and the oxyferrous complex (417 nm). With Arg, no clear intermediates could be observed under the same conditions. In the presence of the BH4-competitive inhibitor 7,8-dihydrobiopterin (BH2), intermediates with maxima at 417 and 425 nm were formed in the presence of Arg and NOHLA, respectively. In the presence of 4-amino-BH4, the maxima of the intermediates with Arg and NOHLA were at 431 and 423 nm, respectively. We ascribe all four spectra to oxyferrous heme complexes. The intermediates observed in this study slowly decayed to the high-spin ferric state at −30 °C, except for those formed in the presence of 4-amino-BH4, which required warming to room temperature for regeneration of high-spin ferric NOS; with Arg, regeneration remained incomplete. From these observations, we draw several conclusions. (1) BH4 is required for reductive oxygen activation, probably as a transient one-electron donor, not only in the reaction with Arg but also with NOHLA; (2) in the absence of redox-active pterins, reductive oxygen activation does not occur, which results in accumulation of the oxyferrous complex; (3) the spectral properties of the oxyferrous complex are affected by the presence and identity of the substrate; (4) the slow and incomplete formation of high-spin ferric heme with 4-amino-BH4 suggests a structural cause for inhibition of NOS activity by this pteridine.

The mechanism by which nitric oxide synthase (NOS;[1] EC 1.14.13.39) generates NO from L-arginine, O_2, and NADPH-derived electrons has been the topic of intensive research (reviewed in refs *1*−*4*). The reaction takes place in two stages. The first step consumes two electrons and results in formation of N^G-hydroxy-L-arginine (NOHLA), which is subsequently oxidized to L-citrulline and NO in a reaction requiring one more electron. Both reactions consume 1 equiv of O_2. There are structural and mechanistic similarities between NOS and cytochrome P450. In both enzymes, catalysis takes place at a heme that has thiolate sulfur as a proximal ligand, and the hydroxylation of Arg resembles mono-oxygenations as catalyzed by cytochrome P450. Consequently, the mechanism of the first step was thought to be adequately described by the catalytic cycle originally proposed for cytochrome P450. For the second step, different mechanisms have been suggested.

Unlike cytochrome P450, NOS requires tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-L-biopterin, BH4] as an additional cofactor. The role of BH4 in catalysis has been the object of numerous studies, but is not yet fully elucidated (reviewed in refs *3*−*6*). In the aromatic amino acid hydroxylases, BH4 is a dissociable cofactor that is involved in reductive oxygen activation and undergoes two-electron redox cycling. In NOS, BH4 is tightly bound, and there is no evidence for two-electron redox cycling. BH4 was shown to stimulate NOS dimerization, substrate binding, and the low-to-high-spin heme transition (*7*−*12*), but all of these phenomena occur to a lesser extent in the absence of BH4 as well, whereas the dependence of NO synthesis on BH4 is absolute. Since this implies an as yet unidentified function of BH4, the idea that it plays a redox role in NOS catalysis was never surrendered completely. Studies with pterin

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[1] Abbreviations: NOS, nitric oxide synthase; nNOS and eNOS, neuronal and endothelial isoforms of NOS, respectively; BH4, tetrahydrobiopterin [(6*R*)-5,6,7,8-tetrahydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin]; BH2, 7,8-dihydrobiopterin; 4-amino-BH4, 4-aminotetrahydrobiopterin [(6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pteridine]; 5-methyl-BH4, 5-methyl-6,7,8-trihydrobiopterin; NOHLA, N^G-hydroxy-L-arginine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EPR, electron paramagnetic resonance.

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analogues offered support for such a role, since tetrahydropterins were shown to sustain NO synthesis, whereas redox-inactive dihydropteridines were inhibitory, although they mimicked the structural and allosteric effects of BH4 (*8, 13*−*15*).

In a previous low-temperature spectroscopic study, we demonstrated that BH4 is required for reductive activation of the oxyferrous complex in the first step of catalysis (*16*). In the absence of either Arg or BH4, the reaction between ferrous NOS and O_2 resulted in formation of an intermediate with an absorbance maximum at 415/7 nm, as is typical of cytochrome P450 oxyferrous heme complexes (*17*−*21*). Only when Arg and BH4 were both present did the reaction exhibit a blue-shifted intermediate (λ_{max} 404/5 nm) that we ascribed to a higher valency iron oxygen complex. In line with this interpretation, NOHLA was formed in a 0.5 per heme stoichiometry under those conditions. Based on those observations, we postulated that BH4 serves as a one-electron donor to the oxyferrous complex. X-ray crystallographic evidence in support of one-electron redox cycling of BH4 during NOS catalysis has since then been presented (*22*). Recently, the formation of a $BH3^{\bullet}$ radical during the reaction of reduced NOS with O_2 in the presence of Arg and BH4 was directly demonstrated with electron paramagnetic resonance (EPR) spectroscopy (*23,* P. P. Schmidt, R. Lange, A. C. F. Gorren, B. Mayer, and K. K. Andersson, unpublished observations).

Our previous study was limited to the effects of Arg and BH4 on the reaction of neuronal NOS (nNOS) with O_2. Here we extend these studies to the alternative substrate NOHLA, several BH4 analogues, and the oxygenase domain of the endothelial isoform (eNOS).

MATERIALS AND METHODS

Materials. Recombinant rat brain full-length BH4-containing (1 BH4 per dimer) and BH4-deficient (<0.1 BH4 per dimer) nNOS were purified from baculovirus-infected insect cells as previously described (*24*−*26*). The eNOS and nNOS oxygenase domains were obtained using standard molecular biological techniques (*16*). The cDNA fragments coding for the oxygenase domains of nNOS and eNOS were kind gifts from Drs. D. S. Bredt and S. H. Snyder, and from Dr. W. C. Sessa, respectively. BH4 and NOHLA were from Alexis Biochemicals (Lausen, Switzerland); 7,8-dihydro-L-biopterin (BH2), 4-aminotetrahydrobiopterin [(6*R*)-2,4-diamino-5,6,7,8-tetrahydro-6-(L-*erythro*-1′,2′-dihydroxypropyl)pteridine, 4-amino-BH4], and 5-methyl-BH4 (5-methyl-6,7,8-trihydrobiopterin) were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). L-Arginine and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were from Sigma. Sodium dithionite was obtained from Fluka. Oxygen (99.995% pure) was from Air Products (Paris, France). Other reagents were purchased from Merck.

Low-Temperature Spectroscopy. Absorbance spectra were recorded from 350 to 700 nm with a Cary 3E (Varian) spectrophotometer in the double beam mode (slit width 1.5 nm). Data acquisition was in steps of 0.5 nm, with an acquisition time of 0.033−0.5 s per data point. The instrument was equipped with a home-built double sample compartment which allowed spectral recordings at low temperature as previously described (*16*). To prevent freezing of the samples, the experiments were performed in 1:1 (v/v) mixtures of ethylene glycol/water, containing 50 mM KP_i (pH 7.4), 1 mM CHAPS, 0.5 mM EDTA, and 1 mM 2-mercaptoethanol. The pH of this mixed solvent is fairly insensitive to temperature changes, and the presence of ethylene glycol did not affect the spectral properties of NOS. The sodium dithionite stock solution (23 mM) was prepared in the same solvent. Prior to use, argon was bubbled through the solutions for 30 min. The NOS samples were diluted in anaerobic buffers at final concentrations of 2−4 μM in total volumes of 1−2 mL in Teflon closed cuvettes. Reduction was achieved at 15 °C by addition of 10−20 μL of a concentrated solution of sodium dithionite (final concentration 230 μM) using a Hamilton syringe. After lowering the temperature to −30 °C, precooled oxygen (2−5 mL) was bubbled through the enzyme solution with a syringe. Absorbance spectra (recording time 2 min) were measured immediately after oxygen addition, and after intervals as specified.

Additional Methods. 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 (*27*).

BH4 binding was assessed with [^{3}H]-BH4 according to a published procedure (*8, 28*) with slight modifications. The enzyme (30 nM eNOS oxygenase domain) was incubated for 10 min at 37 °C in the presence of 50 mM triethanolamine (pH 7.4), 10 nM [^{3}H]-BH4, 0.1 mM Arg, 5 mM dithiothreitol, and various concentrations of unlabeled BH4 (0−1 mM) in a total volume of 0.1 mL, followed by vacuum filtration (MultiScreen Assay System, Millipore) and liquid scintillation counting of bound radioactivity. Since no bound BH4 could be detected in the presence of 50% ethylene glycol, due to the low pterin-affinity of the enzyme under these conditions (vide infra), we repeated the experiments with 2 μM eNOS oxygenase domain and 0.2 μM [^{3}H]-BH4 in total volumes of 22.5 μL.

RESULTS

Effect of Ethylene Glycol on NOS Activity and Pterin Affinity. All experiments reported here were performed in the presence of 50% ethylene glycol. We found previously that nNOS is still able to catalyze the transformation of L-arginine into L-citrulline under those conditions (*16*). In the course of the present study, we noticed that the properties of nNOS in the presence of exogenous BH4 were always indistinguishable from those of nNOS containing 1 equiv of BH4 per dimer. Since this suggests that under the present conditions nNOS was unable to bind a second equivalent of BH4, we decided to look into the effects of ethylene glycol on NOS activity in greater detail.

In one series of experiments, we determined how the activity of BH4-deficient nNOS (~6% per heme) depended on the concentration of exogenous BH4. In the absence of ethylene glycol, BH4 induced an increase in the rate of citrulline formation from 21 ± 17 to 371 ± 4 nmol·mg^{-1}·min^{-1} with an EC_{50} of 18 ± 4 nM, in agreement with previous reports (*10*). In 50% ethylene glycol, BH4 increased the activity from less than 2 to 84 ± 7 nmol·mg^{-1}·min^{-1} with an EC_{50} of 39 ± 14 μM.

In another series of experiments, we determined how 5-methyl-BH4 affected the activity of BH4-containing

Table 1: Properties of Absorption Spectra Obtained Immediately after O_2 Addition to Reduced eNOS Oxygenase Domain

substrate	pterin	λ_{max} (nm) Soret	α,β	$t_{1/2}$ (min)	intermediate[a]
Arg	−	410	549	5.5	none
Arg	BH4	406	549	7.7	none
Arg	5-Me-BH4	410	550	6.7	none
Arg	BH2	417	554	19.4	Fe(II)·O_2
Arg	4-Am-BH4	431	560	54.1	Fe(II)·O_2
NOHLA	−	416	554	7.5	Fe(II)·O_2
NOHLA	BH4	395(sh), 417, 439	547, 580(sh)	4.6	Fe(II)·O_2 Fe(III)·NO
NOHLA	5-Me-BH4	391(sh), 418, 440	547, 580(sh)	3.5	Fe(II)·O_2 Fe(III)·NO
NOHLA	BH2	425	556	21.4	Fe(II)·O_2
NOHLA	4-Am-BH4	423	557	24.6	Fe(II)·O_2

[a] The spectra could be simulated satisfactorily as follows: Arg/−, 67% Red + 33% Ox; Arg/BH4, 55% Red + 45% Ox; Arg/5-Me-BH4, 53% Red + 37% Ox + 10% I431; Arg/BH2, 50% Red + 10% Ox + 40% I431; NOHLA/−, 60% Red + 15% Ox + 25% I425; NOHLA/BH4, 10% Ox + 45% Fe(III)·NO + 45% I416; NOHLA/5-Me-BH4, 8% Ox + 55% Fe(III)·NO + 37% I416. Abbreviations: Red, Fe(II); Ox, Fe(III) high-spin; I431, intermediate spectrum observed with Arg and 4-amino-BH4; I425, intermediate spectrum observed with NOHLA and BH2; I416, intermediate spectrum observed with NOHLA without pterin; 5-Me-BH4, 5-methyl-BH4; 4-Am-BH4, 4-amino-BH4.

nNOS (1 equiv per dimer). We recently reported that 5-methyl-BH4 can substitute for BH4 in NOS catalysis (*29*). In the absence of ethylene glycol, 5-methyl-BH4 increased the activity from 560 ± 20 to 1130 ± 20 nmol of citrulline·mg^{-1}·min^{-1} with an EC_{50} of 2.6 ± 0.4 μM, in line with prior observations (*30*). In the presence of 50% ethylene glycol, 5-methyl-BH4 increased the activity from 17 ± 1 to 150 ± 20 nmol·mg^{-1}·min^{-1} with an EC_{50} of 1.8 ± 0.7 mM.

Finally, we determined the effect of ethylene glycol on the pterin affinity of the eNOS oxygenase domain directly in radioligand binding studies. Control experiments without ethylene glycol yielded a K_D for BH4 of 0.05 ± 0.01 μM in the presence of 0.1 mM Arg, in accordance with previous observations with full-length eNOS (*28*). In the presence of 50% ethylene glycol, we observed a K_D of 7 ± 3 μM.

These results demonstrate that NOS activity decreased considerably in the presence of ethylene glycol. More importantly, the pterin affinity of the enzyme was greatly diminished. It has been demonstrated that NOS binds BH4 anticooperatively, with 1 equiv remaining tightly bound under most conditions (*10*). The presence of 50% ethylene glycol resulted in an activity decrease by 97% for nNOS containing 1 equiv of BH4 per dimer, and by at least 90% for nNOS containing 6% BH4. These observations suggest that the tightly bound endogenous BH4 is lost in the presence of 50% ethylene glycol. Exogenous BH4 partly restored activity, but the EC_{50} values indicate that ethylene glycol lowered the pterin affinity by 3 orders of magnitude; direct binding studies confirmed this dramatic decrease in affinity. Moreover, ethylene glycol reduced the maximal activities in the presence of exogenous pterin by more than 50%. This suggests that only the high-affinity pterin-binding site is occupied under these conditions, in line with the observation that prompted these experiments, i.e., that exogenous BH4 does not affect the results obtained with nNOS containing 1 equiv of BH4. Consequently, it seems that the species obtained in the presence of 50% ethylene glycol contains maximally one pteridine per dimer.

Reactions of Dithionite-Reduced NOS with O_2 in the Presence of Arg. We have previously reported that the reaction of reduced full-length nNOS (λ_{max} 412 nm) with O_2 in the combined presence of Arg and BH4 proceeds via a blue-shifted intermediate (λ_{max} 404.5 nm), whereas a red-shift (λ_{max} 415 nm) was observed for the pterin-free enzyme

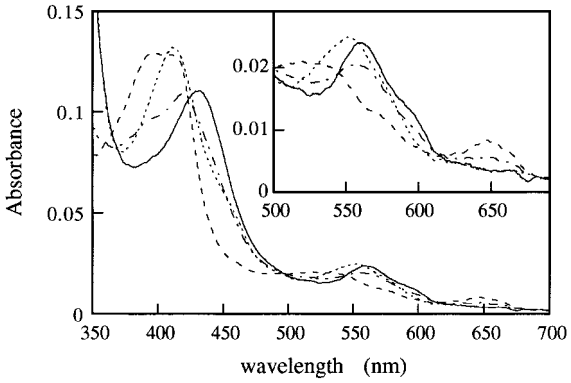


FIGURE 1: Reaction of dithionite-reduced eNOS oxygenase domain with O_2 in the presence of Arg and 4-amino-BH4 at −30 °C. Shown are the optical absorption spectra of the anaerobically reduced eNOS oxygenase domain (•••), the spectrum immediately after O_2 addition (—), the spectrum after 60 min incubation at −30 °C (— • —), and the spectrum after transient warming to room temperature (- - -). Experimental conditions: 2 μM eNOS oxygenase domain, 50 mM KP_i (pH 7.4), 1 mM CHAPS, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 50% ethylene glycol, 0.2 mM Arg, and 25 μM 4-amino-BH4. See Materials and Methods for further details.

(*16*). With the isolated nNOS oxygenase domain, no intermediate could be detected (*16*). Here we found that the transformation of reduced eNOS oxygenase domain to high-spin Fe(III) (λ_{max} 395 nm) in the presence of Arg also occurred without accumulation of an intermediate (Table 1). Similar observations were made with the NO synthesis-supporting pteridines BH4 and 5-Me-BH4 (Table 1).

Different results were obtained in the presence of the inhibitory pteridines BH2 and 4-amino-BH4. In the presence of BH2, an intermediate spectrum typical of an oxyferrous complex was formed (λ_{max} 417 nm, Table 1). This complex slowly decomposed to the ferric high-spin form. With 4-amino-BH4, the intermediate spectrum was strongly red-shifted (λ_{max} 431 nm, Figure 1). The maximum then shifted slowly to 420 nm, but remained stable afterward. Only after warming the sample to room temperature and readjusting the temperature to −30 °C, the final ferric species, a mixture of high- and low-spin heme, was obtained. It should be noted that ferric eNOS oxygenase domain in the presence of 4-amino-BH4 and Arg was completely high-spin before reduction. Similar observations were made with nNOS oxygenase domain (not shown).

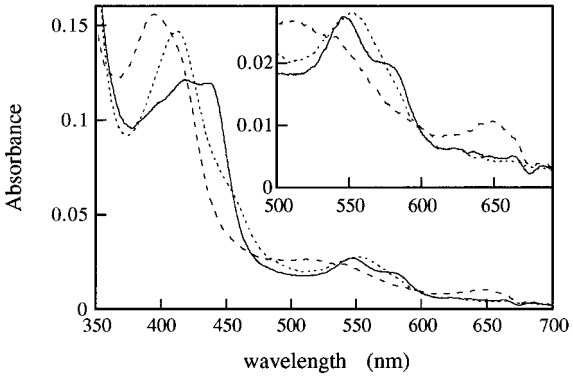


FIGURE 2: Reaction of dithionite-reduced eNOS oxygenase domain with O_2 in the presence of NOHLA and BH4 at −30 °C. Shown are the optical absorption spectra of the anaerobically reduced eNOS oxygenase domain (•••), the spectrum immediately after O_2 addition (—), and the spectrum after 60 min incubation (- - -). Experimental conditions: 2 μM eNOS oxygenase domain, 50 mM KP_i (pH 7.4), 1 mM CHAPS, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 50% ethylene glycol, 0.5 mM NOHLA, and 25 μM BH4. See Materials and Methods for further details.

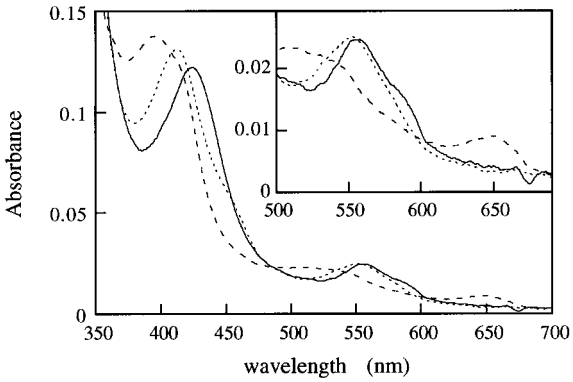


FIGURE 3: Reaction of dithionite-reduced eNOS oxygenase domain with O_2 in the presence of NOHLA and BH2 at −30 °C. Shown are the optical absorption spectra of the anaerobically reduced eNOS oxygenase domain (•••), the spectrum immediately after O_2 addition (—), and the spectrum after 60 min incubation (- - -). Experimental conditions: 2 μM eNOS oxygenase domain, 50 mM KP_i (pH 7.4), 1 mM CHAPS, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, 50% ethylene glycol, 0.5 mM NOHLA, and 25 μM BH2. See Materials and Methods for further details.

Reactions of Dithionite-Reduced NOS with O_2 in the Presence of NOHLA. In the presence of NOHLA and BH4, addition of O_2 to reduced eNOS oxygenase at −30 °C resulted in the immediate appearance of a spectrum with contributions from three compounds: the Soret region exhibited maxima at 417 and 439 nm, and a shoulder at 395 nm (Figure 2). The shoulder most likely reflected formation of the oxidized species. The 417 nm peak probably originated from the oxyferrous complex of the BH4-free subunit. The species absorbing at 439 nm could be identified as a ferric NO complex. Indeed, by subtracting spectral contributions from the ferric species and the oxyferrous complex of 10% and 45%, respectively, a homogeneous spectrum was obtained with properties typical of an Fe(III)•NO complex (Soret band at 439.5 nm and visible bands at 545 and 582 nm, not shown). The conversion of the composite spectrum to high-spin ferric heme was monophasic. In the presence of 5-methyl-BH4, the spectral changes were similar (Table 1). Identical observations were made with full-length nNOS and nNOS oxygenase domain (not shown).

In the absence of pteridines, intermediates with a Soret peak at 416 nm were formed with full-length nNOS (not shown) and with eNOS oxygenase domain (Table 1). In the presence of BH2 (Figure 3) and 4-amino-BH4 (Table 1), red-shifted intermediates (λ_{max} 425 and 423 nm, respectively) were formed. The species obtained with BH2 was transformed into high-spin ferric heme at −30 °C, but decay of the intermediate observed with 4-amino-BH4 yielded a spectrum indicative of the low-spin ferric state (λ_{max} 417 nm), which was converted to high-spin only after warming the sample to room temperature and readjusting to −30 °C (not shown).

DISCUSSION

Intermediates Formed in the Presence of BH2. BH2 binds to NOS, but does not support NO synthesis (*8*). It was therefore of interest to establish how this competitive inhibitor of NO synthesis affects the reaction of reduced NOS with O_2. With the eNOS oxygenase domain, and in the presence of Arg, oxyferrous heme (λ_{max} 417 nm) accumulated, which provides strong support for our hypothesis (*16*) that BH4 functions as an electron donor during reductive activation of the Fe(II)•O_2 complex,[2] a role that BH2 cannot fulfill.

When Arg was replaced by NOHLA, we also observed an oxyferrous heme intermediate, but in this case the Soret peak was red-shifted by 8 nm (λ_{max} 425 nm). The spectral properties of the NOS oxyferrous complex have been the object of some controversy. In stopped-flow studies with nNOS oxygenase domain, Stuehr and co-workers observed a maximum at 427 nm, independent of the presence of Arg or BH4 (*31*−*33*). This peak position is significantly red-shifted in comparison to the corresponding cytochrome P450 species, which display maxima between 416 and 423 nm (*20*). A peak position within the latter range (λ_{max} 415−417 nm) was found by us in low-temperature studies with full-length nNOS (*16*). In confirmation of our observations, rapid-scan spectra with full-length nNOS yielded a Soret peak at 417 nm in the absence of BH4 and Arg (*34*); a red-shifted spectrum (λ_{max} 427 nm) was obtained in the presence of Arg and BH4 in the same study. Similar spectral properties as observed by us (λ_{max} 419 nm) were also found by others in low-temperature studies of nNOS oxygenase domain in the presence of BH4 and the substrate analogue N^G-methyl-L-arginine (*35*).

One cause for the variations in peak positions observed by us might be that some of the spectra represent mixtures of the intermediate species and the initial and final states. Indeed, several of the intermediate spectra that exhibited Soret maxima at wavelengths below 420 nm could be constructed from combinations of the initial (deoxy-ferrous)

[2] There is considerable variation in the literature of cytochrome P450 and NOS concerning the representation of the intermediates in the reaction of reduced heme with O_2. The proper formal notation for the complex of dioxygen with reduced heme is Fe(III)−$O_2^{\bullet}$, which is how we designated the compound in prior publications (*4*, *16*). However, in our experience this sometimes gave rise to confusion regarding the true nature of the complex. Therefore, we opted for Fe(II)•O_2 in the present paper; we use this representation and the term 'oxyferrous complex' in a general sense, without referring to a specific structure of the complex.

and final (high-spin ferric) spectra, together with a contribution from one of the more red-shifted intermediate spectra. For example, the intermediate spectrum obtained in the presence of Arg and BH2, which displayed a maximum at 417 nm, could be reproduced in every detail by combining 50% of the deoxy-reduced species, 10% of the high-spin oxidized species, and 40% of the 431 nm intermediate that we observed in the presence of Arg and 4-amino-BH4 (not shown). Other examples are given in Table 1.

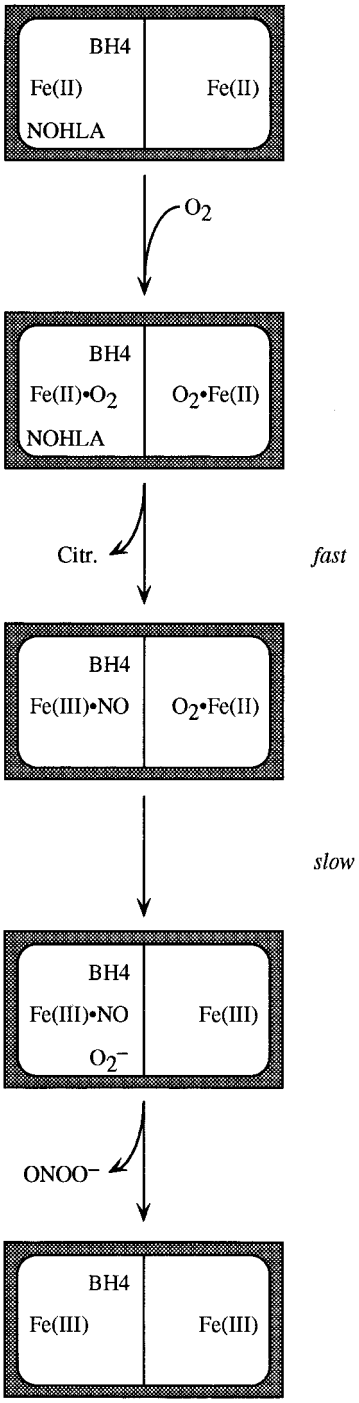
However, incomplete accumulation of the oxyferrous complex cannot explain all spectral differences: The intermediate spectra at 423/425 nm with NOHLA and 4-amino-BH4/BH2, and the spectrum at 431 nm with Arg and 4-amino-BH4 definitely derive from distinct species. The varying peak positions with different substrates are reminiscent of the red-shifts of variable magnitude that were reported with a range of substrates for cytochrome $P450_{CAM}$ (*20*). Apparently, oxyferrous heme can display a fairly broad range of absorbance maxima, depending on the bound substrate and pterin analogues. Definitive confirmation of this observation must await additional studies with different spectroscopic methods.

Intermediates Formed in the Presence of 4-Amino-BH4. Because the electrochemical properties of 4-amino-BH4 do not rule out redox activity a priori (A. C. F. Gorren, unpublished observations), assignment of the intermediate spectra is less straightforward than with BH2, but the similarities of the spectra obtained with 4-amino-BH4 and BH2 suggest that they can all be ascribed to oxyferrous complexes. We attribute the striking red-shift in the presence of Arg and 4-amino-BH4 to conformational or electronic changes around the heme pocket. Perhaps more remarkable than the red-shift is the extremely slow and, in the case of Arg, incomplete recovery of high-spin ferric heme at −30 °C. In the presence of NOHLA, a species absorbing at 418 nm was formed, which most likely represents low-spin ferric heme. The final spectrum observed with Arg at −30 °C (λ_{max} 420 nm) was not due to a separate species, but to a combination of the 431 nm oxyferrous species and the high-spin/low-spin ferric mixture obtained after transient warming to room temperature.

4-Amino-BH4 belongs to the group of pteridines that can act as BH4-competitive inhibitors of NOS activity (*13*, *14*, *36*). Unlike the 7,8-dihydropteridines, however, the redox properties of 4-amino-BH4 resemble those of BH4 (A. C. F. Gorren, unpublished observations). The present observations show that, nevertheless, 4-amino-BH4 does not function as an electron donor to the oxyferrous complex. The sluggish oxidation of reduced NOS in the presence of 4-amino-BH4, and the incomplete regeneration of the high-spin ferric compound with Arg, may be indicative of more extensive structural changes during reoxidation than take place with other pterins. Although the present results do not enable us to elucidate the inhibitory mechanism of 4-amino-BH4, they may hold important clues, since they represent the first case of 4-amino-BH4 behaving differently from both the activity-sustaining pterins (BH4, 5-methyl-BH4) and the redox-inactive inhibitory pteridines (exemplified by BH2).

Intermediates Formed in the Presence of NOHLA and BH4 or 5-Me-BH4. The experiments in the presence of NOHLA and BH4 or 5-Me-BH4 all yielded essentially the same intermediate spectrum, with contributions from three species

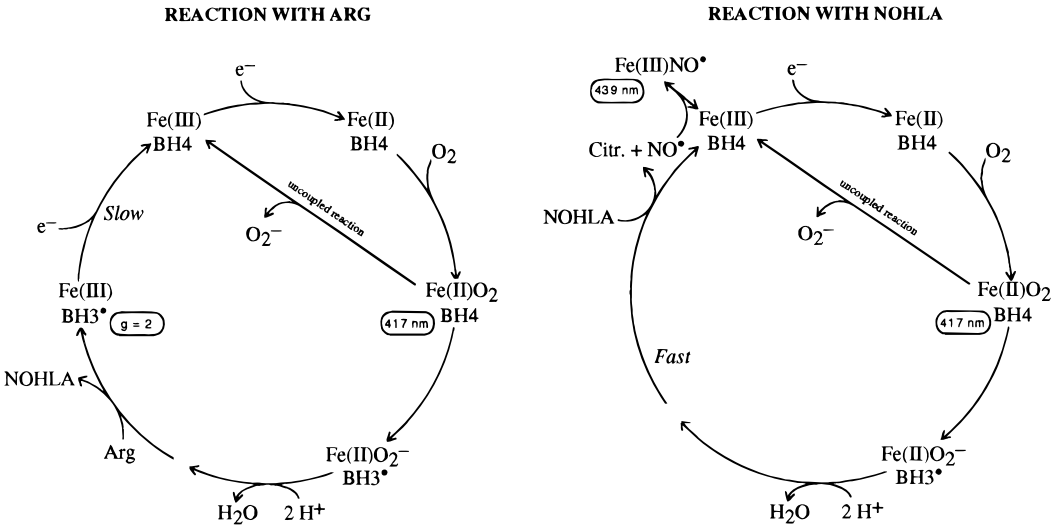
Scheme 1: Hypothetical Explanation for the Concerted Decay of the 417 nm Oxyferrous Species and the 441 nm Ferric−NO Complex[a]



[a] The boxes represent NOS dimers as isolated, with only one BH4 bound per two hemes.

absorbing at 395, 417, and 439 nm, which we ascribe to high-spin ferric heme, the oxyferrous complex, and the ferric NO complex, respectively. In our view, the simultaneous observation of both oxyferrous heme, on the one hand, and a mixture of ferric heme and ferric NO complex, on the other, reflects the stoichiometry of 1 BH4 bound per NOS dimer. In agreement with this proposal, about half of the enzyme is present as the oxyferrous intermediate. These results confirm that the two subunits function independently (*37*, *38*).

Scheme 2: Postulated Role of BH4 in NOS Catalysis in the Reaction Cycles with Arg (Left) and NOHLA (Right)[a]



[a] By allowing the oxyferrous complex to react rapidly with Arg (left) or NOHLA (right), BH4 prevents the uncoupled production of O_2^-. During the reaction cyle with Arg, the pteridine is oxidized to a BH3• radical in the process. This radical can be trapped under the experimental conditions applied in this study, because NOHLA formation requires two electrons, whereas only one electron per NOS monomer is available. The corresponding intermediate in the reaction with NOHLA is not detectable, because one electron is sufficient for citrulline formation. See Discussion for further details. The substrates Arg and NOHLA are bound in the vicinity of the heme throughout the reaction cycle, but were omitted from the scheme for the sake of clarity. Citr.: L-citrulline.

In the presence of BH4 or 5-Me-BH, oxidation was rapid and complete, resulting in formation of the ferric NO complex as the first observable species, whereas in their absence the reaction sequence was halted at the level of oxyferrous heme, which slowly decayed, presumably with concomitant production of O_2^-. These findings demonstrate that the pterin is required for product formation from NOHLA and the Fe(II)•O_2 complex. We therefore propose that, as previously postulated for the reaction with Arg (*16*), BH4 is required for reductive activation of oxyferrous heme in the reaction with NOHLA as well.

Finally, it should be noted that the decay of the two intermediate species formed in the presence of NOHLA and BH4 (oxyferrous compound and ferric NO complex) was monophasic. This implies that relaxation of the ferric NO−heme complex is coupled to decay of the oxyferrous species. Decay of the NO−heme complex could occur by NO dissociation followed by its reaction with O_2, or by a direct reaction of O_2 with the NO−heme complex. The latter reaction was found to underly NOS activity under conditions of maximal autoinhibition by NO (*39*), but may be less prominent under the present conditions, because of the oxidized state of the heme. Neither reaction can explain the observed monophasic decay. However, monophasic relaxation would occur if the ferric NO−heme complex reacted with O_2^- produced by an intrinsically slower reaction of the BH4-free subunit (Scheme 1). Extrapolation of this observation to normal catalysis suggests how half-pterin-saturated NOS might produce NO/O_2^- in a concerted way (*6*).

Role of BH4 as a One-Electron Donor in NOS Catalysis. The question whether BH4 has a redox-active role is one of the most contested issues of NOS catalysis (for recent reviews, see refs *3*−*6*). The best documented effects of BH4 are allosteric and structural in nature: stimulation of the low-to-high-spin heme transition, substrate binding, and NOS dimerization (*7*−*12*). However, unlike NO synthesis, none of these effects absolutely requires the presence of BH4, which implies that the main function of BH4 must be a different one. Moreover, all pterins that bind to NOS mimick the structural and allosteric effects of BH4, but only redox-active pterins can substitute for BH4 with respect to NO synthesis (*8*, *13*−*15*), which suggests that the unidentified function of BH4 involves its electrochemical properties. The recent observation that 5-methyl-BH4 supports NO synthesis (*29*) demonstrates that two-electron redox cycling between BH4 and quinonoid BH2, as occurs during aromatic amino acid hydroxylation, is not required for NOS catalysis, but it does not rule out one-electron redox cycling between BH4 and BH3•.

We previously presented evidence that BH4 provides one electron to oxyferrous heme during the O_2 reaction of dithionite-reduced NOS in the presence of Arg (*16*). The present study suggests that the same occurs in the reaction with NOHLA. Scheme 2 summarizes the hypothetical reactions of reduced NOS with Arg and NOHLA. The key observation of this study, i.e., that the reaction step following formation of the oxyferrous complex in both cycles requires BH4, agrees with recent observations by other investigators (*40*), but the conclusions drawn from those studies are diametrically opposed to ours. Abu-Soud et al. ruled out a redox-active role of BH4 in the reaction with Arg partly on the basis of their observations with NOHLA. Only one electron is required for net NO formation from NOHLA, and most current models have NOHLA itself serving as the electron donor for oxyferrous heme (*1*−*4*, *41*). Consequently, a redox function of BH4 in the second cycle was dismissed and, by analogy, it was deemed unlikely in the first cycle as well (*40*).

One crucial difference between our observations and those of Abu-Soud et al. is that we obtained product formation from reduced NOS and Arg in the presence of BH4 (*16*), whereas they reported citrulline formation from NOHLA, but no NOHLA formation from Arg (*40*). The latter observation would imply that BH4 is not a suitable electron

source in the catalytic cyle with Arg. However, almost stoichiometric formation of NOHLA from Arg was recently confirmed with the iNOS oxygenase domain by Marletta and colleagues (*42*). During the preparation of this paper, Stuehr and co-workers also reported NOHLA formation from Arg, although still with a significantly lower stoichiometry (*33*). The cause for the discrepancy is unclear, but may originate from different detection methods. All three research groups used HPLC for product analysis, but whereas elution in our and Marletta's protocols was performed at pH 7 or lower, Stuehr and co-workers applied a pH of 9.5, and under the latter conditions NOHLA is more labile.

Support for the unprecedented role of pterin as a one-electron donor has come from X-ray crystallographic studies (*22*). Recently, direct evidence for the formation of a pterin radical in the reaction with Arg was obtained with EPR spectroscopy by us (unpublished observations) and Marletta and co-workers (*42*). In the latter study, it was shown that formation of the radical was almost completely quenched with NOHLA as the substrate. From these results, it was concluded that BH4 serves as an electron donor in the first cycle only. However, this leaves the function of BH4 in the second cycle unexplained, and in our view it is unlikely that, with an efficient electron donor to the oxyferrous complex already in place, the reaction would follow different pathways at this stage of the two cycles. Our data suggest rapid rereduction of $BH3^{\bullet}$ as a plausible alternative explanation for the absence of a pterin radical signal (Scheme 2). Rapid regeneration of ferric heme and BH4 in the reaction with NOHLA is likely, since the second cycle consumes only one electron. Rapid completion of the cycle would also explain why no NOHLA-derived radical was detected (*42*), an observation that is hard to account for otherwise. Therefore, we propose that BH4 reduces the oxyferrous complex in both cycles (Scheme 2), but that single-turnover experiments allow detection of the $BH3^{\bullet}$ radical in the first cycle only.

Taken together, the evidence for one-electron transfer by BH4 under the conditions applied in these and similar studies is overwhelming, but whether the same reaction is also an obligatory step under physiological conditions remains to be established. Work is currently in progress in our laboratories to resolve this important issue.

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