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Biochemical and Biophysical Research Communications 338 (2005) 529–535
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Review

Hunting oxygen complexes of nitric oxide synthase at low temperature and high pressure

Stéphane Marchal [a], Antonius C.F. Gorren [b], K. Kristoffer Andersson [c], Reinhard Lange [a,*]

[a] *INSERM U710, Place Eugène Bataillon, Université Montpellier II, Montpellier, France*
[b] *Institut für Pharmakologie und Toxikologie, Karl-Franzens-Universität Graz, Austria*
[c] *Department of Molecular Biosciences, University of Oslo, Norway*

Received 25 July 2005
Available online 22 August 2005

This paper has been dedicated to Dr. Claude Balny in recognition of his pioneering work in the field of enzymology under extreme conditions.

Abstract

The reaction of nitric oxide synthase (NOS) with oxygen is fast and takes place within several steps, separated by ephemeral intermediates. The use of extreme experimental conditions, such as low temperature and high pressure, associated to rapid kinetic analysis, has proven to be a convenient tool to study this complex reaction. Stopped-flow experiments under high pressure indicated that oxygen binding occurred in more than one step. This was further corroborated by the detection of two short-lived oxy-compounds, differing in their spectral and electronic properties. Oxy-I resembles the ferrous oxygen complex known for cytochrome P450, whereas oxy-II appears to be locked in the superoxide form. Subzero temperature spectroscopy, together with an analytical separation method, revealed that the subsequent one-electron reduction of the oxygen complex is carried out by the NOS cofactor tetrahydrobiopterin (BH4). The low-temperature stabilized oxidation product of BH4 was found to be a protonated BH3 radical. Finally, work in the presence of a BH4 analog indicated that proton transfer to the activated oxygen complex is a second essential function of BH4.

Keywords: Nitric oxide synthase; Low temperature; High pressure; Oxycomplex; Rapid-scan stopped-flow spectroscopy; EPR spectroscopy

One of the most intriguing and challenging problems in the field of oxygenases has been the mechanism with which molecular oxygen interacts with the enzyme and becomes activated. The probably best-studied oxygenase is cytochrome P450, which activates dioxygen and carries out monooxygenations of a variety of endogenous and xenobiotic compounds. The importance of these processes for life has led to the development of an exciting area of research aimed at the elucidation of the monooxygenation mechanisms and the identification of reactive intermediate states (selected reviews [1–4]). Closely related to P450 is NO synthase (NOS), a fusion protein of a cytochrome P450 type reductase [5] and an oxygenase domain that spectroscopically [6–9], and functionally [9] resembles cytochrome P450 monooxygenase. Three genetically encoded mammalian isoforms of NOS have been identified: a neuronal form, nNOS, from brain, an endothelial form, eNOS, from endothelial cells, and an inducible form, iNOS, expressed in stimulated macrophages, while mammalian cytochrome P450 have several hundred isoforms. The spectroscopic similarities between NOS and P450 derive from the common nature of their axial cysteinyl sulfur heme ligand [9]. In contrast to P450, NOS requires the presence of two additional cofactors, tetrahydrobiopterin (BH4) and calmodulin/Ca^{2+} complex [10–12].

Since the ferrous dioxygen complex of P450 and NOS is one of the key intermediates in the enzymatic reaction, great effort has been spent to isolate and spectroscopically characterize it [13–18]. As with other oxygenases,

* Corresponding author. Fax: +33 467 14 33 86.
E-mail address: lange@montp.inserm.fr (R. Lange).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.090

dioxygen can bind to the heme iron in its reduced (ferrous) form only. The existence of oxygenated P450 has been known for many years [19], and many of its spectral properties have been determined [20–22]. However, this compound is unstable at room temperature, where it autooxidizes readily to release superoxide/peroxide and to regenerate the ferric resting state [13,23,24]. Further reaction steps, beyond the oxygenated complex, are even more rapid. They are therefore extremely difficult to study. The situation is still worse for NOS, where, depending on the presence of the biopterin cofactor, the half-life of the oxy-complex does not exceed some tens of milliseconds at 10 °C, [16]. It is therefore not astonishing that the spectral properties of the oxyferrous complex are not yet well defined and conflicting observations are reported [17,18,25,26].

Our approach to overcome these difficulties was to apply low temperature and high pressure associated with stopped-flow kinetic techniques. The low temperature approach was used to detect and stabilize ephemeral intermediate states. The high-pressure approach was used to perturb the complex reaction mechanism in order to get insight into elementary steps by altering the rate-limiting step of the reaction. In this review, we will discuss the results obtained under these extreme experimental conditions. Our model was the oxygenase domain of eNOS.

Methods

Low-temperature experiments. Low-temperature UV–visible absorption spectra were recorded with a Cary 3E (Varian, Palo Alto, CA) spectrophotometer, adapted for sub-zero temperature studies, according to previously published procedures [27–29]. To avoid freezing, experiments were carried out in a 1:1 (v/v) mixture of aqueous buffer/ethylene glycol. Stopped-flow experiments were performed at 7 °C using an SFM 300 BioLogic Instrument (Grenoble, France) equipped for rapid-scanning diode array detection [30]. Briefly, anaerobic solutions of ferrous (dithionite containing) eNOS oxygenase domain were oxygenated by bubbling oxygen (in the conventional spectrophotometer) or mixed with equal volumes of oxygen-containing buffer (in the stopped-flow apparatus). The final concentration of oxygen in stopped-flow experiments was varied by the use of three separate mixing syringes: one containing the enzyme, one the oxygen-saturated buffer, and one the argon-saturated buffer.

High-pressure experiments. Single turnover experiments under high pressure were performed using a high-pressure stopped-flow apparatus built in our laboratory (Fig. 1) [31]. The kinetics were determined by mixing equal volumes of the enzyme and ligand solutions in a thermostated high-pressure stopped-flow cell placed in an Aminco DW2 spectrophotometer operating in dual wavelength mode. The experiments were set up with one syringe filled with enzyme in the presence of substrate and cofactor, and the other one containing the O_2- or CO-saturated solution. Enzyme and gas-saturated solutions were prepared as described [28,32].

EPR spectroscopy. Samples were prepared as previously described [27], except that EPR tubes replaced optical cuvettes. After reduction of the enzyme with anaerobic dithionite solution, samples were cooled to −30 °C. The reaction was started by bubbling oxygen gas for 5 s and stopped by flash-freezing in −130 °C pentane of the EPR tube after about 30 s reaction time. Spectra were recorded on a Bruker ESP300E EPR spectrometer equipped with an Oxford Instrument cryostat 900 according to previously published procedures [33,34].

Results and discussion

Evidence for two spectrally distinct oxygenated intermediate species

The wavelength of the Soret band of the oxyferrous and the resonance stabilized iso-electronic ferric-superoxide complex of NOS has proven to be rather variable, with reported peak positions ranging from 416 to 432 nm ([28] and references therein [35]). Several causes appear to underlie these variations. When the rate of formation of the intermediate is not appreciably faster than the rate of breakdown, the reported spectra may be blue-shifted compared to that of the oxyferrous complex by contributions from the initial ferrous (413 nm) and the final ferric (395 or 418 nm) compounds. Furthermore, some of the differences appear to be caused by the presence of different substrates and pterin cofactors. This somehow confusing situation could be clarified by a recent systematic study of the spectral and kinetics properties of formation and decomposition of the oxyferrous complex of eNOS oxygenase domain by rapid-scan/stopped-flow spectroscopy. Two distinct species [30] were observed: in the presence of pteridines without substrate, an intermediate with maxima at 420/421 and 560/561 nm was consistently formed (Fig. 2, solid curve). Under all other conditions, i.e., in the absence of pteridines or in the presence of substrate (with or without pteridines) the intermediate exhibited maxima at 431/433, 563/565, and 595/600 nm with arginine (Arg) (Fig. 2, dotted curve), and at 428/429, 560, and 590/598 nm with N^{ω}-hydroxyarginine (NHA) or without substrate. In corresponding cryogenic studies [28], the same trend was observed, though with some exceptions, as some of the conditions that gave rise to '432-nm' intermediates in rapid-scan studies yielded '420 nm' intermediates in low-temperature spectroscopy. The 420 and 432-nm species were the only, mutually exclusive, intermediates observed, except for the reaction in the presence of BH4 and NHA, which exhibited a Fe(III) · NO product complex that was formed as a second intermediate prior to regeneration of ferric high-spin heme.

As regards the nature of the two spectral species, a trivial interpretation of the 420 nm species as mixtures of the 430 nm compound with the final ferric heme is unlikely, since a red-shifted intermediate was formed with similar kinetics [30]. To correlate the spectral signature of these oxygenated intermediates, with their respective redox state, we studied the ability of the oxycomplexes to form a ferrous CO complex by adding CO immediately after O_2 binding to the reduced oxygenase domain of endothelial NOS at −30 °C. Our rationale was that a ferrous oxygen complex should exchange oxygen readily by CO. However, this might not be the case for a ferric superoxide complex. Against expectation, some of the intermediates which were formed in the absence of redox-active pteridines that we included in our study as oxyferrous controls, turned out not to be able to form a ferrous–CO complex under these

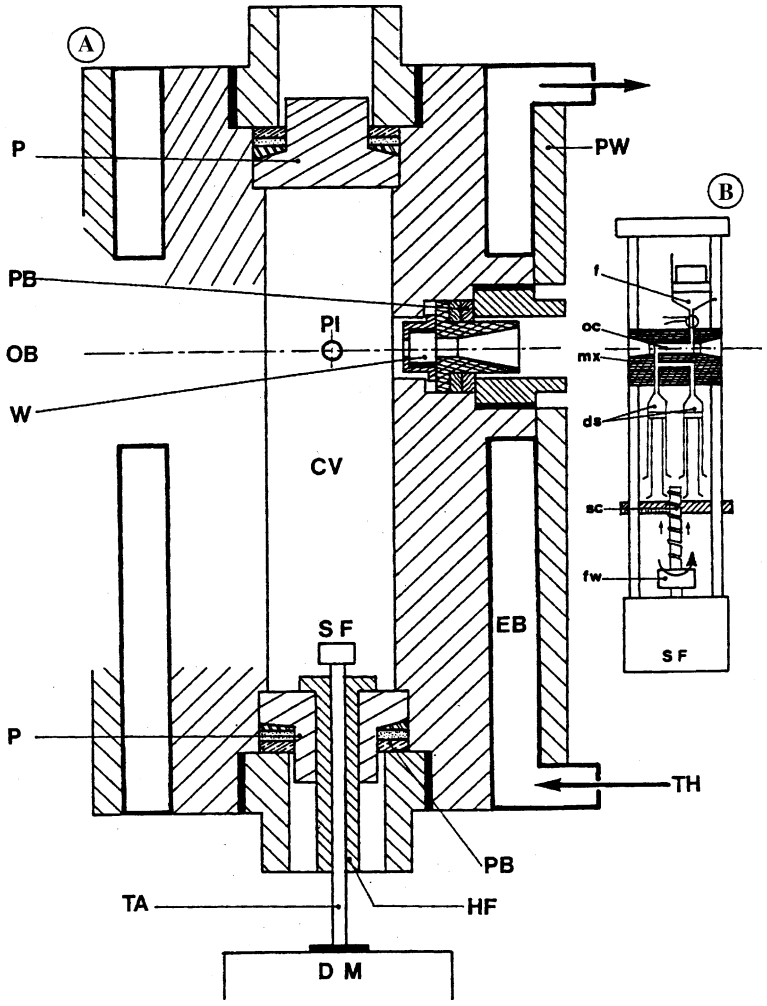

Fig. 1. Schematic view of the high-pressure stopped-flow apparatus. (A) Partial view of the high-pressure cell: P, plugs; CV, central volume containing the stopped-flow device; OB, optical beam; W, sapphire window; PB, packing; TH, thermostatization; PW, isolation; HF, high-pressure fitting; DM, driving mechanism; SF, stopped-flow adaptor; TA, turning axle; PI, pressure inlet; EB, circulating fluid volume. (B) Stopped-flow module which is placed, when operating, in the central volume (CV) of the high-pressure cell: oc, observation chamber; ds, drive syringes; f, waste-syringe; mx, mixing chamber; sc, screw; fw, free wheel.

conditions. Notably, the "432-nm" species, as exemplified by the intermediate obtained in the presence of NHA and BH2, shows direct conversion to the high-spin ferric state without any evidence of Fe(II) · CO formation. The "420 nm" species, on the other hand, as exemplified by the intermediate obtained with NHA in the absence of pteridine, did partially generate the typical 444 nm absorbance peak of a Fe(II) · CO complex. We ascribed the differential behavior of the two species to a different distribution of the electrons between the heme and its oxygen ligand. According to this interpretation, the 420-nm species resembles the oxyferrous complex observed in many hemoproteins, including cytochrome P450, which usually display similar absorbance spectra [36]. Although best described as a Fe(III) · O_2^- complex [37], this species retains sufficient Fe(II) · O_2 character to allow dissociation of O_2 and, consequently, O_2/CO exchange is possible for this compound [38]. However, in the 432-nm species, the oxy-complex appears to be locked in a ferric superoxide state, which is unable to exchange CO for O_2 at −30 °C.

High-pressure studies of O_2 and CO binding

For further characterization of the mechanism of oxygen binding, we applied high-pressure stopped-flow spectroscopy to the reactions of reduced eNOS oxygenase domain and, for comparison, of the reduced F393H mutant of cytochrome P450 BM3 with O_2 and CO [39]. For the cytochrome P450 BM3 mutant, the characteristics of pressure-dependent O_2 binding exactly matched those of CO binding. With eNOS, however, CO binding was slower than O_2 binding at all pressures, and oxygen (but not CO) binding was affected by the identity of substrate and pteridine cofactor. Thus, in the case of NOS, CO binding may be a poor model for oxygen binding, which appears to be more complex. Intriguing information was obtained by substituting the natural NOS cofactor, BH4, by its analogues 4-amino-BH4 (ABH4) and 4-amino-BH2 (ABH2). In the presence of ABH4, the pressure dependence of O_2 binding was found to be biphasic, whereas it was linear in the presence of ABH2. (Fig. 3). A break in the pressure

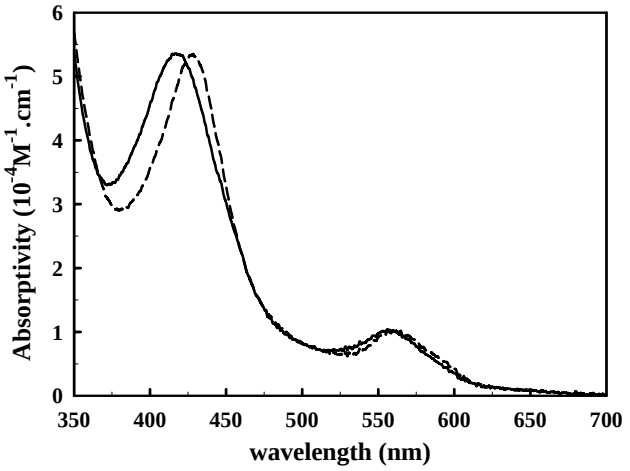

Fig. 2. Absorbance spectra of the heme-oxy intermediates obtained by addition of O_2 to BH4-bound eNOSoxy. Rapid-scan stopped-flow experiments were carried out at 7 °C. An anaerobic solution containing 6 μM reduced enzyme was rapidly mixed with an equal volume of oxygen-saturated buffer. Shown are the absorbance spectra recorded after 2.5 ms of mixing in the absence (heme-oxy-I, solid line) and in the presence of 500 μM NHA (heme-oxy-II, dotted line).

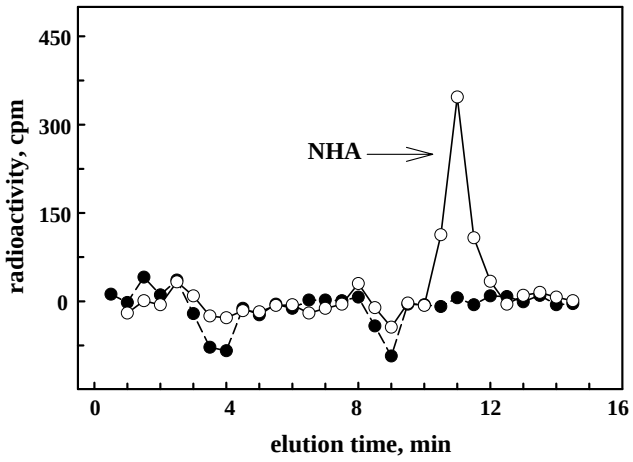

Fig. 4. Effect of BH4 on NHA formation after the addition of oxygen to reduced eNOSoxy. Oxygenation of NOS was carried out in the presence of 500,000 cpm of L-[2,3,4,5-3*H*]arginine solution. After incubation for 5 min in the absence (full circles) and presence of 20 μM BH4 (open circles), the reaction was stopped by addition of HCl. Radiolabeled L-arginine derivatives were separated by cation exchange HPLC and fractions were analyzed by liquid scintillation counting.

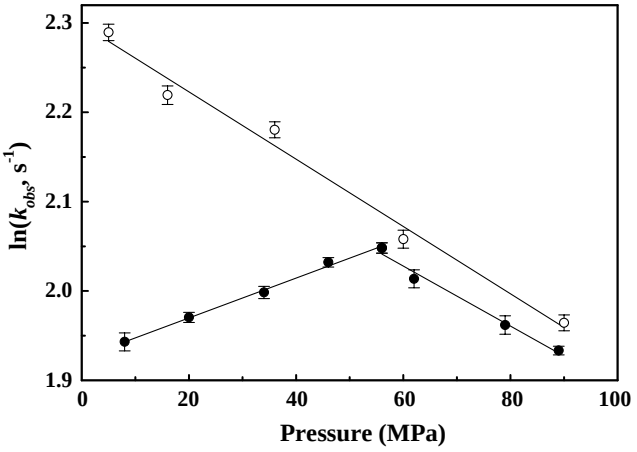

Fig. 3. Pressure effect on the formation of oxygen complex of P450 BM3 and eNOSoxy. The reaction was monitored after rapidly mixing a solution of (●), 7 μM ferrous eNOS, 50 μM 4-amino-BH4, and 500 μM arginine or (○), 2 μM ferrous BM3 and 60 μM arachidonate with an oxygen-saturated buffer solution at 4 °C.

dependence of a reaction rate is suggestive of a change in the rate-limiting step of the reaction. Hence, the results in the presence of the analogue ABH4 indicate that the reaction of NOS with oxygen occurs in more than one step. This information proved to be very useful in the analysis of low-temperature optical and EPR studies of the interaction of NOS with BH4 (see below).

Evidence for redox activity of BH4 in NOS catalysis

Perhaps, the most intriguing puzzle of NOS catalysis is the role of BH4 (for reviews, see [40,41]). BH4 enhances the affinity of NOS for its substrates, changes the state of the heme from low- to high-spin, and stimulates dimerization of the enzyme. However, none of these effects explains the absolute dependence of NO synthesis on BH4. Therefore, some form of redox activity of the pterin cofactor was suspected, partly on the basis of studies with BH4 analogues [42–46]. To determine the role of BH4 during the first steps of arginine mono-oxygenation, we analyzed the temporal evolution of the spectral changes occurring upon oxygen binding to reduced NOS at −30 °C [27]. We showed that, under these conditions, rapid heme reoxidation was observed in the presence of both Arg and BH4, and significant production of NHA could be detected (Fig. 4). In contrast, when either Arg or BH4 was absent, the oxyferrous complex accumulated. On the basis of these observations we proposed that the main function of BH4 is to serve as a one-electron donor to the oxyferrous complex for the first cycle. We found subsequently that the second reaction cycle (with NHA replacing Arg) also requires BH4 to continue beyond the oxyferrous complex [28]. This suggests strongly that BH4 is a one-electron donor in both reaction cycles.

One-electron transfer from BH4 to the oxygen complex: trapping and characterization of a BH4 radical

Although the optical and product-analytical studies provided the basis for the hypothesis that BH4 is a transient electron donor to the oxyferrous complex, they did not permit to characterize spectrally the product of BH4 oxidation. However, this was possible using low-temperature electron paramagnetic resonance (EPR) spectroscopy and the same cryogenic experimental protocol as applied in our previous study [27,28]. Using this low-temperature approach, we demonstrated the formation of a pterin radical with full-length neuronal NOS [33,34], confirming an

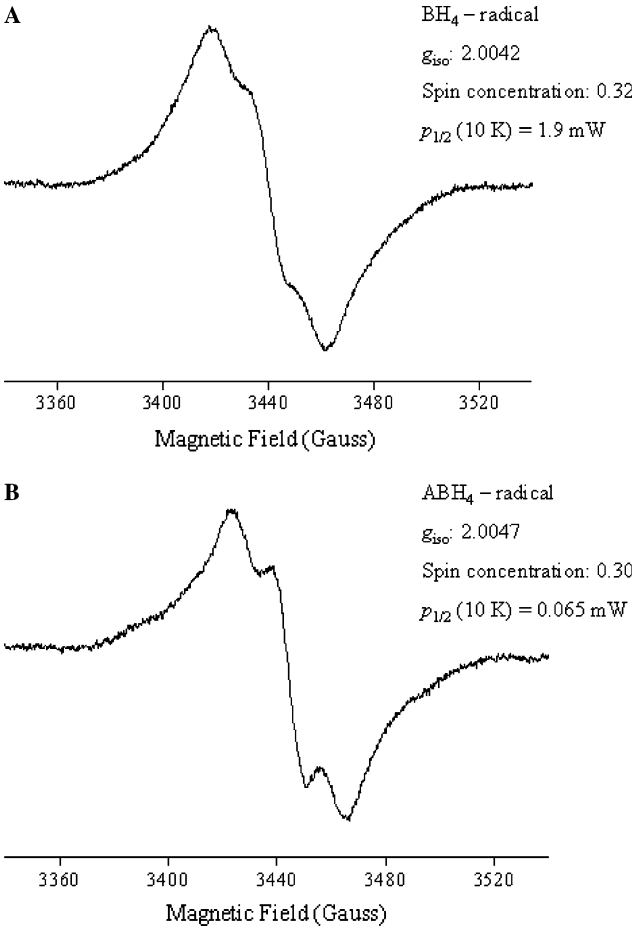

Fig. 5. EPR spectra of the trihydropteridine radicals obtained in the reaction of eNOSoxy with O2 and arginine at −30 °C in the presence of BH4 and 4-amino-BH4. Shown are the radicals observed with BH4 at 10 K (A) and with 4-amino-BH4 at 10 K (B).

earlier report on the generation of a pterin radical by an isolated oxygenase domain of inducible NOS [47]. Later studies showed that the formation of the radical correlates with the decay of the oxyferrous complex [35]. More recently, similar studies demonstrated one-electron redox cycling of BH4 during the second reaction cycle as well [48,49].

Evidence for a key role of BH4 in protonation of Fe(II) · O_2^-

In line with the crucial role of BH4 as an electron donor in NOS catalysis, tetrahydropteridines other than BH4 support NO synthesis (for review [41]), except for ABH4. This BH4 analogue is a potent inhibitor of NO synthesis, although it mimics all allosteric and structural effects of BH4 [43–45]. Importantly, it also exhibits similar electrochemical properties [50], suggesting that the inhibitory action of ABH4 cannot be ascribed to an inability to serve as an electron donor. Indeed, a trihydropteridine ($ABH3^{\bullet}$) radical was observed in the presence of Arg by electron paramagnetic resonance (EPR) spectroscopy at −30 °C (Fig. 5). With NHA instead of Arg, no pterin radical was observed, but an axial ferrous heme-NO EPR signal appeared. The corresponding optical spectra, with Soret bands at 417/423 nm, suggest that the proximal sulfur ligand was hydrogen bonded [51]. Accordingly, ABH4 serves as a one-electron donor to Fe(II) · O_2 with both Arg and NHA but the reaction cycle cannot be completed with either substrate. Therefore, we proposed that BH4 donates a proton to Fe(II) · O_2^- during catalysis and that inhibition by ABH4 may be due to its inability to support this essential protonation step. Our hypothesis was recently corroborated by O_2–CO exchange experiments performed at cryogenic temperatures [52]. This agrees well with the fact that a key hydrogen bond between N_3 of BH4 and one of the propionates of the porphyrin is probably absent in the case of ABH4 [53,54]. Without protonation of Fe(II) · O_2^-, O_2^- rather than H_2O_2 is the immediate product of uncoupled catalysis in the presence of ABH4. Novel results by Agasøster and Andersson (unpublished) indicate that some pterins could have even more complex reactivity.

Conclusion

The combination of stopped flow kinetics, cryobiochemistry, optical and magnetic spectroscopy, and high-pressure techniques allowed unraveling of several new intermediates in the NOS reaction (Fig. 6). This approach was especially

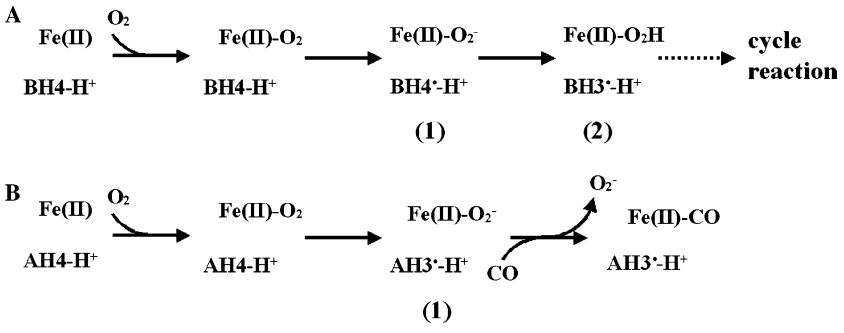

Fig. 6. Hypothetical oxygenated intermediates that could be formed in the presence of BH4 and ABH4. (A) The ability of BH4 to donate one electron and one proton could lead to the successive formation of superoxy (1) and hydroperoxy (2) complexes. (B) In the absence of proton delivery by 4-amino-BH4 (AH4), the oxygenated complex is locked in the ferrous superoxy (1) state. Dissociation of O_2^- and CO binding result in the formation of an Fe(II) · CO complex.

useful in the detection of different oxygen complexes that differ by the electronic distribution between the heme and its ligand. Another benefit was the experimental evidence of two essential catalytic functions of BH4: the one-electron reduction of the ferrous oxygen complex and its subsequent protonation.

Acknowledgments

We thank Bernd Mayer, Morten Sørlie, Armelle V. Agasøster, Toru Shimizu, Peter Schmidt, Hans-Petter Hersleth, and Nicole Bec for their excellent collaboration, with good technical help from F. Henning Cederkvist and Christian Valentin. This work was supported by Human Frontier Science program Contract No. RGP0026/2001 2-M.

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