A Tetrahydrobiopterin Radical Forms and then Becomes Reduced during N$^\omega$-Hydroxyarginine Oxidation by Nitric-oxide Synthase*

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Nitric-oxide synthases are flavoheme enzymes that catalyze two sequential monoxygenase reactions to generate nitric oxide (NO) from l-arginine. We investigated a possible redox role for the enzyme-bound cofactor 6R-tetrahydrobiopterin (H$_4$B) in the second reaction of NO synthesis, which is conversion of N-hydroxy-l-arginine (NOHA) to NO plus citrulline. We used stopped-flow spectroscopy and rapid-freeze EPR spectroscopy to follow heme and biopeterin transformations during single-turnover NOHA oxidation reactions catalyzed by the oxygenase domain of inducible nitric-oxide synthase (iNOSoxy). Significant biopeterin radical (>0.5 per heme) formed during reactions catalyzed by iNOSoxy that contained either H$_4$B or 5-methyl-H$_4$B. Biopeterin radical formation was kinetically linked to conversion of a heme-dioxy intermediate to a heme-NO product complex. The biopeterin radical then decayed within a 200–300-ms time period just prior to dissociation of NO from a ferric heme-NO product complex. Measures of final biopeterin redox status showed that biopeterin radical decay occurred via an enzymatic one-electron reduction process that regenerated H$_4$B (or 5MeH$_4$B). These results provide evidence of a dual redox function for biopeterin during the NOHA oxidation reaction. The data suggest that H$_4$B first provides an electron to a heme-dioxy intermediate, and then the H$_4$B radical receives an electron from a downstream reaction intermediate to regenerate H$_4$B. The first one-electron transition enables formation of the heme-based oxidant that reacts with NOHA, while the second one-electron transition is linked to formation of a ferric heme-NO product complex that can release NO from the enzyme. These redox roles are novel and expand our understanding of biopeterin function in biology.

Nitric-oxide Synthases (NOS, EC 1.14.13.39) are flavoheme enzymes that catalyze oxidation of l-arginine (Arg) to nitric oxide (NO) and citrulline (1–3). The reaction consumes 1.5 mol of NADPH and 2 mol of O$_2$ for each NO formed from Arg and is catalyzed in two steps (Scheme 1). Arg is first hydroxylated to form water and N$^\omega$-hydroxy-l-arginine (NOHA) as an enzyme-bound intermediate. NOHA is then further oxidized to generate water, NO, and citrulline. Both reactions take place in the oxygenase domain of NOS, which contains iron protoporphyrin IX (heme), the essential cofactor (6R)-5,6,7,8-tetrahydro-l-biopeterin (H$_4$B) and the Arg binding sites (4, 5). Electrons derived from NADPH are provided to the NOS oxygenase domain by an attached reductase domain that binds FMN, FAD, and NADPH. A calmodulin binding site is located between the oxygenase and reductase domains and serves to regulate electron transfer (6–8).

The NOS heme iron is ligated by a cysteine thiolate and is responsible for binding O$_2$ and catalyzing its reductive activation during NO synthesis (9, 10). How H$_4$B facilitates this process is a topic of current interest. Investigations have typically employed single catalytic turnover reactions with recombinantly expressed NOS oxygenase domains (NOSoxy). During Arg hydroxylation there is a buildup of a H$_4$B radical in all three NOS isozymes (11–13), suggesting that H$_4$B performs a reductive role in the reaction. Our previous work established that formation of the H$_4$B radical is kinetically coupled to reduction of a heme ferric-superoxide intermediate (Fe$^{II}$O$_2^-$) that forms in NOS after O$_2$ binds to its ferrous heme (14). Electron transfer from H$_4$B to the Fe$^{II}$O$_2^-$ intermediate enables formation of a heme-oxy species that hydroxylates Arg. Moreover, the tempo of Fe$^{III}$O$_2^-$ reduction by H$_4$B is important and must be maintained for efficient Arg hydroxylation. The tempo is regulated by the protein residues that interact with H$_4$B (for example Trp$^{457}$ in mouse iNOS) (15) and by the structure of H$_4$B itself (16).

Although the redox role for H$_4$B during Arg hydroxylation seems established (17), it is still unclear if H$_4$B is redox active during the NOHA oxidation reaction. Theoretically, it is not essential that H$_4$B provides an electron to the Fe$^{III}$O$_2^-$ intermediate in this step (18). Moreover, a model study suggested that NOHA could provide an electron directly to the Fe$^{III}$O$_2^-$ species in place of H$_4$B (19). Only a small amount of H$_4$B radical (≈0.03 per heme) was observed during a NOHA single turnover reaction catalyzed by iNOSoxy (11). However, we and others (20–22) have speculated that this is still consistent with the H$_4$B radical forming and then becoming reduced back to H$_4$B during the time frame of the reaction. Indeed, we have argued that this behavior may be essential for NOS to generate NO instead of nitroxy (23). Other experimental evidence indirectly supports a redox role for H$_4$B during NOHA oxidation. For example, NO synthesis from NOHA does not occur when enzyme-bound H$_4$B is replaced with 6,7-dihydrobiopeterin (H$_4$B) or with the redox-inactive analog 5-deaza-tetrahydrobiopeterin (20, 22). In addition, mutational substitution of a NOS Trp residue that interacts with H$_4$B caused similar changes in the
reaction kinetics and product yields for both the Arg and NOHA single turnover reactions (20). Such common influences are consistent with H_B performing a common redox role in both reactions of NO synthesis.

To further investigate this possibility, we utilized stopped-flow spectroscopy and rapid-freeze EPR to follow in a time-resolved way the heme and H_B transitions that occur during NOHA oxidation as catalyzed by iNOSoxy. We found that a H_B radical does form in conjunction with Fe^{III}O_2^- disappearance, followed by a rapid reduction of the radical, all within the time frame of the single catalytic turnover. Thus, our work suggests that H_B reduces the Fe^{III}O_2^- intermediate in the NOHA reaction and then performs a distinct electron acceptor function at a later point in the reaction. The mechanistic implications of our findings are discussed.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—H_B, H_B, and 5MeH_B were obtained from Schirck’s Laboratory (Jona, Switzerland). All other chemicals were either from Sigma or Fisher Scientific.

**Protein Expression and Purification**—Pterin-free mouse iNOSoxy (amino acids 1–498) containing a six-histidine tag at its C-terminal was expressed in *Escherichia coli* BL21 using the PCWori vector and purified in the absence of H_B and Arg as reported previously (14). The enzyme concentration was determined from the 444 nm absorbance of the ferrous-CO complex by using extinction coefficient of 76 mM⁻¹ cm⁻¹. The final concentrated protein was stored in buffer containing 50 mM HEPES, pH 7.5, 10% glycerol, 2 mM mercaptoethanol, and 0.25 mM NaCl.

**Ferrous Heme Protein Preparation and Rapid Kinetics**—The ferrous iNOSoxy was prepared as described previously (14, 16). The rapid-scanning stopped-flow spectroscopy and rapid-freeze EPR measurements were done as previously (14). EPR spectra were recorded in a Bruker ER 300 electron paramagnetic resonance spectrometer equipped with an ER 035 NMR gauss meter and a Hewlett-Packard 5352B microwave frequency counter. Temperature control was achieved using Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and ITC4 temperature controller. All spectra were obtained at 150 and 4 K using a microwave power of 2 milliwatts, a helium cryostat and ITC4 temperature controller. All spectra were achieved using Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and ITC4 temperature controller. All spectra were achieved using Oxford Instruments ESR 900 continuous-flow liquid helium cryostat and ITC4 temperature controller.

**RESULTS**

**H_B Radical Formation during NOHA Oxidation**—We looked for H_B radical formation during iNOSoxy-catalyzed oxidation of NOHA at 10 °C under single turnover conditions. Reactions were initiated by rapid mixing an anaerobic solution containing ~300 μM ferrous iNOSoxy, 10 mM NOHA, and 2 mM H_B with O_2-saturated buffer (O_2 concentration is ~2.2 mM at 0 °C (11)) in our rapid-freeze instrument. As shown in Fig. 1, a radical species did form during the NOHA reaction that displayed spectroscopic features that were very similar to the H_B radical formed in the Arg hydroxylation reaction (14, 15). The radical signal in the NOHA reaction had a g = 2.0, a peak-to-trough width of ~40 G, and a powder hyperfine structure. In replica reactions run with iNOSoxy that contained 5MeH_B in place of H_B, we detected a distinct radical signal during the NOHA reaction with properties that were similar to those of the 5MeH_B radical formed during the Arg hydroxylation reaction (Fig. 1) (16). No radical signal was observed in control reactions that contained H_B in place of H_B (data not shown).

**Redox State of Bound Biopterin**—We determined the final oxidation state of bound biopterin that remained in the iNOSoxy enzyme following single catalytic turnover reactions, using an established method (25) with modifications. A buffered solution (0.1 ml total) that contained 0.1 mM iNOSoxy, 1 mM H_B, and 10 mM Arg (or NOHA) was incubated at room temperature for 20 min to allow for binding. The solution was then passed through two consecutive Micro-spin P-30 columns (Bio-Rad) to remove free H_B and substrate. Arg (or NOHA) was then re-added to the solution at a final concentration of 10 mM and the sample was made anaerobic by consecutive cycles of vacuum and purging with N_2. The iNOSoxy was then reduced to ferrous form by addition of a near stoichiometric amount of dithionite solution. To initiate the single turnover reaction, 0.1 ml of the ferrous iNOSoxy solution was transferred using a gas tight syringe into an open eppendorf tube that contained 20 μl of O_2-saturated buffer (40 mM EPPS, pH 7.5) and 4 μl of an iodine solution (0.9% (w/v) iodine in H_2O). This iodine solution was needed to oxidize any excess dithionite, which if present was found to reduce oxidized biopterin in the reaction samples and thus confound the results. After vigorous mixing for ~20 s to allow for completion of the single turnover reaction, 50-μl aliquots were transferred either into a vial containing 50 μl of NaOH and 5 μl of alkaline iodine solution (0.9% (w/v) iodine and 1.8% (w/v) KI in 0.1 N NaOH) or into a vial containing 50 μl of 0.2 N HCl and 5 μl of acidic iodine solution (0.9% (w/v) iodine and 1.8% (w/v) KI in 0.1 N HCl). Samples then were incubated at room temperature in the dark for at least 1.5 h. The sample in alkaline solution was then neutralized by adding 10 μl of 1.0 N HCl, and all remaining iodine in all of the samples was quenched by adding 5 μl of freshly prepared 4% (w/v) ascorbic acid solution. The precipitated protein was removed from each sample by centrifuging at 10,000 × g for 10 min. A 5-μl aliquot from each sample was then injected into a 10 × 250-mm Microsorb C18 column (Ranin Instrument Inc.) that was equilibrated with 50 mM ammonium phosphate, pH 3.0. A fluorescence detector (Waters 710) was used to detect biopterin products with excitation wavelength set at 360 nm and emission wavelength set at 460 nm. Total biopterin (oxidized + reduced) was determined from the area of the peak eluting at 21.5 min that had undergone acidic workup, while oxidized biopterin was determined from the area of this peak from the sample that had undergone basic workup (25). The amount of pterin remaining as H_B after the reaction was calculated by subtracting the amount of biopterin present in the alkaline solution from the amount of biopterin present in the acidic solution. Any dilution difference between the acidic and basic condition during the work-up was corrected for in the calculation.

**Scheme 1. Reaction catalyzed by NOS.**

**Fig. 1.** One-turnover reaction (16) of iNOSoxy with L-arginine and NOHA. The resulting isobutylene and NO were isolated and analyzed by gas chromatography.
H4B radical that formed during the NOHA or Arg reactions of H4B and 5-MeH4B radicals were recorded at 60 and 84 ms after respectively, with 0.65 5MeH4B radical formed per enzyme formation and decay were calculated to be 35 and 15 s/H11002 formation and decay in the NOHA reaction of 31 and 8 s/H11002 samples were preincubated with H4B and substrate (NOHA disappeared via an oxidative or reductive process. iNOSoxy enzyme molecules that were actively catalyzing radicals that we observed in the NOHA reaction had formed in respectively. Together, this argues that the transient biopterin model A → B → C (14) gave calculated rates of H4B radical formation and decay in the NOHA reaction of 31 and 8 s⁻¹, respectively, with a calculated yield of 0.55 H4B radical formed per enzyme heme (Table I). Similarly, rates of 5MeH4B radical formation and decay were calculated to be 35 and 15 s⁻¹, respectively, with 0.65 5MeH4B radical formed per enzyme heme. Thus, in the NOHA reaction, 5MeH4B radical formation was only slightly faster than that of H4B, and the 5MeH4B radical disappeared about twice as fast (Table I).

Because a H4B radical also forms in substrate-free iNOSoxy during single turnover reactions (11),² we checked whether the radical signals that we observed during our NOHA reaction might arise from the presence of some substrate-free enzyme. Increasing the NOHA concentration 2-fold did not reduce the radical intensity in our reactions (data not shown), consistent with iNOSoxy being saturated with substrate. In addition, the kinetics of biopterin radical formation and decay seen here in the NOHA reaction are quite distinct from the kinetics observed in reactions with substrate-free iNOSoxy. For example, in a representative substrate-free control reaction, the rates of biopterin radical formation and decay were 9.0 and 1.2 s⁻¹, respectively. Together, this argues that the transient biopterin radicals that we observed in the NOHA reaction had formed in iNOSoxy enzyme molecules that were actively catalyzing NOHA oxidation.

Fate of the H4B Radical—We next determined whether the H4B radical that formed during the NOHA or Arg reactions disappeared via an oxidative or reductive process. iNOSoxy samples were preincubated with H4B and substrate (NOHA or Arg) and then passed through a gel filtration column to remove unbound H4B just prior to setting up the single turnover reactions. The reactions were initiated by manually mixing at room temperature the filtered, anaerobic ferrous enzyme solutions that contained substrate with an O2-

A 

![EPR Signal](image)

5MeH4B

H4B

Gauss

3300 3302 3304 3306 3308 3310 3312 3314 3316 3318 3320 3322 3324 3326 3328 3330 3332 3334 3336 3338 3340 3342 3344 3346

B

![Kinetics of biopterin radical formation and decay during Arg hydroxylation and NOHA oxidation by H4B- or 5MeH4B-saturated iNOSoxy](image)

Fig. 2. Kinetics of biopterin radical formation and decay during Arg hydroxylation and NOHA oxidation by H4B- or 5MeH4B-saturated iNOSoxy. A shows the kinetics of biopterin radical formation and decay during Arg hydroxylation for iNOSoxy containing H4B (solid circles) or 5MeH4B (solid squares). Data are from Ref. 16. B shows the kinetics of biopterin radical formation and decay during NOHA oxidation for iNOSoxy containing H4B (solid circles) or 5MeH4B (solid squares). Inset, radical buildup versus the log of the reaction time. Reactions were setup and run as described in Fig. 1.

Fate of the H4B Radical—We next determined whether the H4B radical that formed during the NOHA or Arg reactions disappeared via an oxidative or reductive process. iNOSoxy samples were preincubated with H4B and substrate (NOHA or Arg) and then passed through a gel filtration column to remove unbound H4B just prior to setting up the single turnover reactions. The reactions were initiated by manually mixing at room temperature the filtered, anaerobic ferrous enzyme solutions that contained substrate with an O2-
saturated solution, and these were then left to react for ~20 s. We then determined the reduction state of the biopterin in each enzyme reaction sample. Fig. 3 contains representative HPLC fluorescent traces that were used to determine the amount of oxidized biopterin present in the NOHA or Arg reaction samples. Results from three experiments are listed in Table II. Almost all of the biopterin was in a fully reduced state (91 ± 2% H4B) at the end of the NOHA reaction, while at the end of the Arg reaction the biopterin was predominantly in an oxidized state (37 ± 8% H4B).³ As controls, we ran reactions with iNOSoxy that contained H2B plus Arg but otherwise underwent identical procedures and with iNOSoxy that contained H4B plus Arg that did not undergo the dithionite reduction step. In both cases, the original oxidation state of the biopterin was maintained at the end of the reaction period, indicating that non-enzymatic reduction or oxidation of the biopterin did not occur (data not shown). Together, these data indicate that H4B radical disappearance in the NOHA reaction was due to an enzyme-mediated reductive process, while in the Arg reaction radical disappearance was due to an oxidative process.

Heme Transitions during NOHA Oxidation—We utilized rapid-scanning stopped-flow spectroscopy to monitor heme transitions that occurred during the NOHA single-turnover reactions catalyzed by iNOSoxy. As in our previous report (20),

³ Up to 50% of the bound H4B may have dissociated from the H4B-saturated iNOSoxy during the time it took to prepare it for the single turnover reaction in the H4B-free buffer (see “Experimental Procedures”). Because free H4B would not have been oxidized in the single turnover Arg reaction, this helps explain why some H4B remained unoxidized at the end of the reaction.

⁴ C.-C. Wei, unpublished data.
Dual Redox Function of \( \text{H}_4\text{Biopterin} \) in the Second Step of NO Synthesis

Anaerobic ferrous iNOSoxy that contained NOHA and \( \text{H}_4\text{B} \) or 5Me\( \text{H}_4\text{B} \) was rapid-mixed with \( \text{O}_2 \)-containing buffer at 10 °C to start the reaction. Subsequent transformations were followed either by stopped-flow rapid-scanning spectroscopy, rapid-freezing EPR spectroscopy.

### Table I

| Transformation | NOHA | 5Me\( \text{H}_4\text{B} \) | Arg | 5Me\( \text{H}_4\text{B} \) |
|---------------|------|----------------|------|----------------|
| \( \text{Fe}^{II}\text{O}_2 \) formation (s\(^{-1}\)) | 47.5 ± 3.7 (4) | 71.9 ± 5.5 (4) | 52.7 ± 2.2 (4) | 52.7 ± 6.0 (4) |
| \( \text{Fe}^{II}\text{O}_2 \) disappearance (s\(^{-1}\)) or \( \text{Fe}^{II}\text{NO} \) formation (s\(^{-1}\)) | 56.7 ± 1.1 (4) | 40.9 ± 1.2 (4) | 12.5 ± 0.2 (4) | 34.7 ± 2.2 (4) |
| \( \text{Fe}^{III}\text{NO} \) disappearance (s\(^{-1}\)) or \( \text{Fe}^{III} \) formation (s\(^{-1}\)) | 2.3 ± 0.1 (4) | 3.23 ± 0.04 (4) | 11.1 (2) | 40 (2) |
| Bipterin radical formation (s\(^{-1}\)) | 31 ± 3.2 (4) | 35 ± 3.3 (4) | 0.71 (2) | 0.2 (2) |
| Bipterin radical decay (s\(^{-1}\)) | 8.3 ± 2.7 (4) | 15 ± 1.2 (4) | ~100 | ~100 |
| Theoretical yield of bipterin radical (%) | 55 ± 3.8 (4) | 65 ± 4.5 (4) | ~100 | ~100 |

\(^a\) Data are from Ref. 16.
\(^b\) Species are not applicable to Arg hydroxylation.

Fig. 3. Reverse phase HPLC chromatograms of bipterin remaining in reaction samples after NOHA oxidation (upper panel) or Arg hydroxylation (lower panel). Reactions were setup as described in the experimental section. The chromatograms were performed on samples processed under acidic conditions (traces a and c) and processed under alkaline conditions (traces b and d). The peaks with retention time ~21.5 min was that of bipterin. The peak area difference for the two processing conditions indicates the amount of reduced \( \text{H}_4\text{B} \) that was present in the completed reaction sample.

### Table II

| Experiment | \( \text{H}_4\text{B} \) content after Arg hydroxylation | \( \text{H}_4\text{B} \) content after NOHA oxidation |
|-----------|---------------------------------|-------------------|
| Exp. 1    | 28                              | 91                |
| Exp. 2    | 40                              | 89                |
| Exp. 3    | 44                              | 93                |

\(^a\) The experiments were performed using the same batch of iNOSoxy.
\(^b\) The \( \text{H}_4\text{B} \) content was determined as described in the experimental section.

We undertook single turnover studies to investigate redox functions of \( \text{H}_4\text{B} \) in the second reaction of NO synthesis. We found that \( \text{H}_4\text{B} \) is first oxidized to its radical and then is reduced back to \( \text{H}_4\text{B} \) during enzymatic conversion of NOHA to NO. The same process occurred in an iNOSoxy reaction that contained 5Me\( \text{H}_4\text{B} \) in place of \( \text{H}_4\text{B} \). Our measures of the amount of pterin radical formed per heme argue that these pterin redox transitions are significant and take place in a majority of enzyme molecules during the NOHA single turnover reactions. Thus, \( \text{H}_4\text{B} \) appears to undergo two redox transitions during the second step of NO synthesis.

The pterin radicals formed in the NOHA reaction displayed unique kinetic properties. For example, 5Me\( \text{H}_4\text{B} \) and \( \text{H}_4\text{B} \) radicals formed at similar rates in the NOHA reactions, whereas in the Arg reactions the rate of radical formation by \( \text{H}_4\text{B} \) is 4 times slower than 5Me\( \text{H}_4\text{B} \) (see Table I). Both pterin radicals also disappeared at least 10 times faster in the NOHA reactions compared with the Arg reactions. This is consistent with the pterin radicals being actively reduced during enzyme catalysis in the NOHA reaction, whereas the radicals are left to oxidize following completion of the Arg reaction. Together, these differences establish that the pterin operates under unique circumstances in each reaction of NO synthesis.

### DISCUSSION

Mechanistic Implications—To understand the potential significance and roles of \( \text{H}_4\text{B} \) redox transitions during NOHA oxidation, it helps to view their timing in relation to the heme transitions that occur during the same reaction. Our stopped-
flow study detected a minimum of three consecutive heme transitions that involve four heme species; the initial ferrous iNOSxy species binds O₂ to generate an FeIIIO₂ intermediate, which then appears to convert to an FeIII NO product complex, which subsequently dissociates into ferric iNOSxy and free NO (Fig. 5).

Our stopped-flow analysis showed that the majority of oxygen binding to ferrous heme occurs within 30 ms after initiating the NOHA reactions. The FeIIIO₂ intermediate then disappears by 100 ms, and this transition occurs coincident with buildup of the pterin radical. Their tight kinetic relationship is consistent with H₄B radical formation representing transfer of one electron from H₄B to the FeIIIO₂ intermediate, as also occurs in the Arg reaction (14, 15). Disappearance of the FeIII NO species is also coincident with buildup of a heme-NO product complex that we have identified as ferric based on its spectral signature. Thus, H₄B electron transfer appeared to form a heme-based oxidant that quickly reacts with NOHA and leads to buildup of an immediate heme-NO product complex. Next there occurred a reduction of the H₄B radical, beginning at around 100 ms and becoming essentially complete by 300 ms. This reductive transition is unique to the NOHA reaction. Unfortunately, reduction of the H₄B radical was not associated with discernable buildup of a distinct heme spectral species. Our attempts to fit the spectral data so as to incorporate a new intermediate within this time range were not successful. The final transition (from about 300 ms onward) represents dissociation of NO from the ferric heme-NO complex and regenerates the ferric enzyme. Together, our analysis suggests that the NOHA reaction may actually contain at least four consecutive heme transitions, although only three of these transitions are distinguishable by our stopped flow analysis (Fig. 5). Importantly, two of the transitions involve separate one-electron redox transformations by H₄B.

Our current work reveals that H₄B may perform a common function in both reactions of NO synthesis, namely, to provide an electron to the FeIII NO intermediate. However, in the NOHA reaction there also occurs a subsequent reductive transition whereby the H₄B radical receives an electron. We believe that this function may be essential for NO synthesis. As discussed previously (23), if the H₄B radical does not take back an electron from the system, then NOHA would undergo a two-electron oxidation instead of a three-electron oxidation and generate nitroxy, the one-electron reduced form of NO. This process actually appears to take place in H₄B-free NOS enzymes that are catalyzing NADPH-driven NOHA oxidation, as judged by buildup of a ferrous heme-NO complex during catalysis (23, 26). Thus, NOS would generate nitroxy instead of NO if the electron transfer back to the H₄B radical were blocked.

What enzyme intermediate might donate an electron back to the H₄B radical? This remains an open question. Our current data indicate that the electron transfer needs to occur between 100 and 300 ms (in a reaction run at 10 °C) to be associated with the observed reduction of the H₄B radical. As has been discussed previously in detail (12, 20, 21, 23), there are at least three putative intermediates that could serve as the electron donor. Both we (23) and others (12, 21) have considered a putative ferrous heme-NO intermediate as one of the possible donors. Because the light spectra of NOS ferrous and ferric

![Image](https://example.com/image.jpg)
heme-NO complexes are similar, it is conceivable that oxidation of a putative ferrous heme-NO complex to generate the observed ferric heme-NO complex might occur during the reaction without our detecting it by stopped-flow spectral analysis. Although the NOS ferrous heme-NO complex exhibits a unique EPR signal (24), we did not observe buildup of this particular signal in our stopped-freeze EPR samples that were collected within the appropriate time range (50–200 ms, data not shown). Thus, at present we have no data to discern which enzyme species reduces the H₄B radical during the NOHA reaction. This interesting problem requires further study.

**Relationship to Other Reports**—Our findings differ from two earlier studies that reported little or no H₄B radical buildup during a NOHA single turnover reaction (11, 12). We suspect that this discrepancy may reflect the inherent difficulty in detecting the transient H₄B radical during the NOHA reaction. In any case, it is useful to consider the details. In one study buildup of 0.03 H₄B radical per heme was observed (11), and its rate of formation and decay were 15 and 0.2 s⁻¹/H₁₁O₂, respectively. Another study reported no H₄B radical buildup during NOHA oxidation (12). However, in this case the reactions were run in 50% polyethylene glycol at cryogenic temperatures, initiated by manual mixing of an oxygenated solution, and followed by manual sample collection and freezing. Thus, our different results could be due to the use of a cryosolvent and to the poor time resolution inherent in the mixing and sample collection.

Related studies show that this particular cryogenic system bestows unique properties to NOS enzymes, as manifested by differences in spectral properties of the heme-dioxy intermediate, relative stabilities of other heme-oxy species, yields of Arg hydroxylation in single turnover reactions, and yields of H₄B radical in reactions run with substrate-free enzymes (27–29). Therefore, meaningful comparisons may not be possible in this case.

**Summary**—We provide evidence of a new redox function for H₄Biopterin in the second step of NO synthesis.

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