Reaction of Neuronal Nitric-oxide Synthase with Oxygen at Low Temperature

EVIDENCE FOR REDUCTIVE ACTIVATION OF THE OXY-FERROUS COMPLEX BY TETRAHYDROBIOPTERIN*

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The reaction of reduced NO synthase (NOS) with molecular oxygen was studied at −30 °C. In the absence of substrate, the complex formed between ferrous NOS and O2 was sufficiently long lived for a precise spectroscopic characterization. This complex displayed similar spectral characteristics as the oxyferrous complex of cytochrome P450 (λmax = 416.5 nm). It then decomposed to the ferric state. The oxidation of the flavin components was much slower and could be observed only at temperatures higher than −20 °C. In the presence of substrate (L-arginine), another, 12-nm blue-shifted, intermediate spectrum was formed. The breakdown of the latter species resulted in the production of N^•-hydroxy-L-arginine in a stoichiometry of maximally 52% per NOS heme. This product formation took place also in the absence of the reductase domain of NOS. Both formation of the blue-shifted intermediate and of N^•-hydroxy-L-arginine required the presence of tetrahydrobiopterin (BH4). We propose that the blue-shifted intermediate is the result of reductive activation of the oxygenated complex, and the electron is provided by BH4. These observations suggest that the reduction of the oxyferroheme complex may be the main function of BH4 in NOS catalysis.

Nitric-oxide synthase (EC 1.14.13.39; NOS)1 catalyzes the oxidation of L-arginine to L-citrulline and nitric oxide (NO) in two reaction cycles. The first one leads to the formation of N^•-hydroxy-L-arginine (NOHLA), and the second one leads to the formation of L-citrulline and NO (1, 2). The latter reaction is not specific for NOS; the formation of L-citrulline and nitrite, the corresponding reactions for NOS have not been reported, except for a very recent stopped-flow study, which demonstrated the transient formation in the oxidation of ferrous NOS by O2 of a compound absorbing at 427 nm (16). In this work, we used a different approach to study the reaction between molecular oxygen and reduced neuronal NOS (nNOS). In order to trap possible intermediates, most of the experiments were carried out at subzero temperatures in the presence of 50% ethylene glycol as anti-freeze solvent (17). The same procedure was employed previously for the study of P450 oxygen complexes (11, 18, 19). Special attention was paid to the role of BH4 in the reduction of the oxygen complex in the presence of L-arginine. Our results throw more light on the function of BH4 in NOS catalysis.

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2 The abbreviations used are: NOS, nitric-oxide synthase; NO, nitric oxide; nNOS, neuronal nitric-oxide synthase; NOHLA, N^•-hydroxy-L-arginine; BH4, (6R)-5,6,7,8-tetrahydro-L-biotperin (tetrahydrobiopterin); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials—Enzymes used for molecular biological procedures were from New England Biolabs (supplied by Biotrade, Vienna). The TA cloning kit from Invitrogen was from the same vendor. The Sequenase kit was obtained from Amersham Pharmacia Biotech (supplied by Med-Pro, Vienna). Oligonucleotides were synthesized by the Institute of Microbiology and Genetics at the Biocenter of the University of Vienna. Glutathione-agarose beads, thrombin, and all other chemicals were from Sigma. BH4 was from Alexis Biochemicals (Switzerland); 7,8-dihydro-L-biotperin (BH3) was from Sigma-Aldrich (Vienna); calmodulin, L-arginine, and NOHLA were from Sigma; 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography.
Activation of Oxygen by Nitric-oxide Synthase

Activation of Oxygen by Nitric-oxide Synthase—Combinatorial rat brain nNOS was purified from baculovirus-infected insect cells as described previously (20). The heme concentrations of cytochrome P450 were determined from absorption coefficients of the oxidized and the ferrous-CO complex according to Sono et al. (21).

Preparation of Cytochrome P450—Cytochrome P450 CYP2B4 from rabbits, which had been phenobarbital treated for 1 week, was prepared according to the procedure of Coon et al. (22). The protein was kept frozen as a 69 μM solution in 10 mM potassium phosphate buffer, pH 6.8, containing 20% glycerol and 1 mM EDTA. The purified enzyme was electrophoretically homogeneous, and in the oxidized state it displayed an absorbance ratio of R$_{453/420}$ = 1.06. Neither NOS nor cytochrome P450 showed P420 characteristics.

Low Temperature Spectroscopy—Base line-corrected absorbance spectra in the 350–700 nm range were recorded with a Cary 3E (Varian) spectrophotometer in the double beam mode, using a 1.5-nm slit width. Data acquisition was in steps of 0.5 nm, with an acquisition time of 0.033–0.5 s/data point. The instrument was equipped with a home-built double sample compartment, which allowed spectral recordings at low temperatures (down to −50 °C). Sample and reference cuvettes were placed in a block made of aluminum, which was thermostatted by circulation of ethanol, thermoregulated in a Haake F3-Q bath. Inside the cells, the temperature was monitored continuously with a thermocouple connected to an AOPF voltmeter. For thermal insulation, the sample compartment was surrounded by polyvinyl chloride walls, equipped with double quartz windows. Formation of ice and condensation of water on the windows was prevented by a flow of dry nitrogen.

Formation of Intermediate Oxygen Complexes—To prevent freezing of the sample, the experiments were done in mixed organic solvents. If not specified otherwise, we used ethylhexyl glycerol/water (1:1, v/v). This solvent did not change significantly the spectral properties of NOS, it did not induce a transition to the P420 state, and in its presence the enzyme was still active in a standard assay (23). The buffer was 50 mM potassium phosphate, pH 7.2, containing 1 mM CHAPS, 0.5 mM EDTA, and 1 mM 2-mercaptoethanol. It contained no calmodulin, and, unless specified, no BH$_4$ was added. For CYP2B4, the solvent was 50% glycerol, and the buffer was sodium phosphate, 50 mM, pH 7.4. The pH of these mixed solvents is known to vary only little as a function of temperature (17). The sodium dithionite stock solution (23 μM) was prepared in the same solvent. Prior to use, argon was bubbled through the solutions for 30 min. The enzymes were diluted in the oxygen-free buffers at final concentrations of 3 μM in Teflon closed cuvettes in total volumes of 2 ml. They were reduced at 15 °C by the addition of 20 μM of a concentrated solution of sodium dithionite (final concentration 3 μM) using a Hamilton syringe. When reduction was complete, the temperature was decreased to the desired value (typically −30 °C). 2–5 ml of precooled oxygen were then bubbled in the enzyme solution with a syringe. This procedure took about 5 s, and thereafter the spectra were recorded in intervals of 2 min.

Product Analysis—Reduction and oxygenation of NOS and its heme domain were carried out as described above, except for the presence of 500,000 cpm of 200 μM [1-3,4,5-3H]arginine. After incubation for 5–60 min at the specified temperature, the reaction was stopped by the addition of 75 μl HCl (1 M) to the reaction mixture (1 ml). After centrifugation with a bench-top centrifuge, 100 μl of the supernatant were injected into a 25-cm Nucleosil 10A 5 HPLC column from Macherey & Nagel. The arginine derivatives were separated at neutral pH with a temperature of 6.5 °C, at a flow rate of 1.5 ml/min. Fractions of 750 μl were collected for 30 min, and radioactivity was determined in each fraction by liquid scintillation counting. NOHLA was identified by comparison with an authentic standard (24). Its formation was quantified from its integrated elution peak relative to the total radioactivity. In one control experiment, we used NADPH and not dithionite to reduce nNOS. In that case, the phosphate buffer was replaced by triethanolamine, 50 mM, pH 7.4, and a procedure similar to that described by Abu-Soud et al. (25) was used. First, the heme iron of nNOS was reduced by 200 μM NADPH in the presence of 14 μM calmodulin and 1 mM calcium chloride in aqueous buffer at 15 °C under the experimental conditions we had employed for reduction by dithionite.

RESULTS

Oxygen Binding to Reduced nNOS in the Absence of Substrate—Under anaerobic conditions, 230 μM dithionite reduced the NOS sample within 10–15 min at 15 °C. Spectrally, the reduction was characterized by a loss of the flavin absorbance, a shift of the Soret band from 400 to 410 nm, a shoulder at 455 nm, and a maximum at 554 nm. During the subsequent temperature decrease to −30 °C, the spectrum of Fe(II) nNOS remained essentially the same, except for a slight sharpening of the Soret band. After the addition of oxygen at −30 °C, the spectrum changed within 1 min. As shown in Fig. 1, the amplitude of the Soret band was slightly decreased, and its maximum was red-shifted to 416.5 nm. In the visible region, the main effect was a broadening of the absorption band. As shown in Fig. 2A, the oxidized minus reduced difference spectrum showed a strong maximum at 426 nm, two sharp minima at 401 and 458 nm, and an isosbestic point at 415 nm. Furthermore, two broad maxima appeared around 500 and 600 nm, as well as a minimum at about 550 nm.

At −30 °C, the slow decomposition of the oxygen complex led to an oxidized heme with reduced flavins (Fig. 1). As shown in Fig. 2A, the oxidized minus oxygen complex difference spectrum grew with maxima at 387.5, 418.5, 516.5, and 650 nm, minima at 442.5 and 558 nm, and an isosbestic point at 427 nm. The observed changes and the resulting spectrum are indicative of a mixture of high and low spin ferriheme but with a much higher fraction in the low spin state than before the experiment at 15 °C. Finally, when the temperature was raised above 20 °C, the flavins became also oxidized, as evidenced by a broad absorbance increase between 350 and 420, and between 470 and 550 nm (not shown). The latter reaction is probably due to a direct (nonphysiological) reaction of the reduced flavins with oxygen or with hydrogen peroxide resulting from the reaction of oxygen with dithionite.

As shown in Fig. 2, the spectral characteristics of formation and decomposition of the oxygen complex were similar to those observed with cytochrome P450. An exception was the trough at 458 nm in the difference spectrum (intermediate − reduced state, Fig. 2A), which was absent in P450. However, a comparison of the spectra of the reduced states shows that this spectral trough originates from the properties of the reduced state of NOS rather than from its oxygen complex. Indeed, the spectrum of reduced NOS, but not reduced P450, exhibits a shoulder at this wavelength. The differences observed during the autoxidation of the oxygen complex as compared with the analogous reaction in P450 (Fig. 2B), i.e., additional absorbance increases at 387.5, 516.5, and 650 nm, can be ascribed to the formation of a significant fraction high spin ferriheme.

The kinetics of oxygen binding and autoxidation were followed at 442.5 nm. Oxygen binding to reduced NOS was very rapid even at −30 °C. Subsequent oxidation of the heme was monoeponential (τ = 6.5 min at −30 °C). Oxidation of the

| Step | Reaction |
|------|----------|
| 1    | Fe$^{2+}$ + O$_2$ → Fe$^{3+}$O$^-$ |
| 2    | Fe$^{3+}$O$^-$ + 2H$_2$O $→$ R-H + OH$^- +$ |
flavins could only be observed at considerably higher temperatures (\(t = 15.6\) min at \(-11^\circ\text{C}\)).

**Oxygen Binding in the Presence of Substrate**—The addition of L-arginine (200 \(\mu\)M) resulted in the well known shift of the heme iron spin equilibrium toward more high spin. This effect persisted in the presence of ethylene glycol and at low temperatures. Reduction gave rise to a spectrum very similar to that of substrate-free reduced nNOS (Fig. 3A). In this case, too, mixing of reduced nNOS with oxygen at \(-30^\circ\text{C}\) produced a detectable intermediate, but its Soret band was blue-shifted (\(\lambda_{\text{max}} = 404.5\) nm) (Fig. 3B). In the visible region, the intermediate spectrum was similar to that obtained in the absence of L-arginine. At \(-30^\circ\text{C}\), its decay kinetics resulted within 37 min in an oxidized type high spin enzyme. The difference spectra reflecting formation (intermediate \(- \text{Fe}^{2+}\)) and reoxidation (reoxidized \(- \text{Fe}^{2+}\)) of the intermediate. The experimental conditions were those of Fig. 1.

**BH4-free NOS**—In the absence of L-arginine, binding of oxygen to BH4-free NOS at \(-30^\circ\text{C}\) resulted in similar spectral changes as those observed with BH4-bound NOS (cf. “Discussion” and Table II); the autoxidation of the oxygen complex (\(\lambda_{\text{max}} = 415\) nm), resulted in low spin ferric heme. In contrast, the presence of L-arginine did not induce the blue-shifted oxygen complex observed in BH4-bound NOS, and the spectral characteristics of the oxygen complex were close to those observed in the BH4-free NOS in the absence of L-arginine. As for the BH4-bound NOS in the presence of L-arginine, the autoxidation of this complex resulted in high spin ferric heme.

**The Oxygenase Domain**—The spectral changes upon oxygen addition to the reduced oxygenase domain were recorded at \(-30^\circ\text{C}\) as described above, in the absence and presence of BH4 (20 \(\mu\)M) and L-arginine (200 \(\mu\)M). In all cases, a direct transition from the reduced to the oxidized state was observed, and no intermediate state could be detected. Whereas the cofactors did not significantly affect the spectrum of the reduced state, the

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**Fig. 1. Spectral changes of reduced NOS (\(\text{Fe}^{2+}\)) after the addition of oxygen at \(-30^\circ\text{C}\).** Reduced full-length nNOS (3 \(\mu\)M) was cooled to \(-30^\circ\text{C}\), and then precooled oxygen was bubbled through the solution. The buffer, 50 mM potassium phosphate, pH 7.2, contained 1 mM CHAPS, 0.5 mM EDTA, 1 mM 2-mercaptoethanol, and 50% ethylene glycol. Spectra immediately after oxygen addition (\(\text{Fe}^{2+} + \text{O}_2\)) and 45 min later (reoxidized) are shown.

**Fig. 2. Comparison with the oxyferrous complex of cytochrome P450.** The P450 LM2 (\(\cdots\)) difference spectra were normalized with respect to those of nNOS (\(\cdots\)). A, formation of the oxygen complex; difference spectra between the oxygen complex minus the reduced state. The experimental conditions of nNOS were those of Fig. 1. For P450, 2 \(\mu\)M, the buffer was sodium phosphate, 50 mM, pH 7.4, containing 50% glycerol. B, decomposition of the oxygen complex; difference spectra in the course of reoxidation relative to the oxygen complex, 17 min (NOS) and 25 min (P450) after addition of oxygen.

**Fig. 3. The oxygen complex in the presence of L-arginine.** A, reduced states of nNOS and P450 LM2 at \(-30^\circ\text{C}\). nNOS in the absence (\(\cdots\)) and in the presence (\(\cdots\)) of 200 \(\mu\)M L-arginine, P450 LM2 (\(\cdots\)). B, spectral evolution of nNOS in the presence of L-arginine after the addition of oxygen at \(-30^\circ\text{C}\): reduced state (\(\text{Fe}^{2+}\)), immediately after oxygen addition (intermediate), and 37 min later (reoxidized). C, difference spectra of NOS reflecting formation (intermediate \(- \text{Fe}^{2+}\)) and reoxidation (reoxidized \(- \text{Fe}^{2+}\)) of the intermediate. The experimental conditions were those of Fig. 1.
endogenous BH$_4$, which is not readily displaced by added BH$_2$. Other experimental conditions were those of Fig. 1. The reaction times are indicated in the figure. The blue shift of the Soret band is indicated by an arrow.

The Oxygen Complex of Reduced nNOS in the Absence of Substrate—By the use of subzero temperature spectroscopy, it was possible to detect an intermediate complex between reduced nNOS and molecular oxygen. This technique was employed previously to trap the analogous complex of various cytochrome P450 forms. The spectral characteristics of the complexes of both enzyme classes are quite similar, and the differences observed during complex formation (a trough at 458 nm) are due to a spectral particularity of deoxyferrous nNOS. The shoulder at 458 nm persists in reduced nNOS in the absence of the reductase domain and is not affected by BH$_4$. The shoulder, which was noted before by others (21), may be due to the presence of a sixth ligand in a fraction of the enzyme. The additional absorbance increase at 380–390 nm suggests a hyperporphyrin spectrum, as has been reported for many ferrous complexes of cytochrome P450 (27, 28). The identity of the putative ligand cannot be established on the basis of the spectral changes, since most exogenous ligands other than O$_2$ induce a red shift upon binding to ferrous P450 type heme.

The spectral similarity of nNOS and P450 oxygen complexes suggests that both complexes have a similar electronic configuration. Most evidence in P450 points to a structure of Fe(III)O$_2$R, and this configuration is also expected for NOS (29). Since the same intermediate spectrum was obtained also in the absence of BH$_4$, it may be concluded that, in substrate-free nNOS, BH$_4$ does not perturb the electronic structure of the oxygen complex.

Stability of the Oxygen Complex—The oxygen complex of nNOS is rather unstable, and it was necessary to lower the temperature to $-30$ °C to prevent its immediate autoxidation. In that aspect, nNOS resembles microsomal cytochrome P450 (18). The oxygen complex of mitochondrial P450$_{sec}$ (more stable (several hours at $-30$ °C (19))) is a reasonable stability at $-30$ °C (20)). The most stable one, the complex of soluble P450 (21), can be detected without difficulty above 0 °C (at 25 °C, $\tau = 90$ s (14)). It appears thus that the protein environment plays a significant role in the stability of the oxygen complex. This idea is further supported by the fact that with the nNOS oxygenase domain the complex could not be detected, indicating that in this case autoxidation of the complex was much faster than its formation. Since reoxidation of the oxygenase domain was actually slower than that of the full-length enzyme, formation of the oxyferrocomplex must be considerably slower for the oxygenase domain. The origin of the effect remains to be established. One possibility might be that, in the full-length enzyme, the reductase domain enhances the oxygen association rate. Alternatively, the differences may somehow originate from the different expression systems used, since the full-length enzyme and the oxygenase domain were obtained from baculovirus-infected cells and from overexpression in E. coli, respectively.

The Oxygen Complex in the Presence of L-Arginine and BH$_4$—The spectral shift of the nNOS oxygen complex in the presence of L-arginine and BH$_4$ may be interpreted in two ways. One possibility is that the substrate and BH$_4$ interact strongly with the Fe(III)O$_2$R complex and modify sufficiently the energy of its electronic orbitals to induce the blue shift. Examples for an effect of substrates on the Fe(III)O$_2$R complex are found in cytochrome P450$_{sec}$, which interacts with cholesterol, (22R)-hydroxycholesterol and (20R,22R)-dihydroxycholesterol. These substrates induce significant changes of the ferric spin state and thus of the P450 Soret absorbance spectrum. However, the $\lambda_{max}$ values of the respective oxyferrous complexes range between 423 and 416 nm, while that of the complex in the absence of substrate lies at 420 nm (31), so that the substrate-induced
Fig. 5. Effect of BH4 on NOHLA formation after the addition of oxygen to reduced nNOS oxygenase domain. 4.5 μM, in the presence of 200 μM L-[3H]arginine. The buffer composition was that used in Fig. 1. After incubation at 15 °C for 5 min in the absence (full circles) and presence (open circles) of 20 μM BH4, the reaction was stopped by the addition of HCl (see “Experimental Procedures”). Radiolabeled L-arginine derivatives were separated by cation exchange HPLC, and fractions were analyzed by liquid scintillation counting (right scale). Left scale, background subtracted profiles; the background was the HPLC profile of L-[3H]arginine.

Possible configurations of such a reduced oxygen complex are shown in Fig. 6. They comprise a peroxo- (or hydroperoxo-) Fe(III) (2), an oxyferryl porphyrin σ-cation (3), or a hydroxyferryl complex (4). Forms 2 and 3 have been characterized for different cytochromes P450 and peroxidases. An oxyferrous complex reduced by one electron maintaining an intact dioxygen bond was recently reported for the D251N mutant of complex reduced by one electron maintaining an intact dioxygen bond was recently reported for the D251N mutant of cytochromes P450 and peroxidases. An oxyferrous form at 15 °C for 5 min in the absence (full circles) and presence (open circles) of 20 μM BH4, the reaction was stopped by the addition of HCl (see “Experimental Procedures”). Radiolabeled L-arginine derivatives were separated by cation exchange HPLC, and fractions were analyzed by liquid scintillation counting (right scale). Left scale, background subtracted profiles; the background was the HPLC profile of L-[3H]arginine.

Table I

| Enzyme                   | Concentration μM | Temperature °C | NOHLA formation μM | NOHLA:heme stoichiometry |
|-------------------------|------------------|----------------|---------------------|--------------------------|
| nNOS                    | 2.8              | 15             | 1.0                 | 0.36                     |
| nNOS                    | 8.4              | 15             | 3.0                 | 0.36                     |
| nNOS                    | 2.8              | −30            | 0.8                 | 0.29                     |
| nNOS in aqueous buffer  | 2.8              | 15             | 1.28                | 0.46                     |
| nNOS + 20 μM BH4        | 2.8              | 15             | 0.42                | 0.15                     |
| nNOS (oxidized)         | 2.8              | 15             | ND                  | ND                       |
| Oxygenase domain        | 4.5              | 15             | ND                  | ND                       |
| Oxygenase domain + 20 μM BH4 | 4.5          | 15             | 2.34                | 0.62                     |
| Oxygenase domain + 20 μM BH4 | 4.5            | 15             | 0.1                 | 0.02                     |

* ND, not detectable.

The shift never exceeds 4 nm. In contrast, the nNOS oxygen complex in the presence of substrate is blue-shifted by 12 nm. Alternatively, the blue-shifted intermediate spectrum in nNOS may reflect a further intermediate state (reduced by a second electron) in the course of the activation of oxygen. The observation that the decomposition of this enzyme state yields NOHLA, the formation of which requires two electrons, provides strong supporting evidence in favor of this possibility. Similarly, with cytochrome P450, product formation is only possible if the oxygen complex is reduced by a second electron.

Possible configurations of such a reduced oxygen complex are shown in Fig. 6. They comprise a peroxo- (or hydroperoxo-) Fe(III) (2), an oxyferryl porphyrin σ-cation (3), or a hydroxyferryl complex (4). Forms 2 and 3 have been characterized for different cytochromes P450 and peroxidases. An oxyferrous complex reduced by one electron maintaining an intact dioxygen bond was recently reported for the D251N mutant of the D251N mutant of complex reduced by one electron maintaining an intact dioxygen bond was recently reported for the D251N mutant of complex reduced by one electron maintaining an intact dioxygen bond was recently reported for the D251N mutant of cytochromes P450 and peroxidases. An oxyferrous form at 15 °C for 5 min in the absence (full circles) and presence (open circles) of 20 μM BH4, the reaction was stopped by the addition of HCl (see “Experimental Procedures”). Radiolabeled L-arginine derivatives were separated by cation exchange HPLC, and fractions were analyzed by liquid scintillation counting (right scale). Left scale, background subtracted profiles; the background was the HPLC profile of L-[3H]arginine.

The Role of BH4 in the Reductive Activation of the Oxygen Complex—Our results clearly show that the decomposition of the oxygen complex of nNOS in the presence of both L-arginine and BH4 results in the formation of NOHLA. This reaction requires that the Fe(III)/O2-L-arginine complex be reduced by one electron to Fe(III)/O2-L-arginine, which would then decompose in several steps to Fe(III) and NOHLA. But where does the second electron, necessary to reduce Fe(III)/O2-L-arginine, stem from? For several reasons, it is highly unlikely that it originates from dithionite. (i) For the related cytochrome P450 system, the second electron is exclusively provided by a specific reductase, the analogous counterpart of the reductase domain in NOS. (ii) Replacement of dithionite by NADPH did not affect the yield of NOHLA formation. (iii) Reduction of nNOS by dithionite was much slower than the formation of NOHLA. In control experiments, we measured the reduction rate between 5 and 30 °C (data not shown) and determined an activation energy of Ea = 115 kJ/mol. Extrapolation of the linear Arrhenius plot to −30 °C yielded a prohibitively slow reduction rate (τ = 2 weeks), whereas NOHLA was formed in less than 5 min at the same temperature.

The fact that we performed our experiments in the absence of calmodulin, essential for electron transfer from the flavins to the heme (9) and that NOHLA formation was also observed with the isolated oxygenase domain rules out the flavins as the electron source. Theoretically, the two hemes within one dimer might be able to provide the electrons to account for the observed NOHLA-to-heme stoichiometry. However, since most
evidence suggests that the hemes operate independently (30, 39) and that electron transfer between the hemes does not occur (40), this option is unlikely. This leaves BH₄ as the prime candidate for the donor of the second electron, in perfect agreement with the observation that NOHLA formation does not take place in its absence.

The present results are therefore relevant with regard to the role of BH₄ in catalysis. It has long been suspected that the redox properties of BH₄ are crucial to its function, although direct evidence for its participation in the redox process is still lacking (reviewed by Mayer and Werner (41) and Hemmens and Mayer (42)). The best evidence to date in favor of a redox-active role is the observation that redox-inactive BH₄ analogues can mimic the effects of BH₄ on NOS dimerization, substrate affinity, and heme spin state but fail to sustain the latter question, it was reported that oxidized BH₄-containing BH₄ lacking (reviewed by Mayer and Werner (41) and Hemmens et al. (46) contained traces of reductant, e.g. reduced flavins.

The present results demonstrate that BH₄ is absolutely required for L-arginine oxidation. If our assignment of the blue-shifted intermediate as the oxyferryl complex (3) is correct, the observation that its formation is only observed in the presence of BH₄ implies that BH₄ is required for oxygen activation. The simplest explanation and the one favored by us is that BH₄ furnishes the electron needed to reduce Fe(III)-O₂-OH (2), which immediately transforms then to the Fe(IV)=O porphyrin π-cation radical (3). Species 3 could then react with bound L-arginine via hydrogen abstraction to form species 4, the decomposition of which would liberate NOHLA and Fe(III). In the absence of BH₄, the uncoupled reaction (47, 48) will occur, and compound 1 will dissociate to Fe(III) and O₂ (Fig. 6).

In this way, our model also explains why the redox activity of BH₄ is crucial for coupling NADPH oxidation to NO formation, although it is not required for O₂ reduction per se. Since only one electron is transferred in this reaction, we denote the resulting state of the biotin domain as Fe(IV)BH₄. However, we cannot exclude the possibility that the state that is actually formed is the quinonoid-BH₂ (6,7-dihydro-L-biopterin), which is the usual product in enzymatic oxidations of BH₄ (41, 49) and which can be reduced to BH₄ by NOS (50).

The NOHLA-to-heme ratios of ≤0.5 suggest that in nNOS as isolated, which contains approximately one equivalent of BH₄ per dimer, only the BH₄-containing subunit produces NOHLA. This confirms that electron transfer between the two subunits in a NOS dimer does not take place (40) and agrees with the role postulated by us for BH₄ in catalysis. Similarly, in experiments with the oxygenase domain, where BH₄ was added (20 μM), the NOHLA-to-heme ratio did not reach unity, although it was a little higher (0.52 instead of 0.36 under the same buffer conditions). This may be explained by an incomplete binding of BH₄, which could result from a relatively high Kₛ of the second BH₄ binding site and an increase of Kₛ due to the presence of ethylene glycol. The latter idea is supported by our observation that with full-length nNOS the ratio is somewhat decreased in the presence of ethylene glycol.

**Conclusion**—The observations presented in this study provide important clues to the role BH₄ plays in NOS catalysis. The hypothesis that electron transfer from BH₄ is required for oxygen activation is the simplest explanation of our results. It does, however, need further corroboration, and it remains also to be established if the same holds true for the second step in NO catalysis, the oxidation of NOHLA to L-citrulline.

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**TABLE II**

| Enzyme                  | Experiment                        | λₘₐₓ  | Attributed to species no. in Scheme 1 and Fig. 6 | Reference |
|-------------------------|-----------------------------------|-------|-----------------------------------------------|-----------|
| nNOS                    | Fe(II) + O₂ (stopped-flow)        | 427   | 1                                             |           |
| nNOS                    | Fe(II) + O₂ (low temperature)     | 416.5 | 1                                             | This work |
| nNOS BH₄-free           | Fe(II) + O₂ (low temperature)     | 415   | 1                                             | This work |
| nNOS + l-arginine        | Fe(II) + O₂ (low temperature)     | 404.5 | 3                                             | This work |
| P45₀₉₀₀                  | Oxyferrrous + putidaredoxin        | 405   | 3                                             | 36        |
| P45₀₉₀₀ D251N mutant     | (Fe(II) + putidaredoxin) + O₂     | 421   | 2                                             | 33        |
| P45₀₉₀₀                  | Fe(III) + peroxycolesterol        | 405   | 3                                             | 37        |
| Horseradish peroxidase   | Oxyferrrous peroxidase            | 417   | 1                                             | 51        |
| Horseradish peroxidase   | Compound 1                        | 405   | 3                                             | 51        |
| Chloroperoxidase         | Compound 1                        | 367   | 3                                             | 52        |

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**FIG. 6.** Proposed mechanism of BH₄-dependent reductive activation of oxyferrous NOS leading to the formation of NOHLA. The numbers correspond to the intermediates cited in the text. 1 is observed in the absence of BH₄, 3 is the probable structure observed in the presence of both L-arginine and BH₄. The dashed line shows the uncoupled pathway in the absence of BH₄. P⁺ indicates porphyrin π-cation radical.
