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Oxidation of Tetrahydrobiopterin by Peroxynitrite or Oxoferryl Species Occurs by a Radical Pathway

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The molecular mechanisms of tetrahydrobiopterin (BH_4) oxidation by peroxynitrite ($ONOO^-$) was studied using ultra-weak chemiluminescence, electron paramagnetic resonance (EPR) and UV–visible diode-array spectrophotometry, and compared to BH_4 oxidation by oxoferryl species produced by the myoglobin/hydrogen peroxide (Mb/H_2O_2) system. The oxidation of BH_4 by $ONOO^-$ produced a weak chemiluminescence, which was altered by addition of 50 mM of the spin trap α-(4-pyridyl-1-oxide)-*N-tert* butylnitrone (POBN). EPR spin trapping demonstrated that the reaction occurred at least in part by a radical pathway. A mixture of two spectra composed by an intense six-line spectrum and a fleeting weak nine-line one was observed when using $ONOO^-$. Mb/H_2O_2 produced a short-living light emission that was suppressed by the addition of BH_4. Simultaneous addition of POBN, BH_4 and Mb/H_2O_2 produced the same six-line EPR spectrum, with a signal intensity depending on BH_4 concentration. Spectrophotometric studies confirmed the rapid disappearance of the characteristic peak of $ONOO^-$ (302 nm) as well as substantial modifications of the initial BH_4 spectrum with both oxidant systems. These data demonstrated that BH_4 oxidation, either by $ONOO^-$ or by Mb/H_2O_2, occurred with the production of activated species and by radical pathways.

Keywords: Tetrahydrobiopterin; Peroxynitrite; Oxoferryl; Chemiluminescence; Electron paramagnetic resonance; Diode array spectrophotometry

Abbreviations: BH_4, tetrahydrobiopterin; BH_2, 7,8-dihydrobiopterin; q-BH_2, quinonoid dihydrobiopterin; Por, porphyrin; Mb, myoglobin; uwCL, ultra-weak chemiluminescence; EPR, electron paramagnetic resonance; POBN, α-(4-pyridyl-1-oxide)-*N-tert* butyl nitrone

INTRODUCTION

Tetrahydrobiopterin (BH_4) is important in mammalians since it is involved in biological processes including amino acid metabolism, brain function, immune response, cell proliferation and

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vascular homeostasis.[1-3] The mechanisms by which BH_4 acts, remains however unclear,[2,4] although BH_4 appears to be a cofactor required for the activation of many heme and non-heme iron enzymes. With non-heme iron enzymes, BH_4 is a redox-active cofactor for the reaction of hydroxylases with aromatic amino acids, such as phenylalanine,[5,6] tyrosine[7] and tryptophan.[8] During this hydroxylation activity, an oxygen molecule is added by a mechanism that is independent of the presence of a heme structure[5,9] to form a peroxy compound, which is oxidized first into quinonoid dihydrobiopterin and finally into dihydrobiopterin (BH_2). BH_2 is then reduced back into BH_4 by an NAD(P)H-dependent reductase.[10] The oxidation of BH_4 is also promoted by heme systems. It is then an oxoferryl species that is held responsible for the oxidation,[11] and the BH_4 oxidation would take place via a process similar to that presented by Patterson.[12] According to this hypothesis, the combination of myoglobin (Mb), an oxygen carrier, and hydrogen peroxide (H_2O_2) forms, via the production of a porphyrin (Por) pi-cation radical, an oxoferryl species with a hypervalent iron capable of oxidizing biological targets as depicted in reaction given below [Eq. (1)]:

$$\begin{aligned} &\text{HMb–Por–Fe}^{\text{III}} + \text{H}_2\text{O}_2 \\ &\quad\rightarrow [\text{HMb–Por}^{+\cdot}\text{–Fe}^{\text{IV}} = \text{O}] \\ &\quad\rightarrow {}^{\cdot}\text{Mb–Por–Fe}^{\text{IV}} = \text{O} + \text{H}_2\text{O} + \text{H}^+ \quad (1) \end{aligned}$$

BH_4 is also an essential cofactor for the activity of the heme enzymes nitric oxide synthases (NOS).[1,10,13] BH_4 has been reported to modify the structure of NOS and to inhibit the production of superoxide anion ($O_2^{-\cdot}$) from NOS by generating oxoferryl species via the formation of heme-peroxo species when L-arginine is present.[14] However, the exact mechanism by which BH_4 is implicated in nitric oxide ($^{\cdot}NO$) biosynthesis still remains obscure. BH_4, by its oxidation into BH_2, surely acts in the electron transfer process,[15] and a mechanism involving free radical species has been recently proposed.[16]

Furthermore, a limitation of the availability of BH_4 would result in an increased production of $O_2^{-\cdot}$ by NOS in endothelial cells, resulting in the formation of potent oxidant species such as peroxynitrite ($ONOO^-$).[17,18] It is well established that $ONOO^-$ is formed *in vivo* from the reaction of $O_2^{-\cdot}$ with $^{\cdot}NO$ and that this reaction seems to be controlled only by the diffusion rate of these two compounds.[19] Just after its formation, $ONOO^-$ is capable of reacting with a variety of relevant biomolecules such as proteins,[20,21] DNA,[22] thiols,[23] lipids,[24] and antioxidant compounds.[25-27]

The activity of NOS can thus be related to the *in situ* production of $ONOO^-$ when NOS activity occurs in a cellular compartment where $O_2^{-\cdot}$ production is also possible, which can certainly occur in activated neutrophils or macrophages. To understand the interactions of $ONOO^-$ with BH_4, a cofactor needed for the early activity of NOS merits further research.[28] Data have been published on the interactions of $ONOO^-$ with the heme moiety of NOS, but the direct interactions of $ONOO^-$ with BH_4 have not been completely studied at the molecular level.

Milstien *et al.*[29] recently reported that BH_4 was oxidized by $ONOO^-$ yielding a quinonoid dihydrobiopterin, further losing its side chain to form 7,8-dihydropterin. By this way, BH_4 promotes its own destruction together with endothelial cell dysfunctions due to a decreased production of $^{\cdot}NO$.

An important unsolved problem is to know if homolytic or heterolytic (or both) pathways are involved in these processes since radical pathways are more and more implicated in the reactions of $ONOO^-$ with biomolecules.[30-32] Thus, there is a strong impetus for studying the molecular mechanism of BH_4 oxidation by $ONOO^-$, a challenge that we faced in this study, comparing the results with those obtained for the reaction of BH_4 with Mb/H_2O_2, a typical oxoferryl producing system. The kinetics of the

reactions were monitored by UV–visible spectrophotometry with diode array detection and by using a combination of ultra-weak chemiluminescence (uwCL) and electron paramagnetic resonance (EPR) spin trapping. Chemiluminescence was used for the global detection of activated species and EPR for radical intermediates detection, a combination of techniques that we previously applied for the reaction of $ONOO^-$ with heterocyclic biomolecules.[32]

MATERIALS AND METHODS

Chemicals

BH_4 [2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-4(3H)-pteridinone] and horse heart myoglobin (Mb) were purchased from Sigma. H_2O_2 and phosphate salts (NaH_2PO_4 and K_2HPO_4) were from Merck (analytical grade). The spin trap α-(4-pyridyl-1-oxide)-*N-tert* butylnitrone (POBN) was purchased from Aldrich. Peroxynitrite was synthesized as described below. All the experiments were carried out in phosphate buffer at pH values ranging from 7.0 to 8.0. The other chemical products were of analytical grade and used as such.

Peroxynitrite Syntheses

$ONOO^-$ alkaline solutions were prepared by three different ways:

- by synthesis from sodium azide and ozone as previously described by Uppu *et al.*[33]
- in a quenched flow system by mixing H_2O_2 and sodium nitrite in acidic conditions as described by Koppenol *et al.*[34]
- by the reaction of tetramethylammonium superoxide with $^{\cdot}NO$, yielding solid tetramethylammonium $ONOO^-$ ($[(CH3)_4N]^+ ONOO^-$) as described by Bohle *et al.*[35] The solid tetramethylammonium $ONOO^-$ used in this study was in part, a gift from the Laboratory of Inorganic Chemistry, ETH Zürich, Switzerland (Professor W.H. Koppenol).

The purity and concentration of $ONOO^-$ samples were determined by spectrophotometry in NaOH aqueous solution, using an extinction coefficient of $1700\,M^{-1}\,cm^{-1}$ for the main peak located at 302 nm.[36] The solutions of $ONOO^-$ were stored at −80°C.

Chemiluminescence Assays

Ultra-weak chemiluminescence measurements were carried out with a Bio-Orbit 1251 luminometer (Turku-Finland) at room temperature, in polystyrene tubes. For the reaction involving $ONOO^-$, BH_4 in phosphate buffer (pH 8.0) was automatically added to an $ONOO^-$alkaline solution with a Hamilton syringe. The final volume was 500 µl and the solution was 1 mM in BH_4 and 1 mM in $ONOO^-$. POBN (final concentration of 50 mM) was added at different times during the uwCL reaction. For the uwCL assays performed with the Mb/H_2O_2 system, H_2O_2 was injected (together with POBN in some assays) to a 500 µl mixture containing 50 µg Mb and 1 mM BH_4 dissolved in phosphate buffer at pH 7.4. The final concentrations were 1 mM for H_2O_2 and 50 mM for POBN. At the end of each assay, the final pH value of the reaction mixture was controlled to be in the range of 7.0–8.0. The uwCL assays were monitored for 30 s and repeated at least three times.

EPR Spin Trapping Experiments

After uwCL measurement, the reaction mixture containing $ONOO^-$, BH_4 and POBN was immediately put in an ice bath, quickly transferred into a quartz flat cell and placed into the TM_{110} cavity of the EPR spectrometer. For the experiments with the Mb(50 µg)/H_2O_2(1 mM) system, the samples were prepared just before EPR measurements as the lifetime of the POBN spin adducts was found

to be very short. In the same way, experiments under nitrogen were performed in order to investigate the influence of oxygen on the oxidative process. All measurements were carried out at room temperature on a Bruker spectrometer ESP 300E (Bruker Karlsruhe, Germany), operating at non-saturating microwave power (20 mW) and 9.75 GHz microwave frequency. The other instrumental settings were as follows: 3480 G center field, 100 G scan range, 40.96 ms time constant, 20.40 ms conversion time, 1.01 G modulation amplitude, 2.10^4 receiver gain. The number of scans was 6 for all the experiments.

UV–visible Diode Array Spectrophotometric Measurements

All the reactions were carried out at room temperature on an HP ChemStation (HP 845× UV–visible software) with an HP 8453 UV–visible spectrophotometer (Hewlett-Packard, Waldbronn, Germany) fitted with a 1024-element diode-array. The monitoring of the reaction was done by following the changes of the spectrum from 190 to 1100 nm (10 scans/s) during 150 min for the BH_4 autoxidation assays or 15 min for the other assays. The measurements were performed in phosphate buffer at pH 8.0 with 1 mM $ONOO^-$ or at pH 7.4 with 10 μg Mb/0.1 mM H_2O_2. In all cases, the concentration of BH_4 was 0.1 mM.

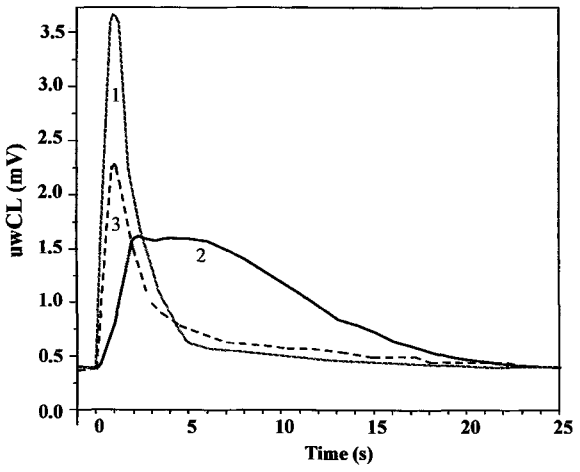


FIGURE 1 Ultraweak chemiluminescence (uwCL) curves resulting from the reaction of 1 mM $ONOO^-$ with 1 mM BH_4 in phosphate buffer at pH 8.0. Curve 1: $ONOO^-$ alone. Curve 2: $ONOO^-$+BH_4. Curve 3: same as curve 2 with addition of 50 mM of the spin trap POBN at time 0.

RESULTS

Oxidation of BH_4 by Peroxynitrite

Production of uwCL by the Reaction of $ONOO^-$ with BH_4

When 1 mM peroxynitrite (synthesized in the solid form) was added to a buffer solution at pH 8.0, a weak uwCL was produced (Fig. 1, curve 1).

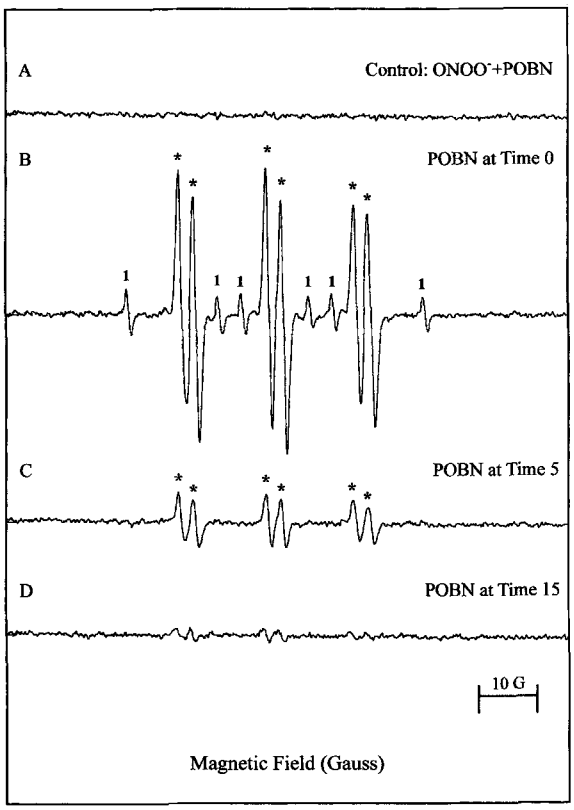


FIGURE 2 EPR spectra of the POBN spin adducts of the reaction of 1 mM $ONOO^-$ with 1 mM BH_4 in phosphate buffer (pH 8.0). Spectrum A: no BH_4 (control). Spectra B, C and D were obtained when 50 mM POBN were added at time 0, 5 or 15 s after starting of the reaction between $ONOO^-$ and BH_4. The *g*-factor value was 2.0028. Total running time: 6 min. The asterisks (*) identify the six-line structure.

The exposure of BH_4 to 1 mM $ONOO^-$ at pH 8.0, in the absence of POBN, produced an uwCL with a profile characterized by a spread peak of light that slowly decreased and returned to baseline after about 25 s (Fig. 1, curve 2).

The addition of 50 mM POBN to the mixture ($ONOO^-$ and BH_4) at time 0 (Fig. 1, curve 3) resulted in a decrease of the spread peak of uwCL compared to curve 2. Addition of 50 mM POBN at time, 5 or 15 s after the start of the reaction also induced a decrease of the uwCL that returned to baseline after about 25 s (data not shown). Similar results were obtained with other $ONOO^-$ solutions prepared from $ONOO^-$ synthesized in liquid form.

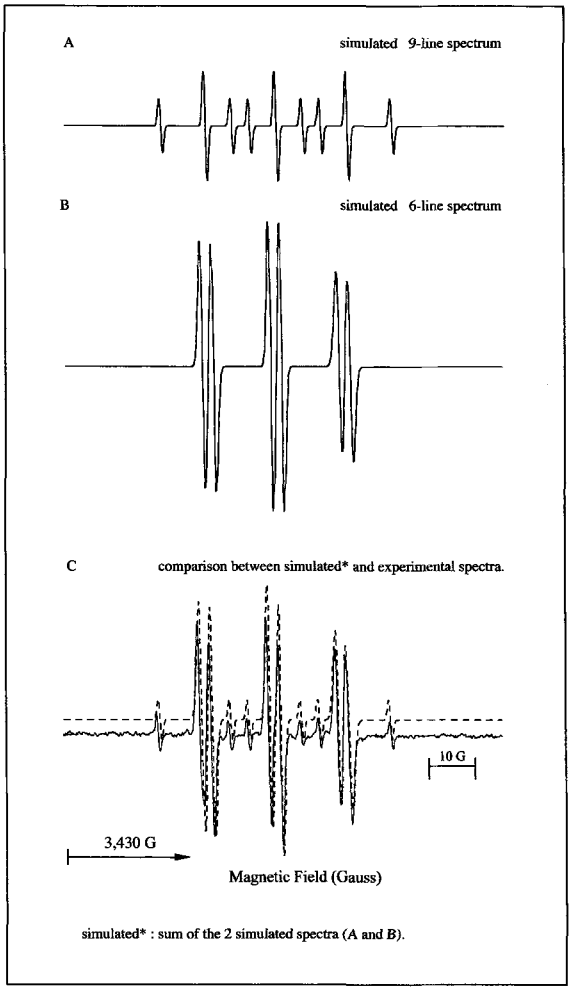


FIGURE 3 Simulated EPR spectra of POBN spin adducts. Spectrum A: Trapping of a H-atom. Spectrum B: Trapping of $^{\bullet}BH_3$ radical. Spectrum C: same as in Fig. 2B (solid line) with the superposition of the spectrum resulting from the addition of simulated spectra A and B (dashed line).

EPR Results

In the absence of BH_4, no EPR spectrum of POBN spin adduct was observed (Fig. 2A). When 1 mM BH_4 was added at pH 8.0 to the solution of $ONOO^-$ (synthesized in the solid form), in the presence of 50 mM POBN, a mixture of two spectra was obtained and characterized by an intense six-line EPR spectrum plus a fleeting nine-line spectrum (Fig. 2B). When POBN was added after 5 and 15 s, we observed a decrease in the intensity of the six-line EPR spectrum accompanied by the disappearance of the nine-line spectrum (Fig. 2C and D).

The nine-line EPR spectrum resulted from the trapping of H-atom radical by POBN with relative intensities (1:2: 1:1:2:1: 1:2: 1). However, the lines of intensity 2 were masked by the more intense six lines (asterisked lines on Fig. 2). To validate these results, simulated spectra resulting from the

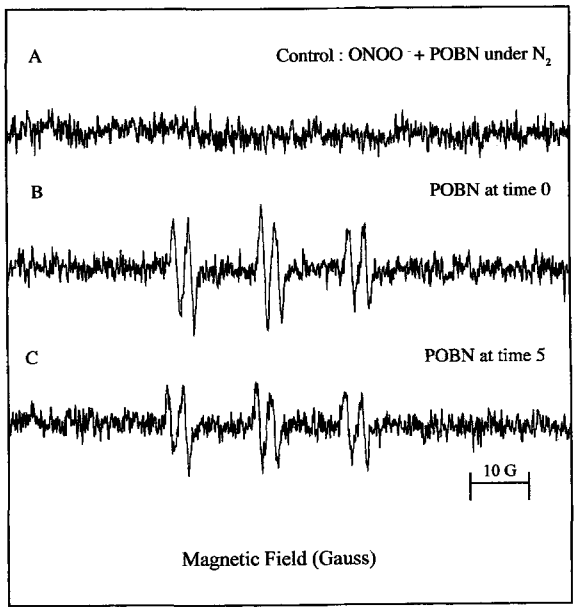


FIGURE 4 EPR spectra A–C, same as in Fig. 2A–C, but the experiment was carried out under N_2 atmosphere. The *g*-factor value was 2.0037. Total running time: 6 min.

trapping either of a H-atom or $^{\cdot}BH_3$ by POBN were performed from experimental data using a WIN Simfonia simulation software and compared with the experimental spectrum (Fig. 3A and B). In addition, both simulated spectra were added and the resulting spectrum was finally compared with the experimental one (Fig. 3C). Since the importance of oxygen in oxidative processes is well known, we performed the same experiments under N_2 atmosphere. In these conditions, the EPR signal was strongly reduced compared to that obtained under air (Fig. 4A–C).

Similar results were obtained with $ONOO^-$ prepared from either hydrogen peroxide and sodium nitrite or sodium azide and ozone (data not shown). These results indicated that the reaction of $ONOO^-$ with BH_4 occurred, at least in part, via a radical pathway.

Oxidation of BH_4 by the Mb/H_2O_2 System

Inhibiting Effect of BH_4 on uwCL Produced by the Mb/H_2O_2 System

At pH 7.4, the reaction of 1 mM BH_4 with 1 mM H_2O_2 resulted in a minute luminescence (data not shown). The addition of 50 μg Mb to 1 mM H_2O_2, in the absence of spin trap POBN and of BH_4, resulted in a light emission of which the lifetime did not exceed 5 s (Fig. 5A). When BH_4 was added, a drastic reduction of the uwCL signal was observed (from 107 to 0.6 mV) (Fig. 5B). This residual uwCL was nearly equivalent to that measured for BH_4+$ONOO^-$ (see Fig. 1). The addition of 50 mM POBN together with H_2O_2 also strongly decreased the intensity of the light emission (Fig. 5C). Addition of 50 mM POBN, after 5 or 15 s, did not modify the spectrum because the reaction was already finished.

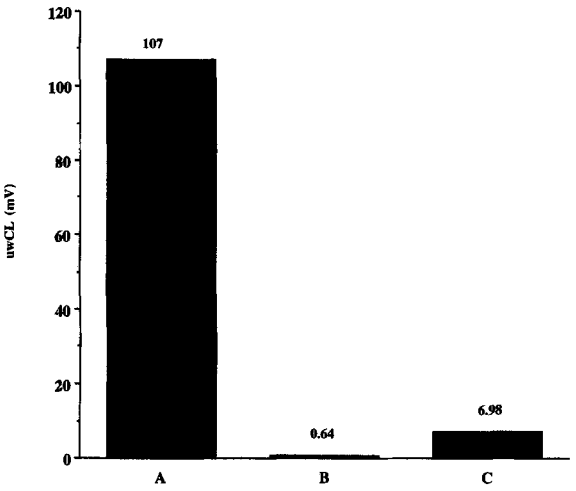


FIGURE 5 Inhibiting effect of BH_4 on the uwCL induced by the Mb/H_2O_2 system at pH 7.4. (A) Mb (50 μg)+H_2O_2 (1 mM); (B) same as A+1 mM BH_4; (C) same as A+ 50 mM POBN injected at time 0.

EPR Results of the Reaction of BH_4 with Oxoferryl Species

The reaction of oxoferryl species (Mb/H_2O_2) with various concentrations of BH_4 (0.1, 0.5 and 1 mM),

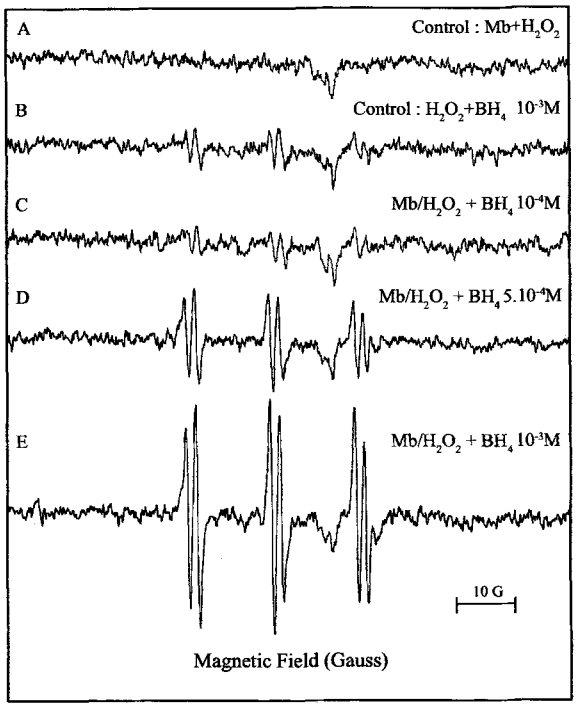


FIGURE 6 EPR spectra of POBN spin adducts of the reaction of the Mb (50 μg)/H_2O_2 (1 mM) system with BH_4. (A) Mb+H_2O_2. (B) H_2O_2+BH_4 (1 mM). Spectra C–E: same as A with increasing concentrations of BH_4 (10^{-4}, 5.10^{-4} and 10^{-3} M, respectively). POBN was 50 mM and pH was 7.4. The *g*-factor value was 2.0032. Total running time: 6 min.

performed in phosphate buffer (pH 7.4) in the presence of 50 mM POBN, resulted in the appearance of a six-line EPR spectrum, and the intensity of this EPR signal increased with the concentration of BH_4 (Fig. 6, spectra C–E). This EPR spectrum was similar to the six-line spectrum observed for the reaction of BH_4 with $ONOO^-$ (see Fig. 2).

Control experiments performed with Mb/H_2O_2 and POBN (no BH_4 addition) showed no EPR spectrum (Fig. 6, spectrum A). In contrast, the control performed with H_2O_2 and 1 mM BH_4 (no Mb addition) showed the same six-line spectrum, that we attributed to a slight oxidation of BH_4 by H_2O_2 (Fig. 6, spectrum B), and of which the intensity remained very weak when compared to spectrum E. The addition of POBN to the Mb/H_2O_2+BH_4 solution at times 5 or 15 s after the start of the reaction, did not produce EPR spectra (data not shown).

UV–visible Spectrophotometric Study of BH_4 Reaction with $ONOO^-$ or the Mb/H_2O_2 System

The spectrum of BH_4 in phosphate buffer (pH 8.0) was composed of two peaks located at 220 and 298 nm, respectively. Exposure of this buffered BH_4 solution to ambient air led to an autoxidation characterized by the appearance of a new peak at 274 nm and by a bathochromic shift of the peaks at 220 and 298 nm to 227 and 333 nm, respectively (Fig. 7). In addition to these observations, 3 isosbestic points (222, 285 and 318 nm) were seen (marked with "I" arrows on Fig. 7). In contrast, when the experiment was carried out under N_2 atmosphere, only the main peak (298 nm) of BH_4 appeared that slightly decreased over time. Figure 8 shows that the oxidation of 0.1 mM BH_4 by 1 mM $ONOO^-$ led to a decay of the peak located at 298 nm, the appearance of two other ones at 277 and 328 nm, and the presence of 3 isosbestic points at 247, 263 and 285 nm (marked with "I" arrows on inset of Fig. 8). All the $ONOO^-$ solutions (prepared with liquid or solid $ONOO^-$) gave the same results. It should be noted that the strong absorbance in the region of 300 nm observed at the beginning of the reaction (Fig. 8, spectra 1–3) was due to $ONOO^-$.

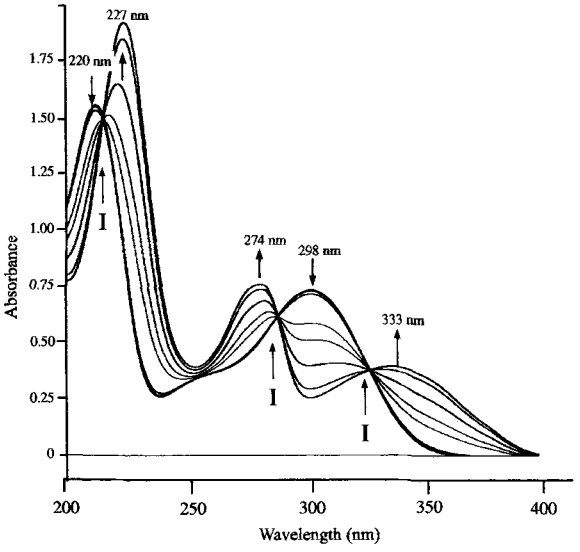


FIGURE 7 Evolution of the UV–visible spectrum obtained for the autoxidation of 0.1 mM BH_4 at pH 7.4 (phosphate buffer). The reaction was followed during 150 min. "I" arrows indicate isosbestic points.

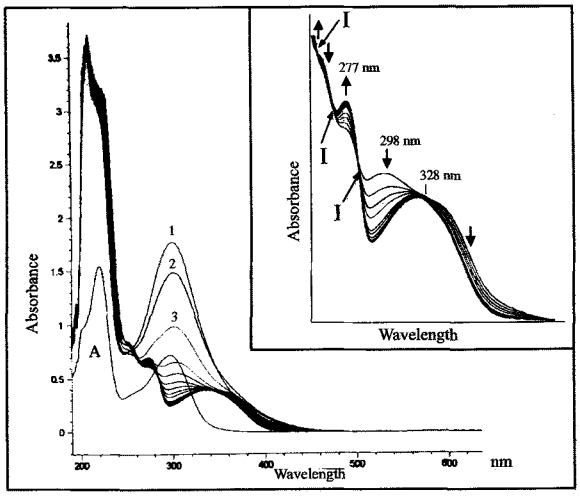


FIGURE 8 Evolution of the UV–visible spectrum of the reaction of 0.1 mM BH_4 with 1 mM $ONOO^-$. The reaction was followed during 15 min. A: spectrum of 0.1 mM BH_4 before the addition of $ONOO^-$. Curves 1,2 and 3: rapid decrease of $ONOO^-$. The inset represents an enlarged view of the latest spectra of the evolution of the reaction. "I" arrows indicate isosbestic points.

The replacement of $ONOO^-$ by 10 μg Mb and 0.1 mM H_2O_2, in a final volume of 2 ml, showed an UV–visible curve slightly different from that obtained with $ONOO^-$ (Fig. 9). In this latter system, the decrease of the 298 nm peak was counterbalanced by the appearance of a new peak at 282 nm with two isosbestic points at 285 and 318 nm (marked with "I" arrows on inset of Fig. 9). In contrast, the peaks located at 274 and 333 nm were not observed.

The difference of the spectral profiles obtained with both oxidant systems ($ONOO^-$ and Mb/H_2O_2) could be explained by a more selective reactivity of myoglobin system compared to the complex reaction possibilities of $ONOO^-$ with BH_4.

DISCUSSION

Tetrahydrobiopterin (BH_4) has been reported to be oxidized by $ONOO^-$[28] to yield three main products: 7,8-dihydrobiopterin as the major one at acidic pH, and 7,8-dihydropterin and dihydroxanthopterin as the major ones at basic pH. Furthermore, it is known that BH_4 is susceptible to autoxidation at neutral pH.[37] We demonstrated that $ONOO^-$ oxidized BH_4, and that the oxidative system Mb/H_2O_2, already described to take part in the *in vivo* redox processes,[38–40] also induced an oxidation of BH_4.

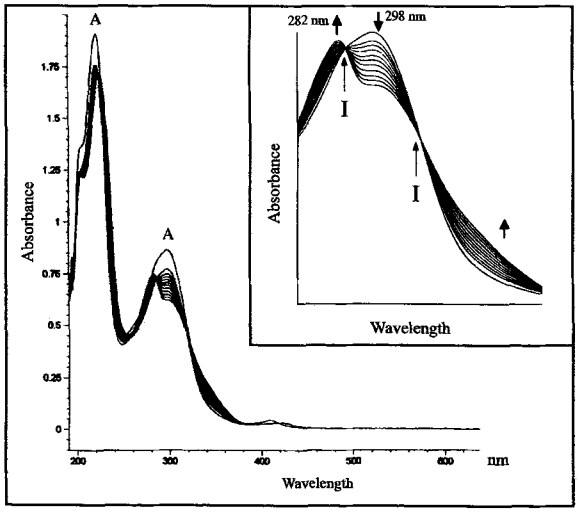
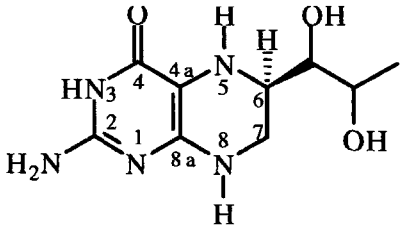


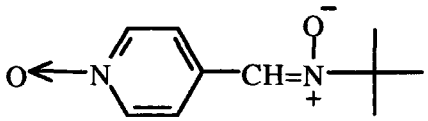
FIGURE 9 Evolution of the UV–visible spectrum of the reaction of 0.1 mM BH_4 with the Mb (10 μg)/H_2O_2 (0.1 mM) system. The reaction was followed during 15 min. A: spectrum of 0.1 mM BH_4 before Mb/H_2O_2 addition. The inset represents an enlarged view of the latest spectra of the evolution of the reaction. "I" arrows indicate isosbestic points.

Firstly, the reactivity of BH_4 towards three different preparations of $ONOO^-$ was investigated. We observed in all cases, the appearance of a spread peak of light indicating the presence of activated species. According to our data, these latter species arose from $^{\cdot}BH_3$ radical rather than from the decomposition of $ONOO^-$ because, at this low pH, $ONOO^-$ had a short lifetime which did not exceed 1 s. When POBN was added together with $ONOO^-$, the more intense but short-living peak of light that was observed (Fig. 1, curve 3) could result from the decomposition of $ONOO^-$ and from the reaction of POBN with excited species resulting from BH_4 oxidation. When POBN was added later, there was a decrease in the chemiluminescence by scavenging the $^{\cdot}BH_3$ radical and yielding a new POBN-centered radical that did not emit light.

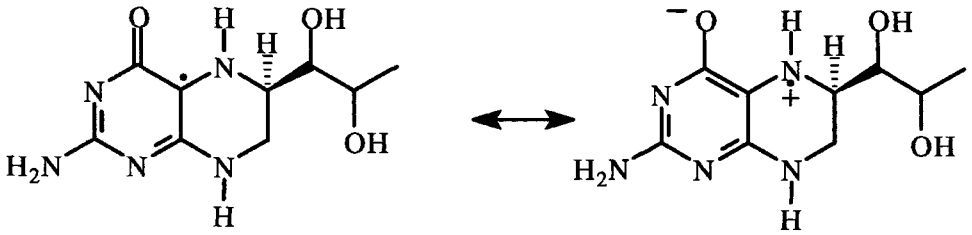
Secondly, BH_4 oxidation by $ONOO^-$ yielded an EPR spectrum characterized by a mixture of two spin trapped components: a six-line spectrum ($a_N = 15.7$ G and $a_H^\beta = 2.6$ G) of higher intensity corresponding to a carbon-centered radical ($^{\cdot}BH_3$) and a weak nine-line spectrum ($a_N = 16.2$ G and $a_H^\beta = 10.2$ G) resulting from an H-atom radical trapping by POBN. This latter spectrum rapidly disappeared by contrast with the six-line one, which still persisted after 5 s (Fig. 2, spectra B and C). Indeed, as described in Scheme 1, the formation of a carbon-centered radical from BH_4 was favoured because the spin density at the C(4a)-atom was the highest.[41] The high intensity observed in the case of the six-line spectrum could be explained by the additional role of oxygen in the oxidative process as described in Scheme 2. These results were confirmed by the experiments carried out



(6R)-Tetrahydrobiopterin



α-(4-pyridyl-1-oxide)-N-tert butylnitrone

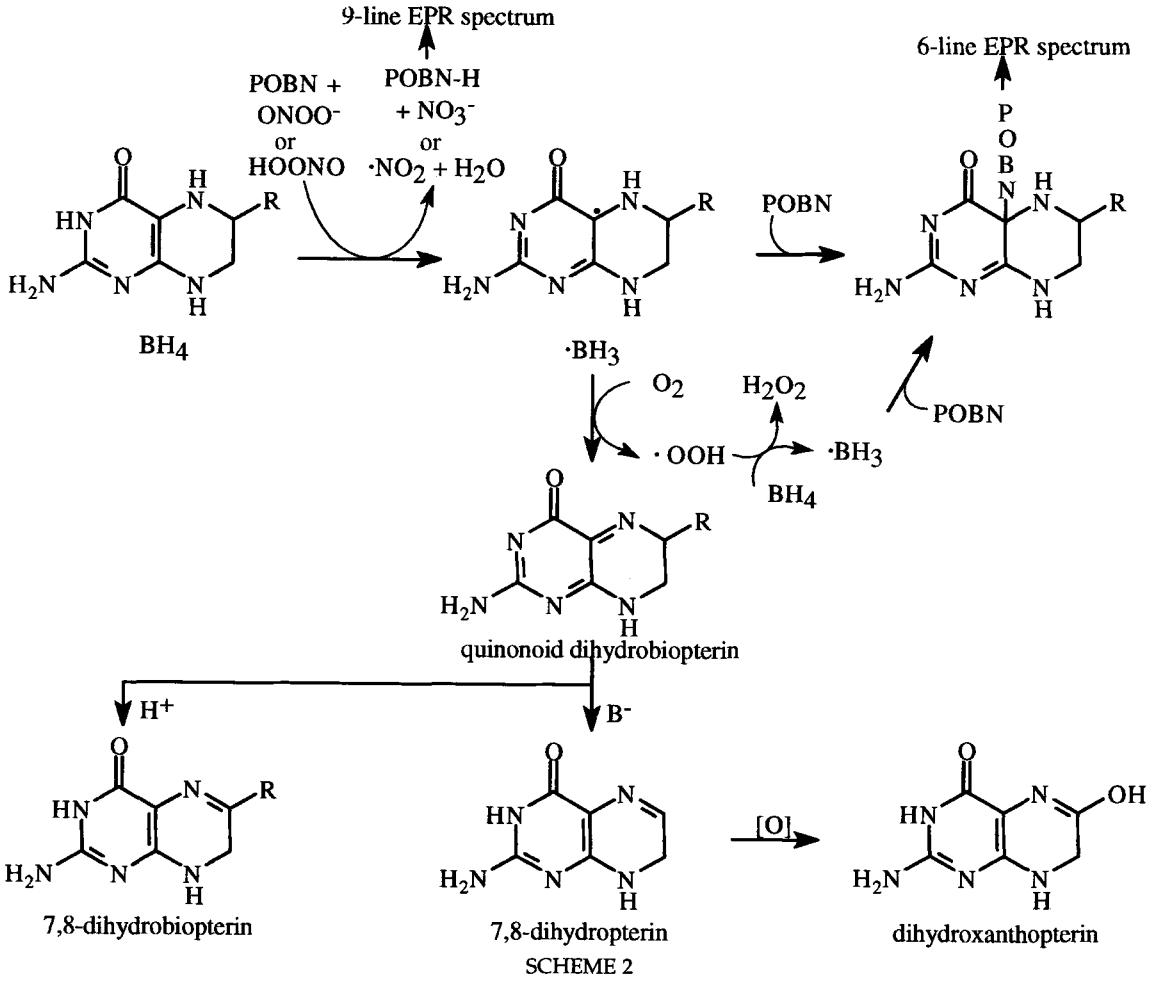


The 2 most contributive resonance forms of the ·BH_3 radical

SCHEME 1

under N_2 atmosphere that showed a strong decrease of this signal (Fig. 4, spectra A–C). However, the 9-line spectrum observed in our experimental conditions and originating from a H-atom radical trapping was more surprising and subject to discussion since little is known about the H-abstraction in peroxynitrite chemistry. It is possible that $ONOO^-$ promoted a homolytic rupture of a N–H bound, e.g. at N(3) atom, yielding a H-atom radical, which could further react with the spin trap agent POBN or with a radical species produced by the decomposition of $ONOO^-$, maybe a hydroxyl radical.[42] This latter reaction would be faster than that with POBN, thus explaining the relatively weak signal intensity of the POBN–H adduct. Our hypothesis on the implication of the H-atom was in agreement with the literature data showing that the H-atom that was generated by sonication of water, could be trapped by POBN ($a_N = 16.4$ G and $a_H^\beta = 10.1$ G).[43] The simulation of the 9-line spectrum of the bimolecular reaction between H-atom and POBN by using the experimental parameters confirmed our findings. The addition of two separate simulated spectra of 9 and 6 lines (Fig. 3A and B) led to a spectrum which fitted well the experimental one. This observation confirms the presence of H-atom and BH_3 species in our experimental conditions (Fig. 3C).

Thirdly, the reactivity of BH_4 towards the Mb/H_2O_2 system was investigated for comparison. The Mb/H_2O_2 system produced an important uwCL. But, when BH_4 was added to the system, a drastic decrease of the uwCL was observed, indicating a reaction of BH_4 with the activated species resulting from the Mb/H_2O_2 interactions. This residual uwCL also suggested that the new activated species so produced were probably similar to those resulting from the reaction of BH_4 with $ONOO^-$ since quite same uwCL intensities were observed in both cases. The intensic limits of the uwCL current devices did not permit the identification of the wavelengths of the activated species. But, it was highly probable that this residual luminescence could be accompanied by a radical state of BH_4



SCHEME 2

produced as summarized in Eqs. (2) and (3):

$$Mb \cdot Fe^{IV} = O + BH_4 \rightarrow {}^{\cdot}BH_3 + H\text{–}Mb\text{–}Fe^{IV} = O \quad (2)$$

$$H \cdot Mb\text{–}Fe^{IV} = O + {}^{\cdot}BH_3 + H^+ \rightarrow BH_2 + Mb\text{–}Fe^{III} + H_2O \quad (3)$$

Indeed, by EPR spin trapping technique, we confirmed this $^{\cdot}BH_3$ production since we observed a six-line spectrum resulting from the trapping of $^{\cdot}BH_3$ radical by POBN, with the following coupling constants ($a_N = 15.4$ G and $a_H = 2.8$ G). This EPR signal did not result from a species directly deriving from the Mb/H_2O_2 system, at least in our experimental conditions, because no EPR spectrum was observed in the absence of BH_4 (Fig. 6, spectrum A). Nevertheless, in particular conditions (direct EPR at low temperature), it was possible to observe an EPR spectrum of Mb-derived peroxyl radical from the Mb/H_2O_2 system alone.[44]

The reaction of BH_4 with H_2O_2 without Mb addition also yielded a weak EPR signal (Fig. 6, spectrum B). This observation has already been described and attributed to a partial BH_4 oxidation by H_2O_2 (about 20% of BH_4 was oxidized after 5 min with 100 μM H_2O_2 addition).[26] Previous observations also demon-

strated that BH_4 oxidation by H_2O_2 led to the production of dihydrobiopterin.[37]

Our results thus support the hypothesis that the oxidation of BH_4 by the Mb/H_2O_2 system occurred according to a radical pathway. It was reported that heme peroxo species reacted with different substrates by two sequential 1-electron oxidations,[39] and a $^{\cdot}BH_3$ radical species has already been described by Hurshmann *et al.*[16] in the reaction of the heme domain of inducible NOS with O_2. Therefore, we hypothesize that the reaction of BH_4 with Mb/H_2O_2 led to the formation of a trihydrobiopterin radical ($^{\cdot}BH_3$) that further rearranged to 7,8-dihydrobiopterin in the same manner as for the reaction of BH_4 with $ONOO^-$ (see Scheme 2).

Recently, Milstien and Katusic[29] suggested that both autoxidation and peroxynitrite-dependent BH_4 oxidation proceeded by the same mechanism, most likely involving reactive oxygen species. These observations were confirmed by our UV–visible results. We observed that BH_4, when dissolved in phosphate buffer at pH 8.0 without buffer deoxygenation, slowly became oxidated with an evolution of the UV–visible profile that was quite similar to that of the BH_4 oxidation induced by $ONOO^-$. The main differences between the two spectra appeared below 250 nm: an isosbestic point was formed at 222 nm for the BH_4 autoxidation, but this isosbestic point was absent for the reaction of BH_4 with $ONOO^-$, because the products of $ONOO^-$ decay strongly absorbed in this wavelength range. In addition, we could not follow the appearance of the isosbestic point at 318 nm because of the interfering effect of $ONOO^-$ in this region of the spectrum.

In summary, as described in Scheme 2, $ONOO^-$ initially oxidized BH_4 into the $^{\cdot}BH_3$ radical. This latter species was then oxidized by atmospheric oxygen (which was reduced to hydrogen to give the quinonoid intermediate q-BH_2. In a further step, q-BH_2 would rearrange to give 7,8-dihydrobiopterin (7,8-BH_2), 7,8-dihydropterin and dihydroxanthopterin in agreement with data previously reported by Milstien and Katusic.[29]

Nevertheless, small differences in the profile of UV–visible spectrum of the reaction of BH_4 with Mb/H_2O_2 system compared to the oxidation of BH_4 by $ONOO^-$ seemed to indicate that only one of the two pathways involving the q-BH_2 rearrangement occured during the oxidation of BH_4 by Mb/H_2O_2, likely, leading to 7,8-BH_2 formation. This different behavior could be explained by a selective pathway directed to the production of BH_2 for the BH_4 oxidation by Mb/H_2O_2, whereas the oxidation by $ONOO^-$ was less selective, giving more than one product.

We previously demonstrated that $ONOO^-$, in the presence of the spin trap POBN, induced the oxidation of tryptophan by a radical pathway.[32] In the same way, Kirsch *et al.* reported the existence of a radical pathway for the reaction of $ONOO^-$ with HEPES.[30] The results reported here thus supported the hypothesis that the oxidation of BH_4 by $ONOO^-$ was also accompanied by a radical process: uwCL assays coupled with EPR spin trapping technique showed EPR spectra characteristic of the trapping of a $^{\cdot}BH_3$ radical that was formed during the oxidation of BH_4 either by oxoferryl species or by $ONOO^-$. In the case of the BH_4 oxidation by $ONOO^-$, a hydrogen atom was also trapped by POBN.

From these results, it can thus be extrapolated that, when BH_4 inhibits the production of $O_2^{\cdot-}$ from NOS or stimulates the formation of heme-peroxo species as reported by Vasquez-Vivar and coworkers,[14] this will occur via radical reaction pathways and has great relevance for biological systems. Nevertheless, further research is needed to fully understand the reaction pathways (and their consequences) if $ONOO^-$ oxidizes BH_4 bound to NOS enzyme *in vivo*.

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