Three Different Oxygen-induced Radical Species in Endothelial Nitric-oxide Synthase Oxygenase Domain under Regulation by L-Arginine and Tetrahydrobiopterin*

Received for publication, April 12, 2004, and in revised form, May 21, 2004
Published, JBC Papers in Press, May 27, 2004, DOI 10.1074/jbc.M404044200

Endothelial nitric-oxide synthase (eNOS) plays important roles in vascular physiology and homeostasis. Whether eNOS catalyzes nitric oxide biosynthesis or the synthesis of reactive oxygen species such as superoxide, hydrogen peroxide, and peroxynitrite is dictated by the bioavailability of tetrahydrobiopterin (BH4) and L-arginine during eNOS catalysis. The effect of BH4 and L-arginine on oxygen-induced radical intermediates has been investigated by single turnover rapid-freeze quench and EPR spectroscopy using the isolated eNOS oxygenase domain (eNOSox). Three distinct radical intermediates corresponding to >50% of the heme were observed during the reaction between ferrous eNOSox and oxygen. BH4-free eNOSox produced the superoxide radical very efficiently in the absence of L-arginine. L-Arginine decreased the formation rate of superoxide by an order of magnitude but not its final level or EPR line shape. For BH4-containing eNOSox, only a stoichiometric amount of BH4 radical was produced in the presence of L-arginine, but in its absence a new radical was obtained. This new radical could be either a peroxyl radical of BH4 or an amino acid radical was in the vicinity of the heme. Formation of this new radical is very rapid, >150 s⁻¹, and it was subsequently converted to a BH4 radical. The trapping of the superoxide radical by cytochrome c in the reaction of BH4(--eNOSox) exhibited a limiting rate of ~15 s⁻¹, the time for the superoxide radical to leave the heme pocket and reach the protein surface; this reveals a general problem of the regular spin-trapping method in determining radical formation kinetics. Cytochrome c failed to trap the new radical species. Together with other EPR characteristics, our data strongly support the conclusion that this new radical is not a superoxide radical or a mixture of superoxide and bioprotein radicals. Our study points out distinct roles of BH4 and L-arginine in regulating eNOS radical intermediates. BH4 prevented superoxide formation by chemical conversions of the Fe(II)O2 intermediate, and L-arginine delayed superoxide formation by electronic interaction with the heme-bound oxygen.

Nitric-oxide synthase (NOS), which catalyzes nitric oxide (NO) biosynthesis, is a complicated cytochrome P450-like enzyme. Three substrates (L-arginine, NADPH, and oxygen) and four products (NO, L-citrulline, NADP+, and water) are involved in the 5-electron oxidation of the guanidino nitrogen of L-arginine to yield NO. Four cofactors, FMN and FAD in the reductase domain and heme and BH4 in the oxygenase domain, participate in catalysis (1, 2). The reductase domain mainly functions as the source of reducing equivalents generated after NADPH binding, and the heme center is the site of the key chemical events. A tight coupling between the reductase and oxygenase domains maximizes NO formation rather than producing side products such as superoxide, hydrogen peroxide, and even peroxynitrite (3–6). When NOS behaves as a superoxide synthase or peroxynitrite synthase, it may cause endothelial dysfunction, arteriosclerosis, and even vascular injury and septic shock (7–10). The two major controlling factors in achieving an optimal redox coupling are the timely supply of L-arginine and the integration of BH4 into the oxygenase domain. Superoxide radical formation in three different NOS isoforms has been studied extensively in the presence or absence of either L-arginine or BH4 mainly by spin-trapping and EPR spectroscopy (3, 4, 11–13). It was found that autoxidation of flavin in the reductase domain dominates superoxide formation in iNOS (11), whereas breakdown of the Fe(III)O2 (or Fe(III)O3) heme intermediate is the main source of superoxide in nNOS and eNOS (3, 4, 12). Furthermore, superoxide formation in nNOS is almost completely inhibited by L-arginine, whereas in eNOS only BH4 appears to suppress superoxide formation fully (3, 4, 12). It is critical to understand these isozyme-specific regulatory processes of eNOS to develop strategies to intervene in NOS-derived vascular dysfunction in an isozyme-specific manner.

Several experimental barriers hamper elucidation of the underlying mechanisms regarding the differential regulation of superoxide formation by L-arginine and/or BH4. First, most previous studies were carried out under steady-state conditions, which involve many complicated chemical steps from both the reductase and the oxygenase domains, and it is difficult to locate the key steps that define the kinetics of superoxide formation. Second, most of the previous studies used excess

* This work was supported by United States Public Health Service Grants GM56818 (to A. T.) and GM55807 (to G. P.) and by Welch Foundation Grant C636 (to G. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center at Houston, Houston, TX 77030

‡ From the Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center at Houston and the Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77030

§ To whom correspondence should be addressed: Division of Hematology, Department of Internal Medicine, University of Texas Health Science Center at Houston, Houston, TX 77025. Tel.: 713-500-6771; Fax: 713-500-6810; E-mail: Ah-lim.Tsai@uth.tmc.edu

¶ D. Xu

∥ R. Wu

§ S. Meyer

¶ J. Wu

†† H.-C. Yeh

§§ G. Palmer

¶¶ A.-l. Tsai

 downloadable from http://www.jbc.org by guest on July 23, 2018
BH4 in addressing the effect of BH4 in regulating superoxide formation. The presence of free BH4 complicates data interpretation because of its side reactions. BH4 reacts with superoxide directly at a respectable rate, $10^7 \text{M}^{-1} \text{s}^{-1}$ (14). This same effect was claimed to be the mechanism by which BH4 prevents NO feedback inhibition of nNOS by channeling NO to peroxynitrite formation (15). Thus, excess BH4 could change the overall steady-state kinetics of NO production. In addition, autoxidation of free BH4 could itself be a source of superoxide, thus causing overestimation of the NOS-derived superoxide. Third, the commonly used spin-trapping EPR measurement for superoxide radical is an indirect determination for superoxide. The rate-limiting radical capturing of spin-traps marks the true rate of superoxide formation, and the trapping efficiency also leads to underestimation of the total amount of superoxide radical (16).

To achieve an unambiguous resolution of the regulatory mechanisms of l-arginine and/or BH4 on superoxide formation, we decided to take direct approaches. We focused on the eNOS oxygenase domain and examined the fate of the Fe(II)O2 heme complex by optical and EPR spectroscopy under single turnover conditions. We thus exclude possible superoxide formation from flavin oxidation. Rapid-freeze trapping/EPR measurement was used to replace spin-trapping to obtain direct structural and kinetic data for superoxide and other radical intermediates (17). More reliable quantification for the amount of radical intermediate(s) was accomplished without the complication of trapping efficiency encountered in spin-trapping and the secondary degradation of spin adduct (16). Only NOS-bound BH4 was evaluated, thus eliminating potential side reactions. In this study, we provide the first direct structural as well as kinetic information for a superoxide, a BH4, and a new radical intermediate occurring during the reaction between ferrous eNOSox and oxygen. This work convincingly shows that BH4 is the factor that dictates the radical structure, whereas l-arginine, on the other hand, has no effect on the radical structure but exhibits substantial influence on the radical kinetics. Our data also substantiate the functional role of BH4 in minimizing the formation of oxygen active species under uncoupled condition of eNOS.

**EXPERIMENTAL PROCEDURES**

**Materials**—BH4, BH2, and 5-methyl BH4 were obtained from Schircks Laboratories (Jona, Switzerland). Horse heart cytochrome c (type III), bovine erythrocyte superoxide dismutase, and other chemicals were obtained from Sigma. Horseradish peroxidase, grade I, was purchased from Roche Applied Science. SEITU was the product of Alexis, San Diego.

**Expression and Purification of Human eNOS Oxygenase Domain (eNOSox)**—Recombinant human eNOSox was prepared using a yeast expression system as described previously (18). The biotin content of the purified eNOSox from yeast expression was less than 0.2/subunit, was measured using the procedure described previously (20). Quantiﬁcation of the secondary degradation of spin adduct (16). Only NOS-bound BH4 was evaluated, thus eliminating potential side reactions. In this study, we provide the first direct structural as well as kinetic information for a superoxide, a BH4, and a new radical intermediate occurring during the reaction between ferrous eNOSox and oxygen. This work convincingly shows that BH4 is the factor that dictates the radical structure, whereas l-arginine, on the other hand, has no effect on the radical structure but exhibits substantial influence on the radical kinetics. Our data also substantiate the functional role of BH4 in minimizing the formation of oxygen active species under uncoupled condition of eNOS.

**EXPERIMENTAL PROCEDURES**

**Materials**—BH4, BH2, and 5-methyl BH4 were obtained from Schircks Laboratories (Jona, Switzerland). Horse heart cytochrome c (type III), bovine erythrocyte superoxide dismutase, and other chemicals were obtained from Sigma. Horseradish peroxidase, grade I, was purchased from Roche Applied Science. SEITU was the product of Alexis, San Diego.

**Expression and Purification of Human eNOS Oxygenase Domain (eNOSox)**—Recombinant human eNOSox was prepared using a yeast expression system as described previously (18). The biotin content of the purified eNOSox from yeast expression was less than 0.2/subunit, was measured using the procedure described previously (20). Quantiﬁcation of the secondary degradation of spin adduct (16). Only NOS-bound BH4 was evaluated, thus eliminating potential side reactions. In this study, we provide the first direct structural as well as kinetic information for a superoxide, a BH4, and a new radical intermediate occurring during the reaction between ferrous eNOSox and oxygen. This work convincingly shows that BH4 is the factor that dictates the radical structure, whereas l-arginine, on the other hand, has no effect on the radical structure but exhibits substantial influence on the radical kinetics. Our data also substantiate the functional role of BH4 in minimizing the formation of oxygen active species under uncoupled condition of eNOS.

**Bioprotein Determination**—Total bioprotein content of purified eNOS was measured using the procedure described previously (20). Quantitation of individual bioprotein redox species in eNOSox was conducted by a combination of chemical quench and HPLC as described recently.6

**Quantitation of Supernatant—eNOSox-released superoxide was determined by the cytochrome c trapping reaction using a difference absorbance coefficient of 21 mdeg cm⁻¹ at 550 nm (29).**

**Stopped-flow Experiments**—Kinetics of the reaction between Fe(II) eNOSox and oxygen was determined using an Applied Photophysics model SX-18MV stopped-flow instrument with a rapid scan diode array accessory (21). The fluid channels were incubated with dithionite solution for several hours and then washed with nitrogen-saturated buffer before sample loading. Reactants were rendered anaerobic by repeated vacuum/argon cycles in a tonometer. The reaction time courses were analyzed by nonlinear regression to single or multiple exponential functions.

Biopterin Radical Formation Kinetics in eNOSox—The procedure was essentially the same as that published recently (18). Ferrous eNOSox was prepared by anaerobic stoichiometric titration with dithionite in a reaction mixture used for structure determination were the same, except for microwave power, 1 mW; frequency, 9.61 GHz; and modulation amplitude, 10 G. The microwave power dependence was analyzed by nonlinear regression to the equation:

$$\log \left( \frac{S}{I(0)} \right) = -b \log (P^{1/2} + P) + b \log (P^{1/2}) + \log K$$

where $P$ is the power, $S$ is the peak to trough amplitude of the EPR signal, $P^{1/2}$ is the power at half-saturation, and $b$ and $K$ are floating parameters (23).

**RESULTS**

Three Radical Species Induced by Oxygen in Ferrous eNOSox—A single turnover reaction between ferrous eNOSox and oxygen was conducted in the presence or absence of BH4 and/or l-arginine. EPR examination of the radical intermediates trapped by rapid-freeze quench revealed three distinct species (Fig. 1). In the absence of both BH4 and l-arginine, a radical with axial symmetry was induced by oxygen (Figs. 1B and 2c). The EPR spectrum of this radical had $g_{xx} = 2.077$ and $g_{yy} = 2.007$ (at zero crossover); it exhibited homogeneous microwave power dependence. The very high $P^{1/2}$ of $50$ mW at 115 K (Fig. 1B, inset) and the EPR line shape were typical of the isolated superoxide radical (24, 25). The same radical species was also observed when l-arginine was included (Fig. 3f).

In the presence of BH4, the freeze trapped radical species showed a distinctly different EPR line shape (Fig. 1, A and C). With both BH4 and l-arginine present, a 40 G wide symmetric spectrum centered at 2.0023 (Figs. 1A and 2a) was trapped and has been documented as a BH4-derived cation or neutral radical (18, 26–28). This radical also showed homogeneous power dependence with a $P^{1/2}$ of $14$ mW (Fig. 1A, inset). In contrast, a previously unidentified radical species was trapped in the presence of BH4 when l-arginine was omitted (Figs. 1C and 2a). This radical exhibited a small broad peak at $g_{xx} = 2.040$ and a dominant symmetric feature centered at 2.0048. This dominant feature was more symmetric than the $g_{xx} = 2.007$ perpendicular component of the superoxide radical but with a broader line width, 20 G, and clear satellites at $g = 1.99$ and 2.023. The microwave power dependence was heterogeneous with the center peak-to-trough showing a $P^{1/2}$ of $4.7$ mW and the $g_{xx} = 1.99$ feature a $P^{1/2}$ of $14.7$ mW (Fig. 1C, inset).

Suspecting that the new radical species shown in Fig. 1C might be a mixture of the superoxide and BH4 radicals, we attempted to reproduce this third species by linear combinations of the superoxide and BH4 radicals. Spectra a–c in Fig. 2 are the individual radical EPR spectra of the BH4 radical, the

---

6 V. Berka, H.-C. Yeh, D. Gao, F. Kiran, and A.-L. Tsai, submitted for publication.
new radical species, and superoxide, respectively, acquired at 1 mW and 115 K. Spectrum $d$ was obtained by subtracting $a$ from $b$ and spectrum $e$ by subtracting $c$ from $b$; the amounts of $a$ and $c$ were adjusted to produce the most plausible resultant spectra. Spectrum $d$ has nominal $g$ values of 2.0023 and 2.045, with the 2.0023 signal significantly more symmetric than that of the superoxide radical. Spectrum $e$ is an isotropic species centered at $g = 2.0023$ with a width of $-47$ G between the two shoulders and a 19 G peak-to-trough width at the center. Spectrum $d$ does not resemble $c$, nor does $e$ resemble $a$. Thus, the new radical species does not appear to be a mixture of superoxide and BH$_4$ radical.
Oxygen-induced Radical Intermediates in eNOS

**Fig. 2.** EPR spectra of oxygen-induced radical intermediates in eNOSox and their arithmetic treatments. EPR spectra recorded at 2 mW and 115 K for the oxygen-induced radicals in 40 μM BH4 (+) eNOS plus 0.2 mM L-arginine (a), BH4 (+) eNOS (b), and BH4 (+) eNOS (c) are shown. Spectrum d was obtained by subtracting proportions of a from b to removal maximally the wing bands signals at g = 1.99 and 2.023. Spectrum e was obtained by removing maximally the g = 2.040 broad feature by subtracting portions of spectrum c from b.

**Fig. 3.** EPR spectra of oxygen-induced superoxide radical of eNOSox in the presence/absence of BH4, BH4, L-arginine, or SEITU. Control EPR spectra were obtained for the reaction of anaerobic ferric L-arginine-free BH4 (+) eNOS versus aerobic buffer (a) and L-arginine-free ferrous BH4 (+) eNOS versus anaerobic buffer (b). Spectra c–g are reactions of ferrous eNOS and versus oxygenated buffer: c, BH4 (+) eNOSox + 0.4 mM L-arginine; d, BH4 (+) eNOSox + 0.2 mM SEITU; e, BH4 (+) eNOSox + 0.2 mM SEITU; f, BH4 (+) eNOSox + 0.4 mM L-arginine; g, L-arginine-free BH4 (+) eNOSox; h, L-arginine-free 5-methyl BH4 (+) eNOSox.

Generation of these three radical species was oxygen-dependent because there was no radical formation when ferrous eNOSox was mixed with anaerobic buffer (Fig. 3a). Only the ferrous form of eNOSox was capable of radical formation because anaerobic ferric eNOSox failed to produce radical upon mixing with oxygen (Fig. 3b). The redox state of boptorin showed a decisive effect on the oxygen-induced radical species. BH4-reconstituted eNOSox failed to generate either the boptorin radical or the new radical when reacted with oxygen. The only radical species observed was superoxide (Fig. 3, e and g). Further addition of L-arginine decreased the maximal radical level by more than 80%, from 0.5 to 0.1 spin/heme. Replacing L-arginine with SEITU, a competitive inhibitor of L-arginine, also led only to superoxide radical formation (Fig. 3, d and e). eNOSox with bound BH4 substantially decreased the amount of superoxide radical formation from 0.3 to 0.06 spin/heme. The new radical species observed in the presence of BH4 and absence of L-arginine (Fig. 1C or 2b) is BH4–specific because replacement by either BH2 or 5-methyl BH4 yielded only superoxide radical (Fig. 3f).

**Kinetic Relationship between Heme Redox Changes and Radical Formation**—The kinetics of heme oxidation, followed in the optical stopped-flow, was compared with the kinetics of radical production, followed by rapid-freeze EPR, at relatively high enzyme concentrations (30–40 μM) to obtain reliable quantitative data for the radicals. We first describe the results of BH4(–) eNOSox. The absorbance changes at 400 nm upon mixing ferrous eNOSox with oxygen were very different in the presence and absence of L-arginine (Fig. 4A). In the absence of L-arginine, there was a biphasic increase that could be fit by a double-exponential function of approximately equal amplitudes and rate constants of 150 and 3.6 s\(^{-1}\). In the presence of 200 μM L-arginine, there was an initial decrease at 207 s\(^{-1}\) followed by an increase at 3 s\(^{-1}\). Similar biphasic kinetics were also observed for the rapid-scan and single-wavelength kinetic measurements at lower enzyme concentrations (–5 μM) (data not shown). However, in the absence of L-arginine the fast phase contributed only 5–10% to the overall absorbance changes at 400 nm.\(^{3}\) The observed biphasic increase at 400 nm at high enzyme concentrations (Fig. 4A) in the absence of L-arginine indicates both fast and slow phases during the oxidation of ferrous heme to the ferric heme. In contrast, with L-arginine present, the Fe(II)/O2 intermediate was stable as an optical intermediate, thus leading to the bimodal changes at 400 nm in opposite directions, indicating a reaction sequence as in Reaction 1.

\[
\text{Fe(II) + O}_2 \rightarrow \text{Fe(III)}
\]

**Reaction 1**

The parallel rapid-freeze quench data without L-arginine showed an initial burst of superoxide radical which is maintained at the level of –0.5 spin/heme for ~300 ms and then slowly decays at a rate of ~2 s\(^{-1}\) (Fig. 4C). This initial burst of radical signal is compatible with the 150 s\(^{-1}\) phase in the optical data after taking into account the 7-ms dead time of our rapid-freeze system (22). In sharp contrast, the superoxide radical kinetic data showed a much slower rate in the presence of L-arginine (16 s\(^{-1}\)) but also leveled off at about 0.5 spin/heme (Fig. 4C). The radical signal subsequently decayed at a rate, 1.8 s\(^{-1}\), similar to that in the absence of L-arginine. In the reaction containing L-arginine, the radical formation rate appeared to be faster than the positive phase of the bimodal kinetics in the stopped-flow data, 14.5 versus 2.4 s\(^{-1}\) (Fig. 4, A versus C, and Table I). The amplitude change of the corresponding heme EPR signal at g = 7 was also quantified for its time-dependent amplitude changes in the same set of rapid-freeze samples and showed a rate constant of 5.1 s\(^{-1}\), closer but still somewhat slower than the rate of superoxide radical formation.

To explore further the identity of the radical intermediate, we included cytochrome c and/or superoxide dismutase in the oxygenated buffer in the comparative kinetic measurements. We monitored cytochrome c reduction at 550 nm as an index of

\(^{3}\) V. Berk and A.-L. Tsai, unpublished results.
A versus B the changes at 400 nm (Fig. 4, dashed line) 400/H9262 minus (H9262 overlay of stopped-flow data at 400 nm obtained during reaction of 35 μM ferrous BH4(−) eNOSox with an air-saturated buffer plus (solid line) or minus (dashed line) 400 μM l-arginine at 24 °C. B, parallel kinetic data obtained from the same l-arginine-free eNOSox at 550 nm in the absence or presence of 200 μM cytochrome c and/or 0.5 unit/ml superoxide dismutase. C, superoxide radical kinetics obtained in a similar set of experiments by rapid-freeze in the l-arginine-free experiment without cytochrome c (open triangles), with cytochrome c (solid triangles), and in the presence of 0.4 mM l-arginine without cytochrome c (open circles), and with cytochrome c (solid circles). Lines are linear regressions (top and bottom horizontal traces) and one-exponential fits to the radical signal intensities. Shown in this figure is one of three similar sets of kinetic data.

**TABLE I**

*EPR characteristics and kinetics parameters for three different radical intermediates induced by oxygen in eNOSox.*

| Conditions | Radical species   | [Spin] | % | mW | k^−1 | k^−2 | k^−3 | k^−4 | + Cyt c^−5 |
|------------|------------------|--------|---|----|------|------|------|------|------------|
| +BH4 + l-Arg | BH4 | 90 ± 7 | 13.8 ± 1 | 15.4 ± 1.7 | 10.9 ± 1.5 | 13.1 ± 2.7 | 2 | ND |
| +BH4 – l-Arg | New | 63 ± 11 | 4.0 ± 1.5 | 166 ± 42 | 13.2 ± 0.4 | ≥166 | Fast | ND | Fast |
| −BH4 – l-Arg | Radical | 42 ± 13 | 54 ± 3 | 164 ± 24 | 3.9 ± 0.7 | ≥164 | Fast | ND | Decay |
| −BH4 + l-Arg | O2 | 47 ± 10 | 51 ± 2 | 2.4 ± 0.9 | 5.1 ± 0.5 | 14.5 ± 7.8 | 1.8 | 14.5 ± 5 |

1 [spin] / [BH4].  
2 Determined by g = 7 signal at 11 K.  
3 Based on g = 2 signal at 11 K.  
4 ND, not done.  
5 Double exponential fit.  
6 [spin] / [heme].

its reaction with superoxide radical (29). In the absence of l-arginine and cytochrome c, there was a biphasic decrease at A550, and the rates and contributions of each phase matched the changes at 400 nm (Fig. 4, A versus B), indicating that these changes originated from eNOS heme. When 100 μM cytochrome c was included, we observed a single exponential increase at A500 with a rate constant of 10 s−1. The total change at 550 nm corresponded to −10 μM reducing equivalents, or the trapping of 10 μM superoxide radical (Fig. 4B). This value agreed with the production of superoxide to a maximum level of 0.5 spin/heme measured by rapid-freeze quench EPR (Fig. 4C). Further, inclusion of superoxide dismutase in the oxygenated buffer substantially reduced the absorbance change at 550 nm, confirming that superoxide radical provided the reducing equivalents to cytochrome c.

Parallel rapid-freeze EPR experiments containing 200 μM cytochrome c yielded a kinetic decay from −0.45 spin/heme down to <0.05 spin/heme superoxide radical at a rate of 14.5 s−1 (Fig. 4C). Further increasing the concentration of cytochrome c did not accelerate the superoxide radical decay rate. This rate is thus the dead time required for cytochrome c to trap superoxide released from eNOSox. In the presence of l-arginine, cytochrome c more effectively trapped the superoxide radical because of the slow rate of generation of superoxide. In this case, only a small amount of superoxide radical was observed even at the earliest time point of freeze quench (=7
A similar set of stopped-flow kinetic data (Fig. 4B) was also obtained for the reactions containing l-arginine (not shown) but the rate of cytochrome c reduction was 4 s\(^{-1}\). This value is lower than that obtained in the absence of l-arginine, 10 s\(^{-1}\), possibly because of a limit by the slow radical formation, 14.5 s\(^{-1}\).

In the case of BH\(_4\) (+) eNOS\(_{ox}\) the stopped-flow kinetics at 400 nm were similar to those for BH\(_4\)(−) eNOS\(_{ox}\), but the rate for the second phase was increased several fold (Fig. 5A). Without l-arginine, the absorbance at 400 nm showed a biphasic increase with rate constants of 166 and 13 s\(^{-1}\) with contributions in total amplitude of 55 and 45%, respectively. The rapid-freeze quench of this sample trapped the new radical species (Figs. 1C and 2b). The radical formation showed an initial burst to the level of 0.54 – 0.61 spin/BH\(_4\) and lasted for ~500 ms before decaying at 2 s\(^{-1}\) (Fig. 5B). There was a gradual time-dependent change in EPR line shape of the radical intermediates from that shown in Fig. 1C to an EPR line shape typical of the BH\(_4\) radical after a few seconds of reaction with oxygen (data not shown). The microwave power dependence determined for the sample trapped at an early stage of the reaction was \(P^\% = 5\) mW, different from that of the sample trapped during the latter stage of the reaction (\(P^\% = 14\) mW) when a line shape typical of BH\(_4\) radical was observed. This result indicates that there was a time-dependent conversion from the new radical species to BH\(_4\) radical.

A biphasic change in absorbance at 400 nm, with rate constants of 207 and 15 s\(^{-1}\) for the decrease and subsequent increase, was observed in the reaction containing l-arginine (Fig. 5A), indicating a transient accumulation of the Fe(II)O\(_2\) intermediate before its subsequent conversion to Fe(III) eNOS\(_{ox}\). Rapid-freeze EPR measurements only detected the BH\(_4\) radical (Fig. 1A or 2a). The rate of BH\(_4\) radical formation was 17 s\(^{-1}\) and plateaued at the same spin level as that in the absence of l-arginine. Because we did not expect BH\(_4\) radical to be released from eNOS\(_{ox}\) within the reaction period, we only tested whether cytochrome c could trap the new radical species. As shown in Fig. 5B, 100 \(\mu\)M cytochrome c failed to quench this new radical in any significant way. Thus, either this new radical was not released from eNOS\(_{ox}\), or this new radical is released without reacting with cytochrome c.

The characteristics of all radical species induced by oxygen are summarized in Table I.

**DISCUSSION**

*What Is New in This Work?*—We demonstrated for the first time that three distinct radical species are generated when reduced eNOS\(_{ox}\) reacts with oxygen. To focus on the role of heme in the formation of radical intermediates including superoxide, we used only the oxygenase domain and not the full-length eNOS. Superoxide is also generated by flavins in the reductase domain of NOS but to a lesser extent than the heme, even though, in eNOS, the role of the reductase domain in superoxide formation is more significant than in the other NOS isozymes (3, 4, 11, 12). We also limited our study to single turnover conditions rather than the steady state to locate the early events and obtain rate constants for individual reaction steps. Only under these conditions and at high enzyme concentration was it possible to determine directly the rate as well as the amount of radical species by the freeze-quench EPR method (Figs. 4 and 5 and Table I). The rapid-freeze quench method has been shown to be superior to the indirect spin-trapping procedure in generating detailed structure information of each radical species (Figs. 1–3), for spin quantification and details of kinetics (Figs. 4 and 5).

This direct radical trapping and quantification method for superoxide radical has not been reported for any NOS isoforms or cytochrome P450 proteins. A particularly novel finding was the substantial amount of each radical species directly trapped, with the superoxide radical as high as 50% of the heme, and the BH\(_4\) radical and the new radical to 80–100% the level of bound BH\(_4\) (Figs. 4 and 5 and Table I). Another novel finding is the dramatic effect of BH\(_4\) on the detected radical species (Figs. 1 and 2) and the significantly decreased rate of radical formation in the absence of l-arginine (Figs. 4 and 5). In contrast to the BH\(_4\) radicals, another radical intermediate instead of superoxide radical was detected in eNOS\(_{ox}\). It was either a BH\(_4\) radical or a...
new radical species depending on the presence or absence of L-arginine (Figs. 1 and 2).

Characterization of Superoxide Radical—The superoxide radical we detected was an anion radical, \( O_2^- \), and not a neutral radical, \( HO_2 \), because of the lack of the 11 G proton hyperfine splitting (25). The typical axial EPR spectrum is the consequence of symmetry of the radical anion with the O-O bond along the z axis (the parallel component) and almost equal x and y components (the perpendicular components). Usually the departure of \( g_z \) of the superoxide radical from the free electron value of 2.0023 (by 0.07–0.11) is much larger than that of peroxo radical, 0.03–0.04. The superoxide radical observed in eNOS\(_{ox}\) has a separation between \( g_z \) and \( g_y \) of 0.07 (Fig. 1B). The variation of this value is caused by an environmental effect on the energy of the \( \pi^* \rightarrow \pi^\# \) transition. Based on Equation 2

\[
g_1 = g_z = g + (2\lambda/\delta^2 + \lambda^2)^{1/2} \quad \text{(Eq. 2)}
\]

and using a value of 190 cm\(^{-1}\) for the spin-orbit coupling constant, \( \lambda \), we calculated that the energy separation between the two \( \pi \) antibonding orbitals, \( \delta \), is 4.886 cm\(^{-1}\), similar to that found for the superoxide radical embedded in a ethanol/isopro- pyl alcohol matrix (24). The superoxide radical can be stabilized by an assembly of molecular dipoles instead of by discrete charged ions. There are several polar residues in the distal heme pocket which could affect the value of \( \delta \) and the lifetime of the radical (30, 31). In this context, it is surprising to see the small size of the shift of \( g_y \), <0.001, in the presence of L-arginine (Fig. 3f). Thus the effect of a single positive charge on the guanidine group of L-arginine is much less than an ensemble of molecular dipoles in the heme pocket. The positive charge on guanidine, however, decreased the rate of superoxide formation substantially, likely by a direct electrostatic interaction with the oxygen dipole, stabilizing the Fe(III)O\(_2\) intermediate and strengthening the Fe-O bond. Once the bond breaks, the superoxide radical generated is influenced more by the whole heme pocket rather than a single cationic guanidine group.

Characterization of the New Radical—Several pieces of evidence indicate that the new radical induced in the BH\(_4\)(+) eNOS\(_{ox}\) without l-arginine is not simply a mixture of superoxide and BH\(_4\) radicals. A direct arithmetic treatment failed to demonstrate that this new radical contains various proportions of either superoxide or BH\(_4\) radical (Fig. 2). The power for half-saturation at the center of the main EPR feature is only 4.7 mW, much smaller than that of the BH\(_4\) radical, 13.8 mW, or that of the superoxide radical, 51 mW (Fig. 1). Cytochrome c failed to trap this new radical species, indicating either that this radical is not released out of the heme pocket or that it does not react with cytochrome c because of unfavorable thermodynamics. In either case, this is a strong indication that this new radical is neither a superoxide nor a mixture containing superoxide and BH\(_4\) radical. The EPR line shape, radical relaxation behavior, and the separation of \( g_z \) and \( g_y \) of 0.036 of this new radical bear a resemblance to the EPR features of certain peroxyl radicals (Fig. 2b) (32–34). Peroxyl radicals usually show lower \( D_{\pi\pi} \) than the superoxide radical because of its lower g anisotropy (smaller orbital magnetism), the principal mechanism for relaxation. Comparison of the time-dependent change of the EPR line shape of this new radical with that of a BH\(_4\) radical indicates a peroxyl radical of biotinyl. Thus a reaction sequence

\[
(BH_4)Fe(II)O_2 \rightarrow (BH_4 - O_2)Fe(III) \rightarrow (BH_4^+)Fe(III) + H_2O_2
\]

provides a plausible explanation for all the existing information (Scheme 1C). However, the possibility that this new radical is a peroxyl radical derived from some amino acid such as the C3-peroxyl tryptophan radical found in different peroxidases (35, 36) and Mb/Hb (37, 38) cannot be ruled out. In BH\(_4\)(+) eNOS there is little H\(_2\)O\(_2\) formation in the absence of l-arginine, whereas a significant amount was found in nNOS under similar conditions (39). This finding matches our observation that instead of H\(_2\)O\(_2\) formation, we found a new radical. H\(_2\)O\(_2\) is expected to be detected only at later stage of the reaction when the radical EPR line shape shifted to that of a BH\(_4\) radical.

Effects of BH\(_4\), L-Arginine, and Their Analogs on the Level of Superoxide Intermediate—The maximum amount of superoxide radical freeze-trapped was affected by the presence of both the l-arginine/l-arginine analog (SEITU) and the BH\(_4\)/BH\(_4\)
analog (e.g. compare BH$_2$–l-arginine or SEITU = BH$_4$ in Fig. 3). The presence of both l-arginine and BH$_4$ or their analogs substantially decreased the level of superoxide formation. This outcome provides further support for our earlier hypothesis that heme, l-arginine, and BH$_4$ "triaz" reinforce each other in rigidifying the heme pocket scaffold via a network of H bonding and thus the Fe-O bond strength in the Fe(II)O$_2$ intermediate (30, 31). Our unpublished data that 5-methyl BH$_4$ reduces the amount of superoxide radical in l-arginine-free eNOS$_{ox}$, and the observed effect of l-thiocitrulline in decreasing superoxide radical formation in eNOS may well be caused by the same mechanism (21, 40).

**Comparison with the Single Turnover EPR Studies of iNOS$_{ox}$.—** Two other groups have performed similar single turnover rapid-freeze experiments on iNOS$_{ox}$, but the results are quite different from our results with eNOS$_{ox}$ (26, 27). With both BH$_4$ and l-arginine present, oxygen also induced BH$_4$ radical formation in iNOS$_{ox}$ (26, 27). It was claimed that BH$_4$ radical was also generated in BH$_4$ (+) iNOS$_{ox}$ in the absence of l-arginine, although actual data were not shown (26). Our data indicate that l-arginine is required for BH$_4$ radical formation in eNOS$_{ox}$ (Fig. 1, A versus C). In contrast to the present study, no radical signal was observed in BH$_4$ (−) or BH$_2$ (−) iNOS$_{ox}$ (26), whereas we observed a substantial amount of superoxide formation (Figs. 1 and 4). Furthermore, no heme oxidation was observed for 5 s after BH$_4$ (−) iNOS$_{ox}$ was reacted with oxygen in the presence of l-arginine (26). This latter finding is at odds with another similar study on iNOS$_{ox}$ where efficient oxidation to ferric enzyme was observed (27, 41). Thus, there are several differences between iNOS and eNOS in the radical species induced by oxygen and their regulation by BH$_4$ and l-arginine. Neither superoxide radical nor the putative peroxy radical found in iNOS$_{ox}$ was observed with iNOS$_{ox}$ under similar reaction conditions. The failure to observe radical species other than BH$_4$ radical in iNOS$_{ox}$ even in the absence of BH$_4$ and/or l-arginine was most surprising as we have found that a bacterial NOS-like protein present in *Staphylococcus aureus*, which has 30% sequence homology with eNOS$_{ox}$ lacking the zinc cluster and BH$_4$ binding, generated a substantial amount of superoxide (up to 0.3 spin/heme) under similar reaction conditions (data not shown). *S. aureus* NOS also exhibited a very similar mechanism for Fe(II)O$_2$ complex formation and decay in the presence and absence of l-arginine (data not shown). We expect a substantial amount of superoxide formation in iNOS$_{ox}$ in the absence of BH$_4$ and l-arginine based on the observation for *S. aureus* NOS because the Fe(II)O$_2$ intermediate should easily dissociate into Fe(III) and superoxide because of a more flexible heme scaffolding.

**Indication for Different Heme Structure in eNOS$_{ox}$ Dimer.—** The biphasic decay of the absorbance at 400 nm in the absence of l-arginine (Figs. 4A and 5A) was not so obvious when the kinetic measurements were conducted at enzyme levels around 2 μM (data not shown). Thus, there was an enzyme concentration dependence in kinetic behavior. This is not so surprising as EPR, and resonance Raman studies have shown more than one heme structures in eNOS and other NOS isoforms either as isolated or present as heme ligand complexes (18, 42, 43). A pH-dependent conversion between two low spin imidazole-heme complexes of eNOS has been observed (18, 42). The interconversion of these two heme structures may also be enzyme concentration-dependent, although the underlying mechanism is unclear.

**Limitation of Superoxide Radical Trapping by Cytochrome c.—** Cytochrome c is a more efficient superoxide-trapping agent, 1.1 × 10$^{6}$ s$^{-1}$ s$^{-1}$, than most of the commonly used spin-traps, <10$^{3}$ M$^{-1}$ s$^{-1}$ (13, 44). With 100–125 μM cytochrome c present in our kinetic experiments, we expected to have a rate of cytochrome c reduction or superoxide radical decay around ~100–125 s$^{-1}$, but we observed only ~10–15 s$^{-1}$ rate for either process (Fig. 4); this is an order of magnitude smaller than the rate of superoxide radical formation in the absence of cytochrome c (Fig. 4C). The most plausible explanation is that the superoxide that is formed is not immediately available for reaction with cytochrome c. The rate of migration of the superoxide formed at the distal heme site to the protein surface is thus about 10–15 s$^{-1}$. Crystallographic data for eNOS$_{ox}$ show that the heme center is located at the end of a 30 Å deep channel away from the surface (30, 31). On the other hand, cytochrome c was able to trap the formed superoxide completely when l-arginine was present (Fig. 4C). In this case, the superoxide formation rate was near or less than the rate needed for radical release from the heme pocket.

**BH$_4$ Effects on Superoxide under Steady-state Conditions: Issues to Be Concerned about.—** The BH$_4$ inhibition of superoxide formation in NOS has been examined extensively under steady-state conditions with spin-trapping methods (3–6, 11–14, 39). An excess amount of 5-methyl BH$_4$, 6-methyl BH$_4$, or 6-methyl PH$_4$ versus heme was also found to inhibit superoxide formation in eNOS. On the other hand, BH$_2$ enhanced superoxide radical formation by competing with BH$_4$ in binding to eNOS (45). These studies led to the proposal that BH$_4$ inhibits superoxide formation by its ability to stabilize the Fe(II)O$_2$ complex (40). Our study here demonstrates that BH$_4$ quenches superoxide radical formation either by providing a reducing equivalent to the Fe(III)$O_2$ in the presence of l-arginine, producing N-hydroxy-l-arginine and water (Scheme 1D), or, in the absence of l-arginine, by trapping the superoxide to form a peroxyl radical intermediate with subsequent reduction, bond breaking, and proton uptake to yield hydrogen peroxide (Scheme 1C). BH$_4$ only indirectly stabilizes the Fe(II)O$_2$ intermediate, possibly by rigidifying the scaffolding of the heme distal pocket in coordination with heme and l-arginine.

Rosen *et al.* (13) showed that activated nNOS generates superoxide with or without BH$_4$, and the final level of superoxide was even higher in BH$_4$ (+) than BH$_4$ (−) nNOS. The results of their multiple turnover experiments were very complicated and appeared to contradict our observations. Our current study indicates that BH$_4$ completely eliminates superoxide formation. Rosen *et al.* did remove excess BH$_4$, thus avoiding the possible complication by free BH$_4$ to scavenge superoxide and focus on the effect of protein-bound BH$_4$. However, the enzyme concentration used in their assay was as low as 4 nM, and a significant amount of BH$_4$ dissociation could occur, with an estimated $k_{dis}$ of 0.06–0.32 min$^{-1}$, during the spin-trapping experiments (41). Further, BH$_4$ oxidation is enhanced at low NOS concentrations, leading to a mixture of BH$_4$, BH$_2$, or even BH$_0$ in every assay longer than 10 min. It is plausible that the high level of superoxide formation in BH$_4$ (+) nNOS detected by Rosen *et al.* was contributed by these two side mechanisms. We have demonstrated recently that BH$_4$ oxidation is the only mechanism for autoinactivation of eNOS and the lost activity could be fully recovered by BH$_4$ reconstitution. Therefore, the redox state of biotin in the assay mixture is vital to the outcome of superoxide spin-trapping. In our single turnover experiments, the sample was always under anaerobic environment and was at high enzyme concentration, thus both BH$_4$ dissociation and BH$_4$ oxidation were prevented. This was confirmed by our recently developed HPLC analysis for the amount and redox states of biotin. 

**Effects of l-Arginine on Superoxide Formation.—** The role of l-arginine on superoxide formation is controversial. It was observed that a saturating level of l-arginine increases superoxide production from BH$_4$ (−) eNOS (3), whereas in nNOS l-arginine was shown to inhibit superoxide formation (45).
Neither of these studies considered the effect of L-arginine on superoxide kinetics. Our current study indicates that L-arginine dramatically decreases the superoxide formation rate (Figs. 4 and 5). Crystallographic and ENDOR data (19, 46) also provide nice support for a direct interaction between oxygen and the guanidine moiety of L-arginine. Our recent data from rapid-scan stopped-flow also showed that L-arginine changed the behavior of oxygen binding to Fe(II)/Fe(III) intermediate and minimizing its back dissociation to reactants.2 Thus, in contrast to BH4, L-arginine directly stabilizes the Fe(II)/Fe(III)O2 complex and leads to slower superoxide formation. This new finding reveals distinct roles of BH4 and L-arginine in superoxide radical formation.

Scheme 1 summarizes the overall mechanism of formation of three different radical intermediates by oxygen and the regulatory roles of BH4 and L-arginine. In the absence of both BH4 and L-arginine, facile cleavage of the Fe-O bond of the Fe(II)/Fe(III)O2 yields superoxide. Once free from the heme pocket, superoxide is oxidized to oxygen with reduction of cytochrome c (pathway A). With L-arginine alone, eNOSox also forms superoxide and peroxo-heme, which react with L-arginine to form H2O2 and peroxynitrite. The further study of the full-length eNOS under multiple turnovers and comparisons with iNOS and nNOS should reveal important differences in regulatory behavior.

Acknowledgment—We thank Dr. Lee-Ho Wang for guidance in developing the eNOS yeast expression system.

REFERENCES
1. Feldman, P. L., Griffith, O. W., and Stuehr, D. J. (1993) Chem. Eng. News 51, 26–38
2. Raman, C. S., Martasek, P., and Masters, B. S. S. (2000) in The Phosphoryn Handbook (Radich, K. M., Smith, K. M., and Guilard, R., eds) pp. 293–339, Academic Press, New York
3. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9220–9225
4. Xia, Y., Tsai, A.-L., Berka, V., and Zweier, J. L. (1998) J. Biol. Chem. 273, 25546–25554
5. Kuzkaya, N., Weissmann, N., Harrison, D. G., and Dikalov, S. (2003) J. Biol. Chem. 278, 25546–25554
6. Stuehr, D., Pou, S., and Rosen, G. M. (2001) J. Biol. Chem. 276, 14533–14536
7. Wang, W., Wang, S., Yan, L., Madara, P., Del Pilar Cintron, A., Wesley, R. A., and Danner, R. L. (2000) J. Biol. Chem. 275, 16899–16903
8. Boudoumis, A., Baurersachs, J., Linn, W., Scholens, B. A., Wiemer, G., Flemming, I., and Busse, R. (1997) Hypertension 30, 934–941
9. Cosentino, F., and Katusic, Z. S. (1995) Circulation 91, 139–144
10. Szabó, C., Zingarelli, B., and Salzman, A. L. (1996) Circ. Res. 78, 1051–1063
11. Xia, Y., Roman, L. J., Masters, B. S. S., and Zweier, J. L. (1998) J. Biol. Chem. 273, 22635–22639
12. Pou, S., Reaton, L., Surichamorn, W., and Rosen, G. M. (1999) J. Biol. Chem. 274, 9573–9580
13. Rosen, G. M., Tsai, P., Weaver, J., Porasuphatana, S., Roman, L. J., Starkov, A. A., Fiskum, G., and Pou, S. (2002) J. Biol. Chem. 277, 40275–40280
14. Vasquez-Vivar, J., Whitsett, J., Martasek, P., Hogg, N., and Kalyanaraman, B. (2003) Free Radic. Biol. Med. 31, 975–985
15. Griscavage, J. M., Fukuto, J. M., Komori, Y., and Ignarro, L. J. (1994) J. Biol. Chem. 269, 21644–21649
16. Jez, E. G. (1946) Methods Enzymol. 105, 188–198
17. Rosen, G. M., Tsai, P., KraIk, P. M., Sonn, M., Dawson, J. H., Masters, B. S., and Hoffman, B. M. (2002) Biochemistry 41, 10375–10381
18. Chen, P.-F., Tsai, A.-L., and Wu, K. K. (1997) Biochem. Biophys. Res. Commun. 235, 6002–6011
19. Davydov, R., Ledbetter-Rogers, A., Martasek, P., Larukhin, M., Sono, M., and Masters, B. S. (2002) Biochemistry 41, 6002–6009
20. Knowles, P. F., Gibson, J. F., Pick, F. M., and Bray, R. C. (1969) Biochem. J. 111, 53–58
21. Hershman, A. R., Kresl, C., Edmondson, D. E., Hus, H. B., and Marletta, M. A. (1999) Biochemistry 38, 15689–15696
22. Wei, C. C., Wang, Z. Q., Wang, Q., Meade, A. L., Hemann, C., Hille, R., and Stuehr, D. J. (2001) J. Biol. Chem. 276, 315–319
23. Schmied, G. P., Lange, R., Goren, A. C., Werner, E. R., and Mayer, B., and Andersson, K. K. (2001) J. Biol. Inorg. Chem. 6, 151–158
24. Kuthan, H., Uhrich, V., and Estabrook, R. W. (1982) Biochem. J. 203, 551–558
25. Braun, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. S., and Pouls, T. L. (1998) Cell 95, 1–20
26. Fischmann, T. O., Hrusa, A., Xiao, D. N., Fossetta, J. D., Lunn, C. A., Dolphin, E., Prongay, A. J., Reichert, P., Lundell, D. J., Narula, S. K., and Weber, P. C. (1999) Nat. Struct. Biol. 6, 233–242
27. Yanex, J., Sevilla, C. L., Becker, D., and Sevilla, M. D. (1987) J. Phys. Chem. 91, 487–493
28. Reddy, S. G., Wong, K. K., Parrat, C. V., Peisach, J., Maglione, R. S., and Konarzich, J. W. (1998) Biochemistry 37, 558–563
29. Nelsen, M. J., Cowling, R. A., and Seitz, S. P. (1994) Biochemistry 33, 4966–4973
30. Hoffman, B. M., Roberts, J. E., Kang, C. H., and Margoliash, E. (1981) J. Biol. Chem. 256, 6556–6564
31. Hu, A. N., Martinez, J. I., Arnac, M., Acosta, M., Turner, D. D., Raven, E. L., and Rodriguez-Lopez, J. N. (2001) Eur. J. Biochem. 268, 3091–3098
32. Sivstuenko, D. A. (2001) Biochim. Biophys. Acta 1546, 365–378
33. DeGray, J. A., Gunthor, M. R., Tcherot-Guth, R., Ortiz de Montellano, P. R., and Masen, R. P. (1997) J. Biol. Chem. 272, 2359–2362
34. Vasquez-Vivar, J., Hogg, N., Martasek, P., Kral, H., Pritchard, K.A., Jr., and Kalyanaraman, B. (1999) J. Biol. Chem. 274, 26736–26742
35. Vasquez-Vivar, J., Kalyanaraman, B., and Martasek, P. (2003) Free Rad. Res. 37, 121–127
36. Wei, C.-C., Crane, B. R., and Stuehr, D. J. (2003) Chem. Rev. 103, 2365–2383
37. Berka, V., Palmer, G., Chen, P.-F., and Tsai, A.-L. (1998) Biochemistry 37, 6136–6144
38. Wang, J., Stuehr, D. J., Ikeda-Saito, M., and Rousseau, D. L. (1993) J. Biol. Chem. 268, 22253–22258
39. Keppens, W. H., Van Burren, K. J. H., Butler, J., and Braams, R. (1976) Biochim. Biophys. Acta 449, 157–168
40. Vasquez-Vivar, J., Martasek, P., Whitsett, J., Joseph, J., and Kalyanaraman, B. (2002) Biochem. J. 362, 733–739
41. Li, H., Raman, C. S., Martasek, P., Masters, B. S., and Pouls, T. L. (2001) Biochemistry 40, 5399–5406
Three Different Oxygen-induced Radical Species in Endothelial Nitric-oxide Synthase Oxygenase Domain under Regulation by L-Arginine and Tetrahydrobiopterin
Vladimir Berka, Gang Wu, Hui-Chun Yeh, Graham Palmer and Ah-lim Tsai

J. Biol. Chem. 2004, 279:32243-32251.
doi: 10.1074/jbc.M404044200 originally published online May 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M404044200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 21 of which can be accessed free at
http://www.jbc.org/content/279/31/32243.full.html#ref-list-1