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Pterin-Centered Radical as a Mechanistic Probe of the Second Step of Nitric Oxide Synthase

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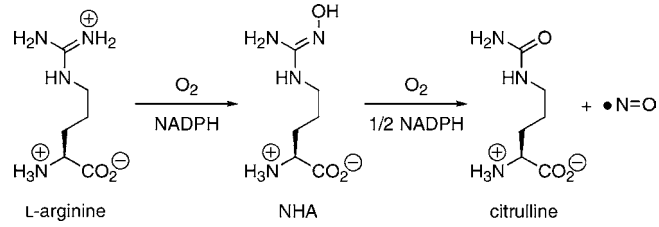
Abstract: The enzyme nitric oxide synthase is both medically relevant and of particular interest from a basic sciences perspective due to the complex nature of the chemical mechanism used to generate NO. The enzyme utilizes multiple redox-active cofactors and substrates to catalyze the five-electron oxidation of substrate L-arginine to citrulline and nitric oxide. Two flavins, a cysteine-coordinated heme cofactor and, uniquely, a tetrahydrobiopterin cofactor, are used to deliver electrons from the cosubstrate NADPH to molecular oxygen, analogous to other P450s. The unprecedented involvement of the pterin cofactor as a single electron donor is unique among P450s and pterin utilizing proteins alike and adds to the complexity of this enzyme. In this report, the peroxide shunt with both Mn- and Fe-containing heme domain constructs of $iNOS_{heme}$ was used to characterize the formation of HNO as the initial inorganic product produced when oxygen activation occurs without pterin radical formation. To recover NO formation, preturnover of the iron-containing enzyme with L-arginine was used to generate the pterin-centered radical, followed by peroxide shunt chemistry. Comparison of NO produced by this reaction with reactions that do not undergo preturnover, do not have peroxide added, or are performed with a pterin unable to generate a radical shows NO production to be dependent on both a pterin-centered radical and activated oxygen. Finally, the chemical HNO donor, Angeli's salt, was used to form the ferrous nitrosyl in the presence of the pterin radical intermediate. Under these conditions, the rate of pterin radical decay was increased as monitored by EPR spectroscopy. In comparison to pterin that aerobically decays, the Angeli's salt treated sample is also significantly protected from oxidation, suggesting ferrous-nitrosyl-mediated reduction of the radical. Taken together, these results support a dual redox cycling role for the pterin cofactor during NOS turnover of NHA with particular importance for the proper release of NO from a proposed ferrous nitrosyl intermediate.

Introduction

Nitric oxide is generated in eukaryotes by the enzyme nitric oxide synthase (NOS). NOS is a multicofactor, multidomain protein that catalyzes the five-electron oxidation of substrate L-arginine to citrulline and NO (Scheme 1). The overall reaction occurs through two successive oxidative transformations, with N^G-hydroxy-L-arginine as a stable intermediate.[1] Each oxidative transformation of substrate requires molecular oxygen (O_2) and electrons derived from the cosubstrate nicotinamide adenine dinucleotide phosphate (NADPH).[2] The molecular details of these transformations are exceedingly complex and have been the focus of intense study. Previous work established that the catalytic oxidation is performed at the cysteine-coordinated heme cofactor, where substrate L-arginine binds in close proximity. Electrons are delivered to the heme through the action of the flavin and pterin cofactors to activate molecular oxygen leading to substrate oxidation. Although the details of the first step are also still debated,[3] the focus of attention here is on the second half reaction.

Scheme 1[a]



[a] Conversion of L-arginine to citrulline and NO occurs over two successive oxidative steps, with N^G-hydroxy-L-arginine (NHA) as a stable intermediate. The conversion of NHA to citrulline and NO requires molecular oxygen and 1/2 equiv of NADPH as cosubstrates.

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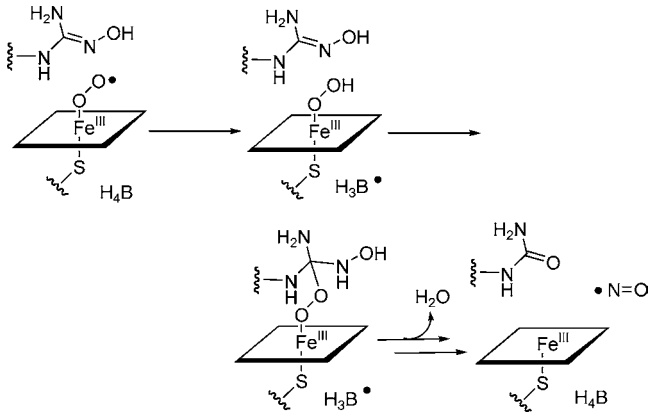
Scheme 2. Proposed Mechanism of NHA Oxidation[a]



[a] The pterin donates an electron to activate heme-bound oxygen to a ferric−peroxo intermediate, resulting in a pterin-centered radical. Nucleophilic attack of NHA by the ferric−peroxo intermediate results in an addition complex, which breaks down to generate citrulline, NO, and the reduced pterin cofactor.

The oxidation of NHA to citrulline and nitric oxide is a reaction that requires the overall input of a single electron, as shown by Scheme 1. Strong evidence now supports the involvement of a ferric−peroxo intermediate as the active oxidant in this transformation.[4] Generation of the active ferric−peroxo intermediate requires two electrons to reduce the cosubstrate O_2. Previous work by us and others has shown that, in the heme domain construct of NOS ($iNOS_{heme}$), turnover of NHA requires the presence of a redox-active pterin and reduced heme.[5−8] These results support the delivery of two electrons to the heme in order to activate molecular oxygen to the ferric−peroxo intermediate, one derived from the reductase domain of the protein and one delivered by the pterin cofactor, analogous to L-arginine turnover. However, in order to form NO and citrulline, one of these electrons must be removed from the intermediates, thereby remaining in the enzyme.

The delivery of a single electron by the pterin cofactor would result in the formation of a pterin-centered radical, just as is observed for turnover of L-arginine to NHA.[5] However, the formation of this radical during NHA oxidation has only been observed by one laboratory, and the rate of its decay was significantly faster in reactions with NHA than with those that involve L-arginine as substrate.[8] Taken together, with our inability to directly detect this radical intermediate, these results suggest transient redox cycling of the pterin cofactor, resulting in very little to no accumulation of the radical. On the basis of these results, we have hypothesized that the delivery of an electron by (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) leads to the formation of a ferric−peroxo intermediate that then reacts with NHA (Scheme 2). Following substrate oxidation, an electron must be removed from the enzyme-bound reaction products to ensure the balance of the electron stoichiometry and the generation of the radical product nitric oxide. The rate-limiting nature of the second electron delivery has hindered characterization of the subsequent intermediates. As a result, we have chosen to employ chemical methods to further define the molecular details of this transformation. One aspect of the work presented here is to provide direct evidence for the dual reductant/oxidant role of the pterin cofactor during turnover of NHA. In addition, we provide evidence for the intermediate that acts to reduce the pterin-centered radical, leading to NO formation.

Materials and Methods

Materials and General Methods: All buffers were made with 18 megOhm water from a Milli-Q Ultrapure water purification system (Millipore) and were filtered using 0.45 μm aqueous filtration units from Millipore. Terrific Broth, isopropyl thiogalactoside (IPTG), hydroxyethyl piperzine ethylsulfonate (HEPES), and dithiothreitol (DTT) were acquired from Research Products International. (6*R*)-5,6,7,8-tetrahydro-L-biopterin (H_4B) and 7,8-dihydrobiopterin (H_2B) were purchased from Schircks Laboratory (Switzerland). Solutions of H_4B were made in a solution of DTT (100 mM) and HEPES (100 mM, pH 7.5). Solutions of H_2B were dissolved in dimethylsulfoxide. Angeli's salt was purchased from Cayman Chemical. Stock solutions of Angeli's salt (AS, $N_2O_3^{2-}$) were dissolved in 10 mM NaOH, and concentrations were determined spectrally at 250 nm ($\varepsilon = 8000\ M^{-1}\ cm^{-1}$). NHA was synthesized as reported previously.[9] All other reagents were purchased from Sigma Aldrich. Protein expression and purification were performed as previously described with the method of Hurshman et al. used to express $iNOS_{heme}$ and Ni-NTA affinity and anion exchange chromatography used to purify $iNOS_{heme}$.[10,11] Heme-substituted $iNOS_{heme}$ was expressed and purified using the recombinant expression method described previously.[11]

Mn and Fe−$iNOS_{heme}$ Peroxide Shunt Reactions: The manganese protoporphyrin IX (Mn-PPIX)-substituted heme domain of murine inducible NOS (Mn−$iNOS_{heme}$) and WT $iNOS_{heme}$ reactions were monitored by UV−visible spectroscopy. Both proteins were reconstituted with H_4B (500 μM) with L-arginine (1 mM) for >1 h on ice. Removal of small molecules was performed using a PD-10 column. Addition of 50 μM excess H_4B to samples ensured complete pterin binding. Samples were finally concentrated to >300 μM $iNOS_{heme}$. For Fe-containing $iNOS_{heme}$, protein (2−7 μM) was mixed with substrate NHA (1 mM) and H_2O_2 (3 or 100 mM). Spectra were recorded on a Cary 3E UV−visible spectrophotometer from 250 to 700 nm for various times at room temperature. For Mn−$iNOS_{heme}$, only the reaction with 100 mM H_2O_2 was performed. Similar protein samples containing NHA but no H_2O_2 were mixed with AS (200 μM), and UV−visible spectra were recorded as described above.

NO formation from peroxide shunt reactions with Mn- and Fe-containing $iNOS_{heme}$ was measured using a Sievers 280i nitric oxide analyzer (NOA). Samples (100 μL) contained $iNOS_{heme}$ (2 μM), H_2O_2 (100 mM), and NHA (1 mM) in HEPES (100 mM, pH 7.5). Reactions were performed in sealed Reacti-Vials (Pierce) and were initiated with the addition of H_2O_2. After 10 min, headspace was removed using a gastight syringe and analyzed by NOA. Each reaction was performed six times.

Mn^{II} and Fe^{II}−$iNOS_{heme}$ Nitrosyl Stability: Ferrous stocks of Mn- and Fe-containing $iNOS_{heme}$ (2−7 μM, 500 μL) were prepared by dithionite reduction in the presence of NHA (1 mM) in anaerobic cuvettes. Anaerobic HEPES buffer (100 mM, pH 7.5) was bubbled on ice for 2 min with NO gas delivered from an NO tank bubbled

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through NaOH (5 M). Once the heme was reduced as determined by UV−visible spectra, NO saturated buffer (5 μL) was added. Mn^{II}−NO or Fe^{II}−NO complexes were opened to aerobic atmosphere, and an equal volume of aerobic buffer was added. UV−visible spectra were recorded until no further change was observed.

$iNOS_{heme}$ Single Turnover, Peroxide Shunt Reactions: $iNOS_{heme}$ was reconstituted on ice in the presence of DTT (5 mM) and H_4B or H_2B (500 μM) for >1 h. Protein was equilibrated into HEPES buffer (100 mM, pH 7.5) using a PD-10 column. After the addition of excess pterin (50 μM), the protein was concentrated to 300 μM, brought into an anaerobic chamber, and stored uncapped at 4 °C for >1 h to equilibrate with the anaerobic atmosphere. Freshly dissolved sodium dithionite was prepared daily. Dithionite was quantified by spectral titration against ferricyanide (A_{420}, ε = 1000). The protein was subsequently reduced with 2 electron equivalents, 1 molar equiv of dithionite. Reduced protein was used to make anaerobic reactions, 20 μL final volume, in 0.3 mL Reacti-Vials that contained ferrous $iNOS_{heme}$ (80 μM) and L-arginine (2 mM). Anaerobic reactions were mixed with an equal volume of aerobic HEPES at room temperature. Control reactions were analyzed by electron paramagnetic resonance (EPR) spectroscopy for the presence of the pterin radical 30 s after the initiation of turnover with oxygen (Supporting Information). Thirty seconds after initiation of the reaction, 5 μL of a solution containing NHA (9 mM) and H_2O_2 (27 mM) was added. Thirty seconds after the addition of NHA and H_2O_2, headspace was sampled (250 μL) using a gastight syringe and injected onto a Sievers 280i nitric oxide analyzer (NOA). Final reactions (45 μL) consisted of 35.6 μM protein, 6.7 μM pterin, 75 μM DTT, 0.89 mM L-arginine, 1 mM NHA, and 3 mM H_2O_2.

Negative control reactions consisted of the same contents as the experimental samples with the use of H_2B-bound protein or with the exclusion of dithionite or peroxide. As a positive control, reduced protein was used to make single turnover reactions of NHA. Samples contained $iNOS_{heme}$ (80 μM) and NHA (2 mM) in Reacti-Vials (20 μL total volume). These samples were mixed with an equal volume of aerobic buffer from which headspace was sampled (250 μL) and analyzed by the NOA 30 s after initiation with oxygen.

To ensure the presence of the pterin radical, samples of 150 μL of reduced $iNOS_{heme}$ (80 μM) with L-arginine (2 mM) were mixed with an equal volume of aerobic HEPES and transferred to an EPR tube. Samples were hand frozen in liquid nitrogen 30 s after initiation of turnover with aerobic buffer. CW EPR was performed at the UC Davis CalEPR Center. Samples were analyzed on a Bruker ECS 106 X band instrument equipped with an Oxford ESR900 liquid He transfer system. Spectra were obtained at a temperature of 10 K with a 500 μW microwave power, a frequency of 9.69 GHz, a modulation amplitude of 7.9 Gauss (0.79 mT), and modulation frequency of 100 kHz.

Single Turnover Angeli's Salt Chase by EPR: To probe the effect of the ferrous nitrosyl on the pterin radical, single turnover reactions with L-arginine were performed followed by Angeli's salt addition. Wild-type $iNOS_{heme}$ was reconstituted with H_4B (500 μM) and L-arginine (2 mM). Samples were desalted in the presence of L-arginine (2 mM) and were concentrated to 300 μM [NOS]. Excess pterin was not added to ensure that the observed signal could be attributed to pterin-bound protein and not free pterin in solution. Protein was reduced with dithionite as described above, and a ferrous stock of $iNOS_{heme}$ (150 μM) with L-arginine (2 mM) was prepared. Reduced protein (150 μL) was mixed with an equal volume of aerobic HEPES (100 mM, pH 7.5). After 30 s, Angeli's salt (2 μL of a 100 mM stock, 4.75 mM final) or HEPES (2 μL) was added to the reactions, and samples were transferred to EPR tubes. Samples were then aged for 0, 30, or 60 s at room temperature before being quenched by submersion of the EPR tube in liquid nitrogen. Each time point was performed in duplicate. EPR spectra were recorded as described, and the pterin radical was quantified by comparison to a known standard of copper-ethylenediaminetetraacetic acid (Cu-EDTA, 300 μM).

Pterin Oxidation State Assay: The oxidation state of bound pterin was assessed using a modified method of Hurshman et al.[10] Turnover reactions were performed as described above using protein that was desalted with a solution containing 2 mM L-arginine in HEPES and that had no additional pterin added following this desalting step. Reactions were set up and performed as described above. Thirty seconds following the secondary addition for each reaction, guanidine HCl (5 μL of 8 M stock) was added to each 40 μL reaction. Samples were mixed vigorously for 30 s, and after an additional 5 min, DTT (5 μL of 1 M stock) was added. Samples were again vortexed and then heated at 90 °C for 2 min to precipitate the protein. Following heat denaturation, the reactions were centrifuged at 14 000 rpm for 10 min to remove precipitated protein, and finally, the eluate was assayed by high-performance liquid chromatography (HPLC) for pterin content as described previously. All manipulations of the samples up to the point of centrifugation were performed in the sealed Reacti-Vials. Pterin recovery was >85% relative to total protein content. Performing the same series of manipulations with pure H_4B and H_2B ensured that the oxidation state is maintained during the sample preparation process.

Results and Discussion

Mn^{II}−NO and Fe^{II}−NO Formation during the Peroxide Shunt: The initial characterization of the peroxide shunt catalyzed by NOS identified N_2O as an inorganic reaction product.[12] This product was hypothesized to arise from the dimerization of nitroxyl (HNO). The formation of HNO would be expected since the input of two electrons to reduce O_2 would not be required when using H_2O_2, thus a pterin radical would not be formed, leaving no avenue to remove an electron from the reaction product. However, the direct detection of HNO has not been observed during the peroxide shunt. Previous work has established that Mn porphyrins offer a chemical means of stably detecting and discriminating HNO from NO and other nitrogen oxides.[13] The Mn^{III} species reacts with HNO to induce the formation of the Mn^{II}−NO complex while exhibiting no reactivity with NO. Because the Mn-substituted $iNOS_{heme}$ can perform the peroxide shunt,[4] this protein was chosen in an attempt to trap and stabilize an HNO intermediate that may initially form during this reaction.

To establish that Mn−$iNOS_{heme}$ could trap HNO as reported for model porphyrins, the chemical HNO donor Angeli's salt ($N_2O_3^{2-}$) was added to the Mn^{III} protein (Figure 1). The addition of 10 molecular equivalents of Angeli's salt led to the complete formation of a spectral intermediate consistent with Mn^{II}−NO over the course of 15 min. This intermediate shows spectral features consistent with those observed upon addition of NO to the Mn^{II} protein and those previously reported for Mn-substituted $P450_{cam}$.[14] The Mn−$iNOS_{heme}$ peroxide shunt reactions were then monitored for the generation of the same spectral intermediate (Figure 2b). Within the time of adding H_2O_2 and taking the first spectrum, the protein shows complete transition to the Mn^{II}−NO complex, providing direct evidence of the formation of HNO during the peroxide shunt.

When the same reaction was performed with the wild-type $iNOS_{heme}$, no buildup of a spectral intermediate was detected;

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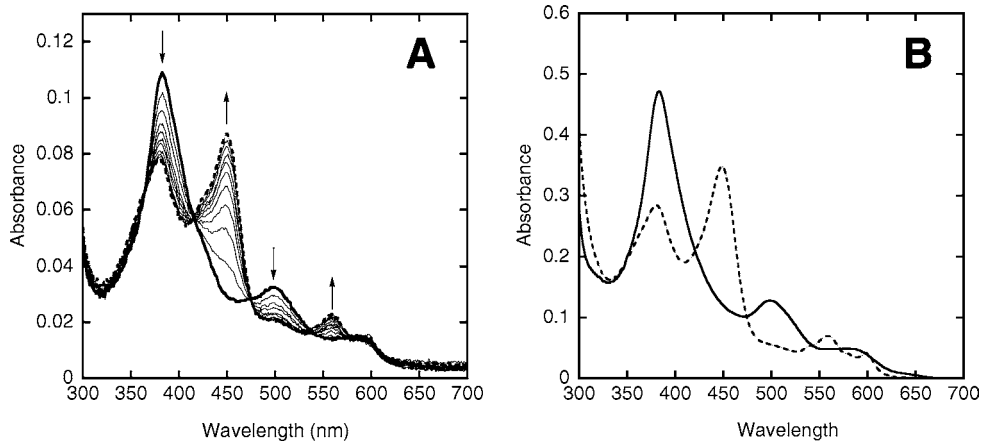

Figure 1. $Mn^{II}-NO$ formation. (A) Addition of a HNO chemical donor to $Mn-iNOS_{heme}$ leads to $Mn^{II}-NO$ formation. Reaction contains $Mn-iNOS_{heme}$ (1.7 μM), NHA (1 mM), and Angeli's salt (170 μM). Spectra of $Mn-iNOS_{heme}$ were recorded over 15 min. Initial spectrum is the dark solid trace, and final spectrum is the dark dashed trace; arrows indicate direction of spectral change from the initial to final spectrum. (B) Peroxide shunt reactions with $Mn-iNOS_{heme}$ show complete formation of $Mn^{II}-NO$ during steady-state turnover. Reactions contain pterin-reconstituted $Mn-iNOS_{heme}$ (7.5 μM), NHA (1 mM), and H_2O_2 (100 mM). Spectra are of protein prior to (solid line) and immediately after the addition of H_2O_2 (dashed line).

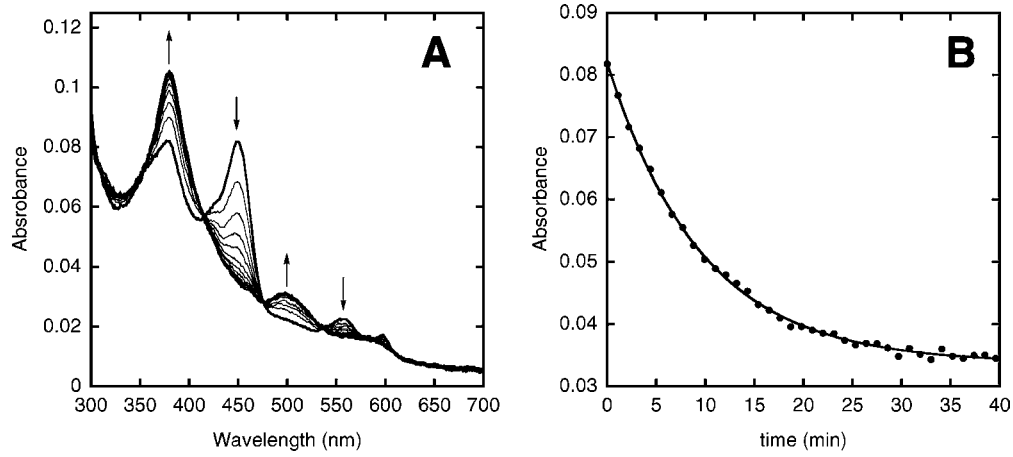

Figure 2. Stability of Mn^{II}-NO under aerobic conditions. (A) $Mn^{II}-iNOS_{heme}$ (1.7 μM) with NHA (1 mM) in an anaerobic cuvette was mixed with NO-saturated buffer (5 μL) to generate the $Mn^{II}-$nitrosyl. Once formed, the sample was opened to the atmosphere, and an equal volume of aerobic HEPES (100 mM, pH 7.5) was added. Spectra were recorded over 40 min to monitor the decay of the $Mn^{II}-NO$ complex. (B) Absorbance trace at 450 nm. Trace was fit to a single exponential decay equation to determine the observed rate of $Mn^{II}-NO$ decay.

rather, continuous bleaching of the heme was observed (data not shown). While buildup of $Mn^{II}-NO$ does support the formation of HNO during the peroxide shunt, the question of why the same spectral intermediate is not observed during turnover with the Fe protein is raised. In model porphyrin systems, the affinity of Fe porphyrins for HNO is nearly an order of magnitude higher than for Mn porphyrins.[13,15] This seeming discrepancy led to the hypothesis that the ability of the Mn-containing protein to accumulate the $Mn^{II}-$nitrosyl may be due to the stability of the complex relative to the Fe protein under the conditions of the reaction.

To test this, the $Mn^{II}-NO$ and $Fe^{II}-NO$ complexes were generated under an anaerobic atmosphere. Even in the presence of excess dithionite, used to scavenge NO in solution, both complexes are completely stable for hours. However, when mixed with aerobic buffer, a significant difference is observed. In the case of the iron protein, oxidation of the $Fe^{II}-NO$ complex is complete within the time it takes to collect the first spectrum, consistent with previous reports that the NOS ferrous$-$nitrosyl is very unstable in the presence of oxygen (k_{obs} = 11.4 min^{-1} at 10 °C[16]). In contrast to the high reactivity observed for the Fe containing protein, the Mn^{II}-nitrosyl complex is quite stable. The complex exhibits single-exponential decay over the course of 45 min with a $k_{obs} = 0.102 \pm 0.002$ min^{-1}, greater than two-orders of magnitude more stable than the Fe^{II} protein under aerobic atmosphere (Figure 2).[16]

Given that the $Mn^{II}-$nitrosyl is much more stable to oxidative decay, we conclude that the inability to detect the $Fe^{II}-$nitrosyl during peroxide shunt turnover is actually due to the inherent instability of the complex and not due to the lack of formation. Because the major oxidant in the peroxide shunt reactions is H_2O_2 (100 mM) and not O_2 (~220 μM), the peroxide concentration in the reaction was lowered to 3 mM. With low peroxide, there is a significant buildup of the $Fe^{II}-NO$ complex within the first few minutes of the reaction (Figure 3a), as evidenced by the change in the Soret from 396 to 433 nm. Furthermore, the spectral changes observed are similar to those seen with $iNOS_{heme}$ in the presence of Angeli's salt (Figure 3b) and when

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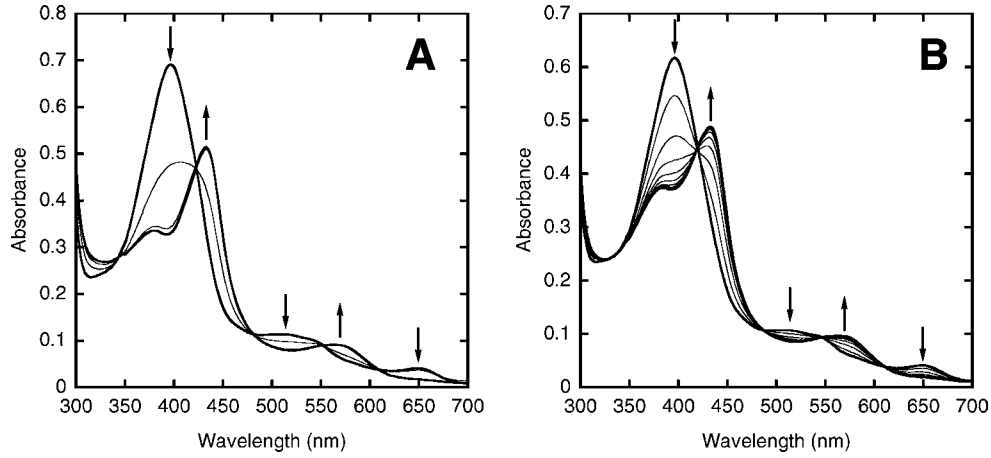

Figure 3. $iNOS_{heme}$ $Fe^{II}-NO$ formation during the peroxide shunt and in the presence of Angeli's salt. (A) Pterin-bound $iNOS_{heme}$ (8 μM) with NHA (1 mM) was mixed with H_2O_2 (3 mM) at room temperature. (B) Pterin-bound $iNOS_{heme}$ (7 μM) with NHA (1 mM) and Angeli's salt (200 μM) at room temperature in an anaerobic cuvette. Spectra were monitored before and after addition of hydrogen peroxide or Angeli's salt. Arrows indicate spectral transitions.

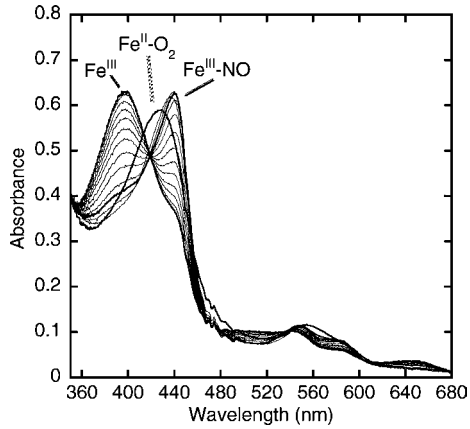

Figure 4. Single turnover NHA reaction by stopped-flow. Dithionite-reduced, pterin-bound $iNOS_{heme}$ (7 μM) with NHA (2 mM) was rapidly mixed with O_2 (2.2 mM) and saturated HEPES (100 mM, pH 7.5) at 10 °C; 96 scans were collected over ~1 s. Observed spectral intermediates are labeled as described previously.[18] Global analysis was performed with Specfit to determine the observed rates for heme transition.

NO is added to the anaerobic Fe^{II} protein, as reported previously.[17] These results show that HNO can also be trapped at the heme during peroxide shunt turnover with Fe-containing protein and supports the hypothesis that HNO represents the initial inorganic product formed in these reactions.

The generation of a ferrous−nitrosyl complex during peroxide shunt turnover is an interesting observation, especially when considered in the context of the single turnover reaction catalyzed by $iNOS_{heme}$ with molecular oxygen. In the presence of substrate and oxygen, the pterin-bound ferrous heme domain of nitric oxide synthase carries out a single turnover reaction, generating NO and citrulline.[5] These reactions can be followed by stopped-flow spectroscopy by rapidly mixing reduced protein with O_2-containing buffer to initiate the reaction.[18] A number of heme transitions are observed during turnover reactions with NHA as substrate (Figure 4). First, a species with a Soret maximum at 428 nm corresponding to the ferrous−oxy complex is observed. This intermediate rapidly converts ($k_{obs} = 70\ s^{-1}$) to an $Fe^{III}-NO$ complex. Finally, NO dissociates ($k_{obs} = 3\ s^{-1}$) to generate the high-spin resting state of the protein with a Soret maximum at 396 nm.

Pterin Radical as a Redox Cycling Cofactor: The observation of a $Fe^{III}-NO$ complex in the natural turnover reaction differs by only a single electron from the $Fe^{II}-NO$ intermediate that is observed during peroxide shunt turnover. As discussed previously, the generation of the ferric−peroxo intermediate from O_2 leads to the formation of a pterin-centered radical. This radical intermediate can act as a single electron oxidant to balance the electron stoichiometry and ensure the formation of the $Fe^{III}-NO$ complex that then dissociates to form NO (Scheme 3a). Because hydrogen peroxide can directly form the ferric−peroxo intermediate, generation of a pterin-centered radical will not occur and an electron cannot be removed from the reaction intermediate, leading to the formation of the $Fe^{II}-NO$ complex that either releases HNO or is oxidized by peroxide to generate nitrite and nitrate (Scheme 3b).

On the basis of this logic, we hypothesized that in the presence of a pterin-centered radical the peroxide shunt reaction should generate NO. To test this, a single turnover peroxide shunt reaction was devised (Scheme 3c). In this reaction, a single turnover is carried out first with L-arginine, oxygen, and ferrous $iNOS_{heme}$. This reaction generates a pterin-centered radical and NHA.[5] Addition of H_2O_2 will then induce peroxide shunt chemistry, oxidizing the substrate NHA. However, if the role of the pterin radical is to act as an oxidant, the presence of a single oxidizing equivalent on the pterin should oxidize a reaction intermediate downstream of the ferric−peroxo species as in normal O_2 turnover, resulting in the generation of NO.

When a single turnover reaction is performed with $iNOS_{heme}$ and L-arginine as substrate, followed by the initiation of peroxide shunt chemistry, there is a significant amount of NO detected in the headspace of the reaction (Figure 5). Three control reactions were performed to establish that (i) NO formation is dependent on the peroxide shunt reaction; (ii) this result is dependent on the preturnover of the protein with L-arginine; and (iii) this is a pterin-dependent process.

Low levels of NO are observed when preturnover with L-arginine is absent (Figure 5, reaction b), controlling for the formation of NO from the peroxide shunt and establishing the dependence of NO production on the preturnover event.

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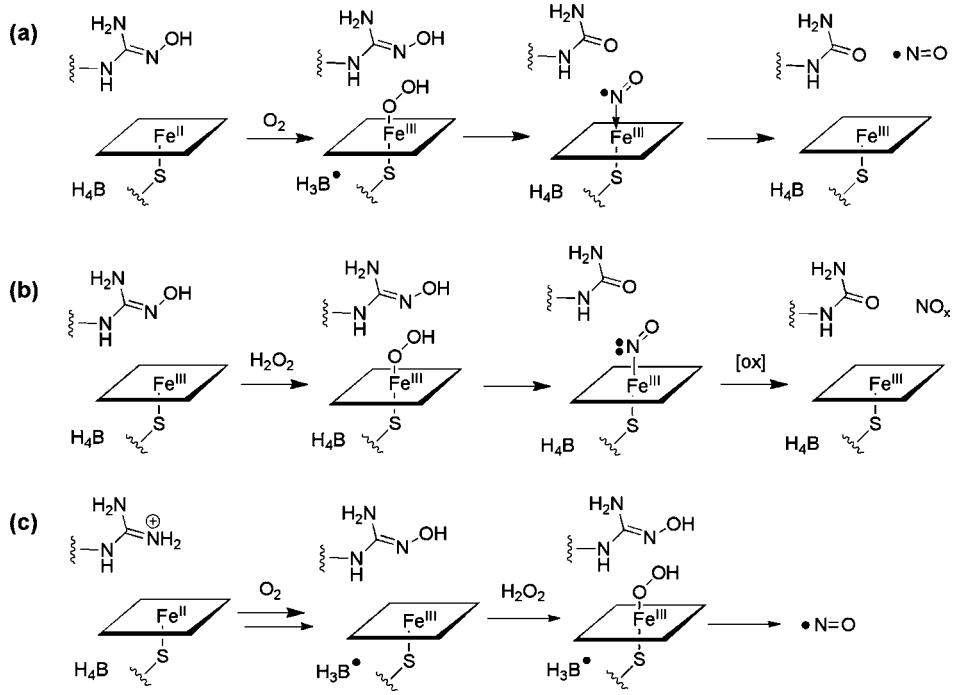
(18) Boggs, S.; Huang, L.; Stuehr, D. J. *Biochemistry* **2000**, *39*, 2332–2339.

Scheme 3[a]



[a] Single turnover and peroxide shunt reactions. (a) During the O_2 and dithionite-driven single turnover of NHA, the pterin donates an electron to the oxygen-bound ferrous heme. Generation of the ferric−peroxo intermediate (not observed by stopped-flow) induces oxidation of NHA to citrulline and an Fe^{III}−NO complex as observed by stopped-flow (depicted using a dative bond to the iron). NO dissociation from the ferric heme forms the final products. (b) During the peroxide shunt, H_2O_2 binding at the ferric heme results in a ferric−peroxo intermediate that does not contain a pterin-centered radical. Oxidation of NHA by this intermediate results in the formation of the Fe^{II}−NO complex (depicted third from the right as an Fe(III) NO^- complex). In the presence of oxidants ([ox]), oxygen and H_2O_2, this intermediate results in a number of nitrogen oxides including N_2O, nitrite, and nitrate. (c) Single turnover, peroxide shunt is a reaction that uses the O_2 and dithionite-driven turnover of L-arginine to generate a pterin-centered radical. Peroxide shunt chemistry is induced to make a ferric−peroxo intermediate with a pterin-centered radical that is predicted to balance the electron stoichiometry and result in NO production.

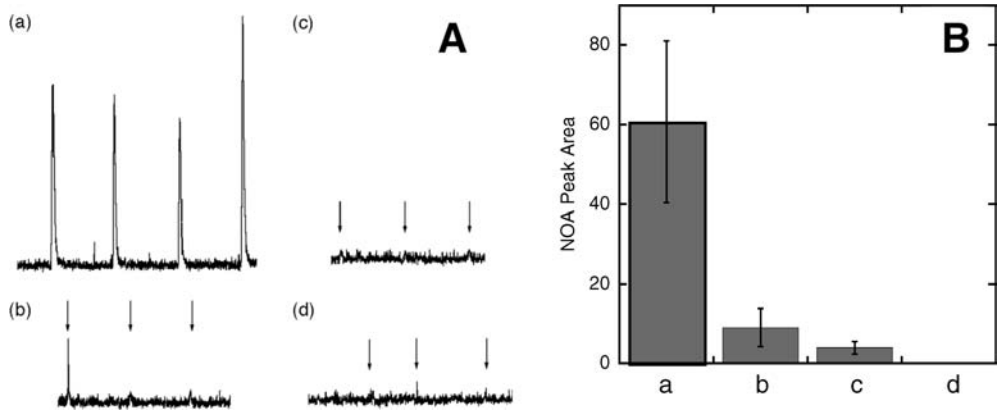
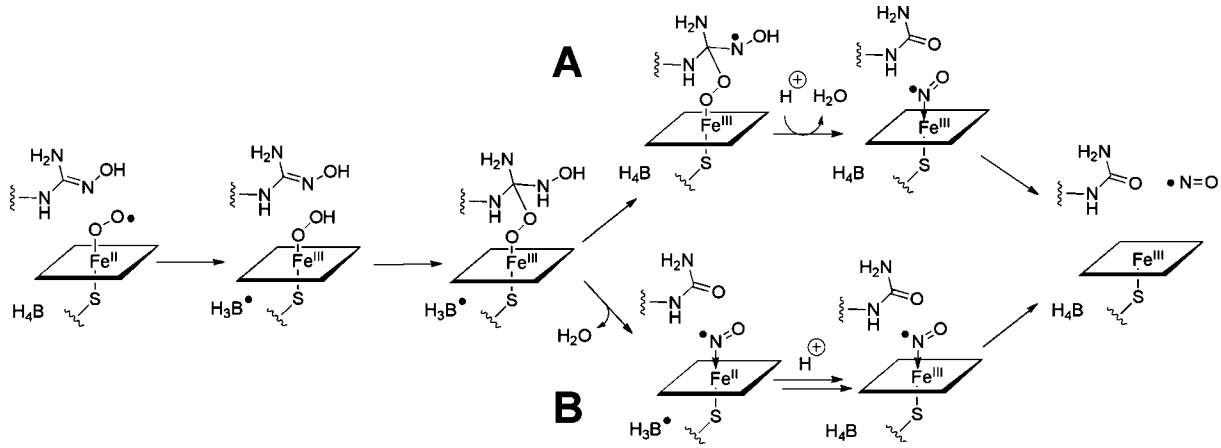


Figure 5. Single turnover, peroxide shunt NO production. (A) Representative NOA chemiluminescence signals for reactions. (B) Integration of NOA signals for each reaction. Values reported as the average ± standard deviation of multiple samples ($n = 6$). (Reaction a) H_4B-bound, ferrous $iNOS_{heme}$ (80 μM) with L-arginine (1 mM) was turned over by the addition of O_2. Peroxide shunt chemistry was initiated by the addition of NHA (1 mM) and H_2O_2 (3 mM). Headspace from reactions was removed and NO content measured by NOA. (Reaction b) Peroxide shunt reaction without preturnover of L-arginine. (Reaction c) Turnover of L-arginine without the addition of H_2O_2. (Reaction d) H_2B-bound, ferrous $iNOS_{heme}$ treated as described in reaction a.

Significantly less NO signal is also observed when peroxide is omitted from the reaction (Figure 5, reaction c), showing that multiple turnover of the protein is not occurring. Finally, single turnover followed by the peroxide shunt with protein reconstituted with the oxidized pterin, 7,8-dihydrobiopterin (H_2B), resulted in no detectable NO signal (Figure 5, reaction d). This result shows the dependence of NO production on the presence of a redox-active pterin that is able to form a pterin radical during L-arginine turnover. Despite the inability to directly observe the pterin radical during NHA single turnover with oxygen and dithionite, formation of NO using these reactions supports the role of pterin as a redox cycling cofactor during this reaction. These results lead to a mechanism in which the pterin initially donates an electron necessary for activation of oxygen to the active ferric−peroxo intermediate. This intermediate supports substrate oxidation, leading to an intermediate that is then oxidized by the pterin radical to ensure proper release of NO.

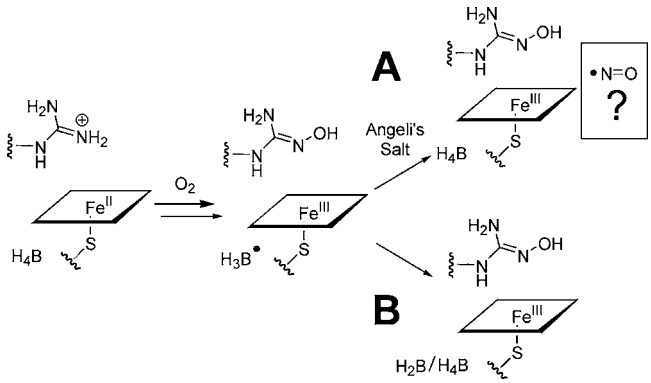
Ferrous Nitrosyl and Pterin Reduction. The above experiments do not address the identity of the intermediate that reduces the pterin radical during the reaction. Invoking nucleophilic participation of the ferric−peroxo intermediate, a number of

Scheme 4. Proposed Mechanisms of Addition Complex Decomposition[a]



[a] Nucleophilic attack of the ferric−peroxo complex results in an addition complex. Two possible mechanisms of decay can be envisioned for this intermediate. In path A, oxidation of the addition complex by the pterin radical leads directly to NO and citrulline. In path B, breakdown of the intermediate generates a ferrous-bound nitric oxide intermediate that is oxidized by the pterin radical to a ferric nitrosyl that directly releases NO. Dative bonds are to more clearly indicate the electron distribution in the bonds.

Scheme 5. Reaction of Angeli's Salt with the Pterin Radical[a]



[a] In the presence of the pterin-centered radical, Angeli's salt addition will generate the ferrous nitrosyl that leads to reduced pterin and NO (path A). Without Angeli's salt addition, the unstable pterin-centered radical could decay to H_2B or H_4B (path B).

possible species could be responsible for this activity (Scheme 4). In one path, the initial tetrahedral intermediate may reduce the pterin and decompose to directly generate a ferric nitrosyl. In a second possible mechanism, the tetrahedral intermediate may undergo decomposition to generate an HNO-bound ferric heme. Deprotonation of this intermediate will lead to the formation of a ferrous nitrosyl and citrulline. Oxidation of the ferrous heme by the pterin radical will regenerate the reduced pterin and lead to NO release.

To discriminate between these two mechanistic possibilities, we chose to investigate the reactivity of the pterin-centered radical in the presence of the NOS ferrous nitrosyl. Addition of Angeli's salt to the heme domain of NOS directly generates a ferrous nitrosyl, albeit quite unstable under aerobic conditions. If path B of Scheme 4 is correct, then the pterin is predicted to act as a single electron oxidant of the ferrous nitrosyl generated upon addition of Angeli's salt to the ferric protein (Scheme 5, path A). Experimentally, the pterin radical formed during the turnover of L-arginine should be reduced in the presence of added nitroxyl to generate H_4B and NO. In the absence of ferrous nitrosyl formation, however, the unstable pterin radical will either disproportionate to yield H_2B and H_4B or be oxidized by oxygen (Scheme 5, path B).

The inorganic product generated by the reaction between the ferrous nitrosyl and $H_3B^{\bullet}$ is predicted to be NO. However, Angeli's salt generates a significant background signal of NO when added to buffer and protein samples alike (data not shown). Given the significant background of NO generated by Angeli's salt and the large error associated with NO measures on a nitric oxide analyzer, definitive formation of NO from Angeli's salt and the pterin radical could not be observed.

Despite the inability to conclusively show NO formation from reaction of the pterin-centered radical with the ferrous nitrosyl, further characterization of this reaction was done to test other aspects of the mechanism. First, formation of the ferrous nitrosyl in the presence of the pterin radical should significantly reduce the pterin signal observed by EPR. As seen, the addition of Angeli's salt to samples containing the pterin radical greatly accelerated the loss of signal (Figure 6a). Although this result shows the ferrous nitrosyl does induce radical decay, it does not report the oxidation state of the pterin after the radical is lost. To address this, the pterin oxidation state was determined by HPLC for samples before L-arginine turnover, after L-arginine turnover, and after L-arginine turnover followed by the addition of Angeli's salt (Figure 6b). Significant oxidation of the pterin is observed in turnover samples with L-arginine relative to protein that did not undergo a turnover event. This is consistent with the unstable nature of the pterin radical leading to oxidation and disproportionation to generate a mixture of H_2B and H_4B following L-arginine turnover (Scheme 5, path B). Samples with added Angeli's salt had significantly less oxidized pterin than those that received buffer only. These results are similar to those previously reported for NHA turnover using oxygen and dithionite, where the pterin remains reduced at the end of the reaction.[8] Importantly, there is no change in oxidation state when the reaction is performed with H_2B containing $iNOS_{heme}$ (data not shown), demonstrating that Angeli's salt cannot directly reduce the oxidized H_2B. These results do not exclude the possible role of the tetrahedral intermediate as the pterin reductant but do show that reduction of the pterin-centered radical by the ferrous nitrosyl is certainly feasible.

Conclusions

Overall, our results are consistent with the pterin cofactor of NOS playing a dual reductant/oxidant role during catalytic

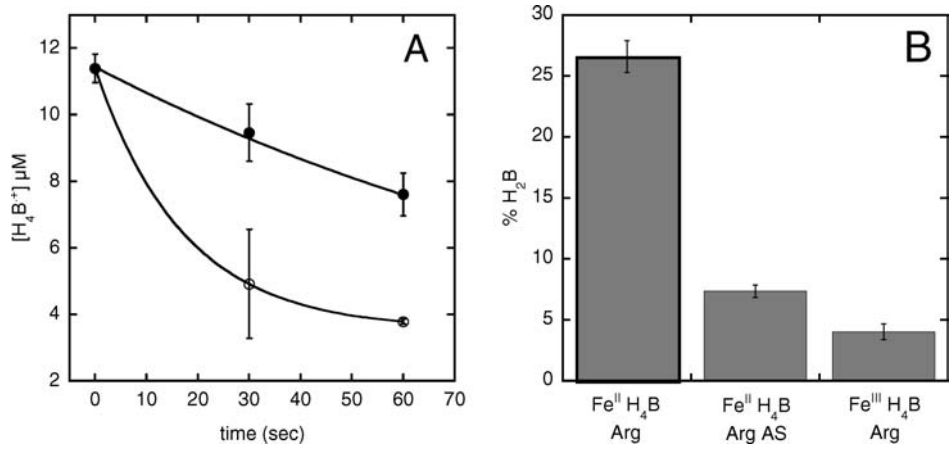
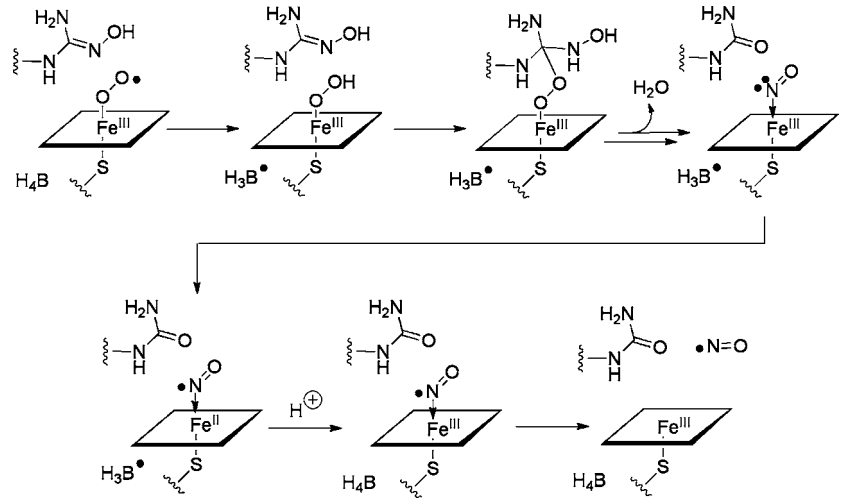


Figure 6. Angeli's salt reduces the pterin-centered radical. (A) Quantified pterin radical signal observed over time in the presence (open circles) and absence (closed circles) of Angeli's salt. Single turnover of ferrous $iNOS_{heme}$ (150 μM) with L-arginine was initiated by the addition an equal volume of aerobic HEPES (100 mM, pH 7.5). Angeli's salt (3 mM final concentration) or an equal volume of HEPES buffer was added to the reactions. Samples were then aged for various times after Angeli's salt addition and were quenched by freezing in liquid nitrogen. EPR spectra were recorded and pterin signal was integrated and quantified using a known Cu-EDTA standard. Values are fit to a single exponential decay. (B) The total oxidized pterin remaining after L-arginine turnover with and without the addition of the Angeli's salt. Reactions contain reduced (H_4B) pterin-bound $iNOS_{heme}$ (40 μM) with L-arginine (1 mM), and aerobic HEPES with or without Angeli's salt (5 mM). Following the reaction, pterin was removed from the protein and analyzed by HPLC. H_2B and H_4B were monitored at their corresponding absorbance maxima, 280 and 297 nm, respectively. Oxidized and reduced pterin content was quantified by comparison to original standards of H_2B and H_4B.

Scheme 6. Proposed Detailed Mechanism of NOS Oxidation of NHA[a]



[a] Activation of molecular oxygen to a heme-bound ferric−peroxo intermediate occurs with one electron derived from co-substrate NADPH and one from the pterin cofactor, generating a pterin-centered radical. Nucleophilic attack of substrate NHA by this intermediate leads to the formation of an initial addition complex. Decomposition of the new complex generates a heme-bound nitroxyl anion, citrulline, and water. Oxidation of the ligand by the ferric iron generates nitric oxide coordinated to ferrous heme. Oxidation of the iron by the nearby pterin radical results in a ferric nitric oxide complex, which can dissociate in an aerobic environment to generate the final product nitric oxide. Dative bonds are shown to more clearly indicate the electron distribution.

turnover of NHA. Initially, the electron-rich cofactor acts as a single electron donor coupled to oxygen activation. Subsequently, the resulting pterin radical oxidizes a downstream intermediate by a single electron, ensuring the release of NO as the final product. This proposal is supported by a number of observations presented here. First, when oxygen activation occurs without the generation of the pterin-centered radical using the peroxide shunt, NOS generates a ferrous−nitrosyl complex. Without the oxidizing equivalent generated on the pterin radical, the final reaction product is off by a single electron and HNO is generated rather than NO. Second, NO production can be restored when oxygen activation and the pterin radical are combined through two independent means, namely, preturnover of L-arginine to generate the pterin radical followed by peroxide shunt chemistry. Although neither reaction is sufficient independently, the combination of activated oxygen, through the use of hydrogen peroxide, and the pterin-centered radical, generated by preturnover of $iNOS_{heme}$ with L-arginine, can recover NO production. Finally, the addition of Angeli's salt to generate a ferrous nitrosyl leads to a decrease in pterin radical signal as monitored by EPR ultimately resulting in reduction of the pterin-centered radical.

In consideration of these results, we favor a mechanism with the initial formation of the ferric−peroxo nucleophilic addition complex (Scheme 6), the breakdown of which generates heme-bound HNO and citrulline. From a chemical standpoint, HNO is an attractive intermediate along the reaction pathway to NO formation. Nitroxyl anion has a very low reduction potential (NO/−NO couple, −810 mV),[19] providing a strong driving force

(19) Shafirovich, V.; Lymar, S. V. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 7340–7345.

for the heme-mediated oxidation to NO by the pterin radical. It is likely that the initial product is the protonated form of nitroxyl ($pK_a = 11.4$), deprotonation of which may be driven by the Lewis acidity of the ferric iron to produce the strongly reducing nitroxyl anion. Deprotonation followed by iron reduction will generate the ferrous nitrosyl. During normal turnover, this intermediate is oxidized by the nearby pterin radical to generate the observed ferric nitrosyl and the reduced pterin cofactor. Finally, NO dissociation and citrulline release complete the reaction.

The use of a pterin radical is unique to NOS catalysis. The activation of oxygen to a ferric−peroxo intermediate requires two electrons. However, stoichiometry dictates that one of these electrons must be removed downstream of the reaction of the ferric−peroxo intermediate to ensure the production of NO during NHA oxidation. Chemically, the heme can be envisioned to perform this chemistry without involvement of a pterin radical, where the ferrous nitrosyl can dissociate NO directly. However, the added challenge of operating in an aerobic environment requires the sequestration of the ferrous heme electron to ensure efficient NO release from the active site without significant formation of other nitrogen oxides (peroxynitrite and nitrate). The radical cycling role of the pterin offers a unique solution to this problem, providing quick delivery of an electron to activate molecular oxygen while acting as a single electron oxidant to ensure proper NO formation.

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Supporting Information Available: Additional figure. This material is available free of charge via the Internet at http://pubs.acs.org.

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