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Reaction of Cytochrome P450$_{BM3}$ and Peroxynitrite Yields Nitrosyl Complex

Rachel K. Behan,[†] Lee M. Hoffart,[‡] Kari L. Stone,[†] Carsten Krebs,[†,‡] and Michael T. Green*,[†]

Contribution from the Departments of Chemistry and Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received June 28, 2006; E-mail: mtg10@psu.edu

Abstract: Peroxynitrite has come into the spotlight in recent years. Its effects on proteins have been implicated in several diseases such as acute lung injury, rheumatoid arthritis, implant rejection, atherosclerosis, Parkinson's disease, and Alzheimer's disease. Peroxynitrite is thought to inactivate a variety of proteins including thiolate-ligated heme proteins such as cytochrome P450 2B1 and PGI_2 synthase, through the nitration of tyrosine residues. In previous studies it was reported that thiolate-ligated heme enzymes react with peroxynitrite to form a ferryl intermediate. In an effort to spectroscopically characterize this species in P450$_{BM3}$, we discovered that the peroxynitrite-generated intermediate is not an Fe^{IV}oxo, but rather an iron−nitrosyl $\{FeNO\}^6$ complex. We present density functional calculations as well as Mössbauer and stopped-flow spectroscopic characterizations of the peroxynitrite-generated intermediate in P450$_{BM3}$.

Introduction

Prostacyclin (PGI_2) synthase, a protein involved in the inflammatory response and platelet accumulation in humans, is a thiolate-ligated heme protein that is inactivated in the presence of low amounts of peroxynitrite (PN).[1] In general, thiolate-ligated heme proteins have proven to be a significant target for PN nitrations, including proteins like cytochrome P4502B1 and nitric oxide synthase, all of which are thought to be inactivated by PN through tyrosine nitration.[2−5] Importantly, these nitrosylated residues appear to be of pathological significance having been detected in Parkinson's and Alzheimer's diseases and neurodegenerative, chronic inflammatory, gastrointestinal tract, and cardiovascular disorders.[6−9]

In an attempt to understand the mechanism by which PN inactivates PGI_2 synthase, Ullrich and co-workers studied the reaction of PN with P450cam, P450nor, chloroperoxidase (CPO), and P450$_{BM3}$.[10−13] Stopped-flow experiments identified spectroscopically similar intermediates during the reaction between PN and these thiolate-ligated heme enzymes. Based on (1) previous reports of PN generated oxos in histidine-ligated peroxidases[14] and (2) comparisons with the UV/visible absorption spectrum of chloroperoxidase compound II (CPO-II), it was concluded that an Fe^{IV}oxo (ferryl) species was a common intermediate in all four reactions. As a result of these stopped flow experiments, it is currently believed that PN can be used to generate P450-II in high yield and that under certain conditions P450-II is more stable than CPO-II.[10,13,15] Moreover, it has recently been reported that the relatively stable P450−PN intermediate can serve as a platform from which P450 compound I can be generated by laser flash photolysis.[16] These reports have stirred considerable interest in the use of peroxynitrite as an alternative oxidant to aid in the study of P450 chemistry.

These results are unusual. P450-II is more reactive than CPO-II. We have prepared P450-II and CPO-II samples for a variety of spectroscopic investigations, and without exception the ferryl yield and stability have been greater in CPO.[17−20] Experience

[†] Department of Chemistry.
[‡] Department of Biochemistry and Molecular Biology.

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suggests that the characterization of the P450−PN intermediate is incomplete. To verify the identity of the P450−PN intermediate, we have applied a combination of density functional theory (DFT) calculations and Mössbauer spectroscopy. Prior investigations of P450 and CPO have shown that this combination of techniques can be used to obtain structural information.[17,19]

Our previous examinations of the ferryl forms of P450 and CPO revealed that the ferryl moiety in both P450-II and CPO-II is protonated. The signature feature of these $S = 1$ $Fe^{IV}OH$ intermediates is an enlarged quadrupole splitting ($\Delta E_Q \approx 2.1$ mm/s) relative to those observed for authentic 6-coordinate ferryl-heme species ($\Delta E_Q \approx 1.4$ mm/s).[17,19] The $Fe^{IV}OH$ assignment obtained from our Mössbauer investigations of thiolate-ligated ferryls is supported by EXAFS and resonance Raman measurements of CPO-II.[18,20,21] EXAFS experiments revealed a 1.82 Å Fe−O bond in CPO-II, while resonance Raman experiments identified an ^{18}O- and ^{2}H-dependent Fe−O stretching mode at 565 cm^{-1}.

If the P450−PN intermediate is indeed P450 compound II, it should contain an Fe(IV)OH center with $\Delta E_Q \approx 2.1$ mm/s and an $S = 1$ ground state. Our investigations, however, reveal that this is not the case. Instead, we find that PN reacts with $P450_{BM3}$ to generate a diamagnetic ($S = 0$) $\{FeNO\}^6$ iron−nitrosyl complex. Similar reactions may be expected in other thiolate-ligated heme systems.

Computational Procedures

DFT calculations were performed on several $P450_{BM3}$ complexes to compare theoretical Mössbauer parameters to the experimentally determined parameters of the $P450_{BM3}$−PN intermediate. These calculations were performed on a large active site model of $P450_{BM3}$, which included a porphine and the first four residues in the axial helix (Cys400-Gln403). All residues except Cys and Gly were converted to Ala. The starting structure for these calculations was taken from the ferric $P450_{BM3}$ crystal structure (1POV).[22] An example of the model used for these calculations can be found in Figure 3.

Geometry optimizations were performed using Gaussian 03 (B3LYP/6-311G).[23] During geometry optimizations the positions of the distal ligand, iron atom, porphyrin nitrogens, alpha carbons, meso carbons, meso hydrogens, and the axial SCH_2CH were allowed to vary. Mössbauer parameters were determined at the optimized geometries. Quadrupole splittings were determined at the B3LYP/6-311G level and isomer shifts were calculated using Neese's core properties (CP) basis set.[24,25] An integration grid of 199 radial shells with 590 angular points was used to determine the theoretical isomer shifts. The electron density at the nucleus was obtained using the Atoms In Molecules (AIM) option in Gaussian 03.

Experimental Procedures

Materials. ^{57}Fe-enriched $P450_{BM3}$ was prepared following previously published procedures.[17] Peroxynitrite was purchased from Calbiochem (250−300 mM in 1 M NaOH) and quantified by UV/visible spectroscopy using the accepted extinction coefficient of $\epsilon = 1670$ M^{-1} cm^{-1} at 302 nm.[10]

Freeze−Quench Samples. The $P450_{BM3}$−PN intermediate was generated using freeze−quench techniques. A 4-syringe ram freeze−quench apparatus from Update Instruments (Madison, WI) was used for all freeze−quench experiments. $P450_{BM3}$−PN was formed by mixing 4 mM ferric $P450_{BM3}$ (^{57}Fe-enriched, 1 M KPhos, pH 6.4) with a 16-fold excess of PN (0.25 M NaOH) for a final pH of 6.8. The syringes containing $P450_{BM3}$ and peroxynitrite were kept at 12 °C by means of a circulating water bath. The reaction was quenched in an isopentane bath (−140 °C) 450 ms after mixing.

Mössbauer Spectroscopy. Mössbauer spectra were recorded on spectrometers from WEB Research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. The spectra were recorded at 4.2 K. For low-field measurements the samples were kept in a Janis SVT400 cryostat (Wilmington, MA). These spectra were recorded in a 53 mT magnetic field applied parallel to the γ-beam. The 12SVT cryostat (Janis), used for high-field measurements, houses a superconducting magnet, which can supply a magnetic field between 0 and 8 T (also parallel to the γ-beam). The reported isomer shifts are relative to the centroid of the spectrum of a metallic foil of α-Fe at room temperature. Data analysis was performed using the WMOSS program from WEB Research. Simulations are based on the following spin Hamiltonian,

$$\mathbf{H} = \beta \mathbf{S}\cdot\mathbf{g}\cdot\mathbf{B} + D\left(\mathbf{S}_z^2 - \frac{S(S+1)}{3}\right) + E(\mathbf{S}_x^2 - \mathbf{S}_y^2) + \frac{eQV_{zz}}{12}[3\mathbf{I}_z^2 - I(I+1) + \eta(\mathbf{I}_x^2 - \mathbf{I}_y^2)] + \mathbf{S}\cdot\mathbf{A}\cdot\mathbf{I} - g_n\beta_n\mathbf{B}\cdot\mathbf{I}$$

in which the first three terms describe the electronic Zeeman effect and zero-field splitting of the electronic ground state, the fourth term represents the interaction between the electric field gradient and the nuclear quadrupole moment, the fifth term describes the magnetic hyperfine interaction of the electron spin with the ^{57}Fe nucleus, and the last term represents the nuclear Zeeman interaction.

$P450_{BM3}$−NO. $P450_{BM3}$−NO is not stable under aerobic conditions. *UV/visible samples* were prepared by degassing 50 μM $P450_{BM3}$ (0.1 M KPhos, pH 7) and diluting it 10:1 with saturated NO buffer (1.9 mM NO in 0.1 M degassed KPhos buffer, pH 7) in an anaerobic cuvette. *Mössbauer samples* were prepared in a similar fashion. ^{57}Fe−$P450_{BM3}$ (≈ 4 mM) was anaerobically mixed (in a glovebox) with NO-saturated buffer in a 1:1 ratio. The $P450_{BM3}$−nitrosyl complex was aliquoted into a Mössbauer cup, placed in an airtight vial, and immediately frozen in liquid nitrogen following removal from the glovebox.

Stopped-Flow Spectrophotometry. The $P450_{BM3}$−PN reaction was studied using stopped-flow spectrophotometry. For these experiments a BioLogic 4-syringe stopped-flow apparatus was used. A solution of 50 μM $P450_{BM3}$ (0.1 M KPhos, pH 6.7) was placed in one syringe and a 128-fold excess of PN (6−7 mM) in 10 mM NaOH in another. These solutions were mixed in a 1:1 ratio (for a final pH of 6.8) at 12 °C. The reaction was monitored from 10 ms to 9 s. The reaction was followed at 302, 417, and 435 nm, representing the consumption of PN (302 nm) and the formation and decay of the PN intermediate (435 nm) and ferric enzyme (417 nm).

Results and Discussion

Stopped Flow of the $P450_{BM3}$−Peroxynitrite Reaction. For comparative purposes, we performed stopped-flow experiments on the $P450_{BM3}$−PN reaction (Figure 1). Our results are similar to those obtained by Ullrich and co-workers.[10] We observe the formation of only one transient intermediate during the reaction. This species has an absorbance maximum at 432 nm, but as with previous investigations[10] we report single-wavelength data at 435 nm. It was the perceived similarity of the 432 nm absorbance to the absorption maximum of CPO-II ($\lambda_{max} = 438$ nm) that led to the initial assignment of the $P450_{BM3}$−PN intermediate as a ferryl species. As will be shown, however, the PN intermediate has the same spectral features (both Mössbauer and UV/visible) as the ferric−nitrosyl complex of $P450_{BM3}$.

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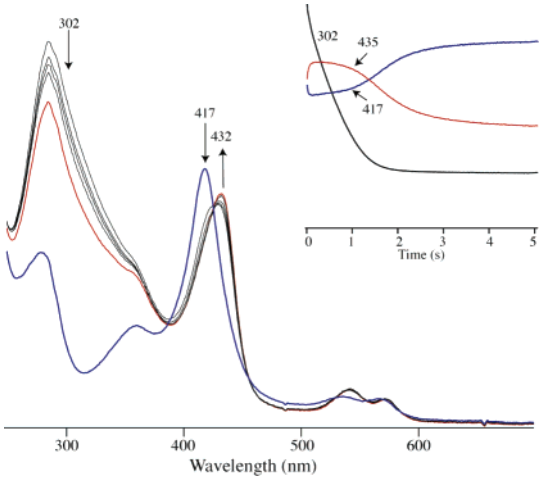

Figure 1. Stopped-flow spectrophotometry experiments on the P450$_{BM3}$ reaction. The blue spectrum corresponds to ferric P450$_{BM3}$ and the red spectrum is fully formed P450$_{BM3}$−PN (300 ms). The time points between the spectra of ferric P450$_{BM3}$ and P450$_{BM3}$−PN are 10, 20, 40, and 70 ms (decreasing in absorbance at 302 nm). The inset shows single-wavelength data at 302, 417, and 435 nm.

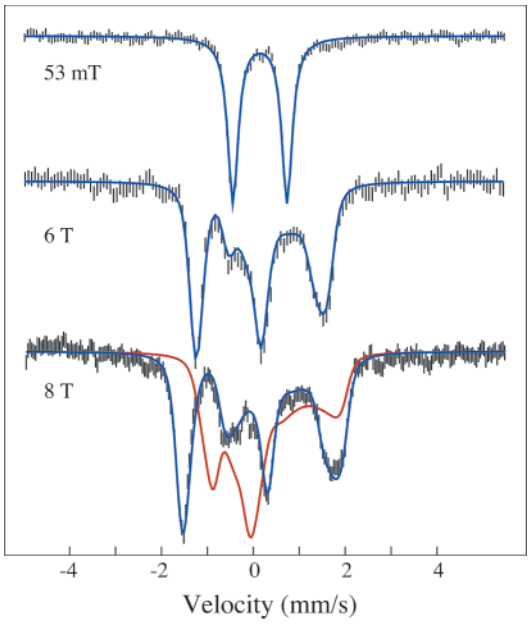

Figure 2. Variable-field Mössbauer spectra of the P450$_{BM3}$−PN intermediate. Spectra were recorded in a 53 mT, 6 T, and 8 T external magnetic field applied parallel to the γ-beam. The solid blue lines represent a spin Hamiltonian simulation with an $S = 0$ ground state. An $S = 1$ simulation (red line, see the text for details) is also shown for the 8 T spectrum.

Mössbauer Measurements (4.2-K/53 mT). The 4.2-K/53 mT Mössbauer spectrum of the P450$_{BM3}$−PN intermediate (pH 6.8) is shown in Figure 2(top). Consistent with EPR measurements (Supporting Information), this spectrum suggests a diamagnetic or integer spin species. Mössbauer parameters for the P450$_{BM3}$−PN intermediate are $\delta = 0.15$ mm/s and $\Delta E_Q = 1.15$ mm/s. The isomer shift is similar to those reported for the peracetic acid (PA)-generated Fe^{IV} centers in P450cam-II, P450$_{BM3}$-II, and CPO-II.[17,19] The quadrupole splitting of the P450$_{BM3}$−PN intermediate, however, is significantly smaller than those observed for the PA-generated ferryls: P450cam-II, P450$_{BM3}$-II, and CPO-II have quadrupole splittings of 2.06, 2.16, and 2.06 mm/s, respectively, at pH 7.[17,19]

Table 1. Mössbauer Parameters for P450$_{BM3}$ Species in mm/s

distal ligand	formal oxidation/ spin state	theory [mm/s] δ	theory [mm/s] ΔE_Q	experiment [mm/s] δ	experiment [mm/s] ΔE_Q
OH^-	IV ($S = 1$)	0.09	2.17	0.13[b]	2.16[b]
O^{2-}	IV ($S = 1$)	0.11	1.05		
NO_2^-					
nitro	IV ($S = 1$)	0.18	2.84		
nitrito	IV ($S = 1$)[a]	0.36	−3.08		
NO_3^-	IV ($S = 1$)[a]	0.28	2.87		
HNO	IV ($S = 1$)[a]	0.21	−2.07		
$O_2^{\cdot -}$	III ($S = 0$)[d]	0.31	−2.19	0.31[c]	−2.15[c]
NO^+	II ($S = 0$)	0.09	1.32	0.15	1.15

[a] These complexes are formally Fe(IV); however, their ground states are better described as Fe(III) radicals. This ferric character is reflected in the isomer shifts. [b] Reference 17. [c] P450$_{cam}$ from ref 32. [d] Superoxide couples to the ferric iron to give an $S = 0$ ground state.

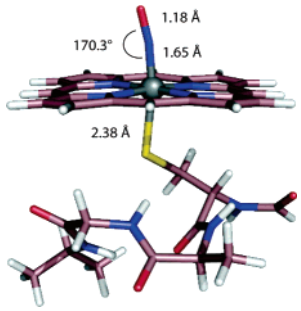

Figure 3. {FeNO}^{6} P450$_{BM3}$ model used in calculations. Calculated Fe−N (1.65 Å), N−O (1.18 Å), and Fe−S (2.38 Å) distances and Fe−N−O (170.3°) bond angle are shown.

The reaction of P450$_{BM3}$ with PA and PN clearly yields different products. The PA-generated intermediate is an $S = 1$ Fe^{IV}OH with $\Delta E_Q = +2.16$ mm/s, while the PN-generated intermediate has a quadrupole splitting ($\Delta E_Q = 1.15$ mm/s) that is closer to those observed for authentic 6-coordinate Fe^{IV}oxo species (i.e., unprotonated ferryls).[19] It seems improbable, however, that protonation of the ferryl moiety is oxidant-dependent.

The inconsistency between the Mössbauer parameters of the PN- and PA-generated intermediates in P450$_{BM3}$ prompted the calculation of other possible species that could result from the reaction of P450$_{BM3}$ and PN. DFT calculations were performed on several complexes that were considered to be reasonable products of the PN−P450$_{BM3}$ reaction. Only diamagnetic or integer spin species were considered since the P450$_{BM3}$−PN intermediate is EPR silent and exhibits a quadrupole doublet in a weak applied field. The results of these calculations are found in Table 1. Of the species examined, only the Mössbauer parameters calculated for the iron(IV)oxo and the {FeNO}^{6} nitrosyl complex compare favorably with our measurements. Calculations reveal that the NO ligand coordinates to P450$_{BM3}$ in a linear fashion ($\theta_{FeNO} = 170.3°$), indicating that the iron−nitrosyl complex is best described as an Fe^{II}(NO$^+$) species (Figure 3). Importantly, this complex has an $S = 0$ ground state.

High-Field Mössbauer Measurements and Comparisons with P450$_{BM3}$−NO: Confirmation That the P450$_{BM3}$-PN Intermediate Is a Nitrosyl Complex. To determine the ground state of the P450$_{BM3}$−PN intermediate, Mössbauer measurements in an externally applied field of 6 and 8 T were performed

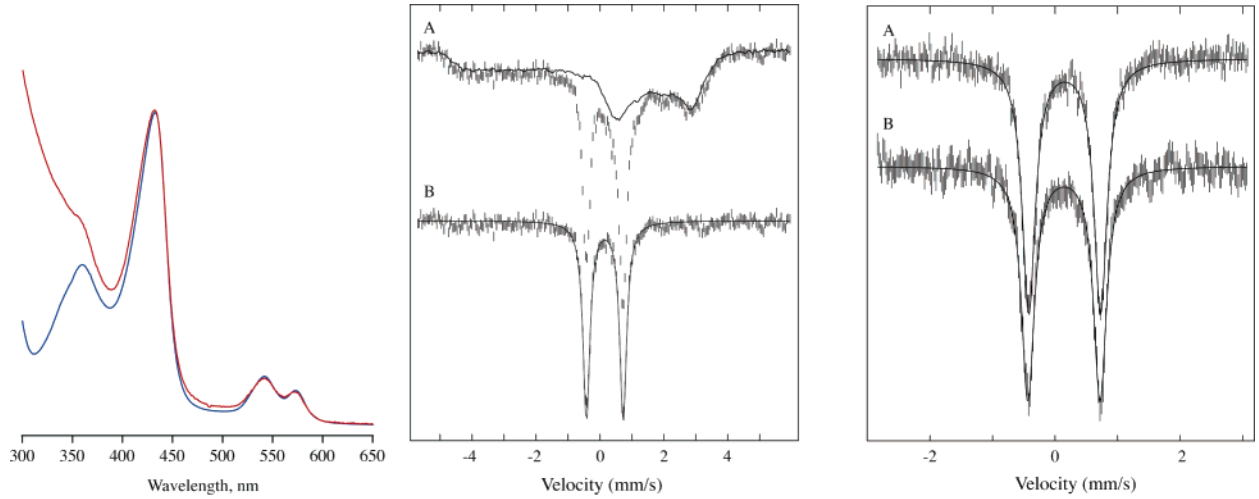

Figure 4. Comparison of $P450_{BM3}$−nitrosyl complex and $P450_{BM3}$−PN intermediate. *Left*: UV/visible spectrum of the $P450_{BM3}$−nitrosyl complex (blue) and the $P450_{BM3}$−PN stopped-flow reaction 300 ms after mixing (red). The increased absorbance around 300 nm is due to excess PN (λ_{max} = 302 nm). *Middle*: 4.2 K/53 mT Mössbauer spectrum of the $P450_{BM3}$−NO complex. Spectrum A is the raw data for a sample containing $P450_{BM3}$−NO and ferric $P450_{BM3}$ (in an ≈40:60 ratio). See Experimental Procedures for sample preparation details. The spectrum of ferric $P450_{BM3}$ is overlaid as a solid line in A. Spectrum B is the $P450_{BM3}$−NO spectrum obtained by removing the contribution of ferric $P450_{BM3}$ from the raw data. *Right:* Comparison of the Mössbauer spectra of (A) $P450_{BM3}$−PN and (B) $P450_{BM3}$−nitrosyl complex (4.2 K/53 mT). The Mössbauer parameters for these two complexes are identical, ΔE_Q = 1.15 mm/s and δ = 0.15 mm/s.

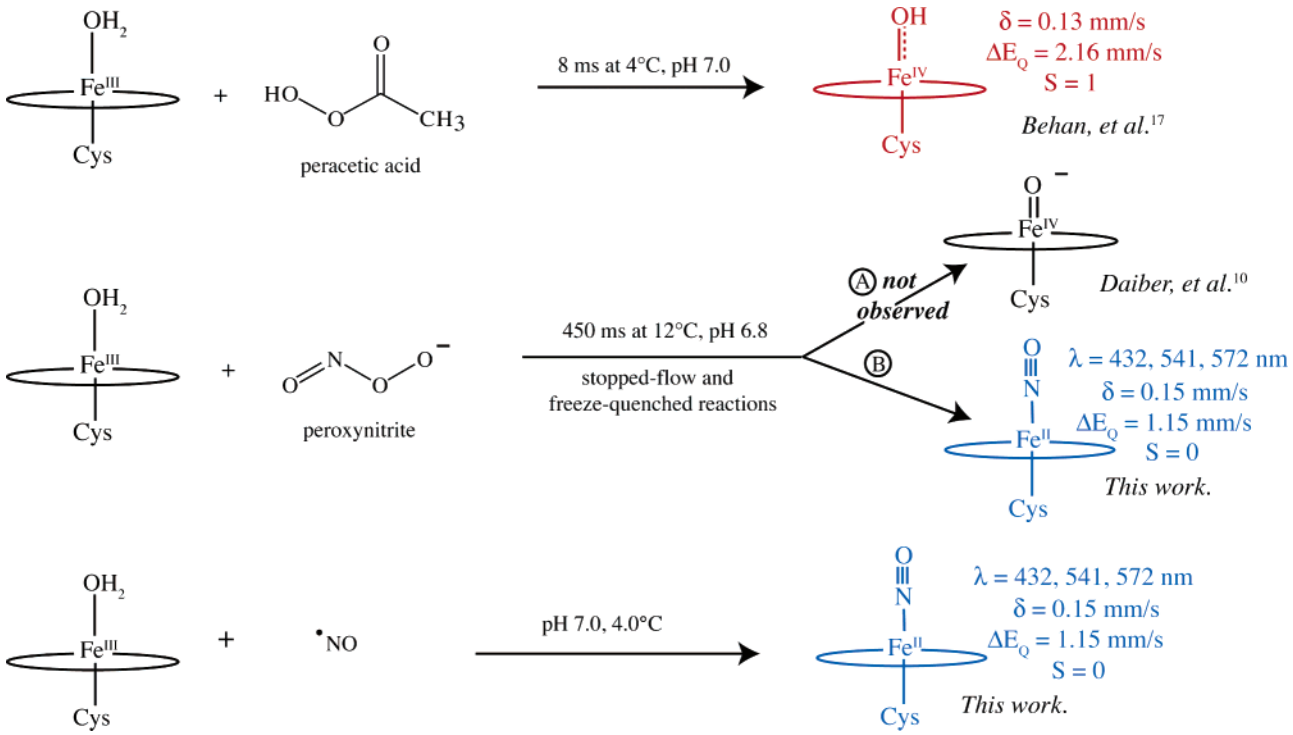

Figure 5. Reactants, reaction conditions, and observable iron-containing products for the reaction of $P450_{BM3}$ with the following: (*Top*) Peracetic acid (PA): The first spectroscopically characterizable intermediate in the freeze−quench reaction of $P450_{BM3}$ with PA is compound II. We have shown that this species is best described as an iron(IV)hydroxide. (*Middle*) Peroxynitrite (PN): The reaction of $P450_{BM3}$ with PN results in the formation of a nitrosyl complex (path **B**). No ferryl species are observed (path **A**). (*Bottom*) Nitric oxide: The ferric $P450_{BM3}$−nitrosyl complex and the $P450_{BM3}$−PN intermediate have identical spectroscopic features.

(Figure 2).[26,27] The experimental data (hash marks) for both fields can be satisfactorily simulated (solid lines) using a spin Hamiltonian formalism. For these simulations, the isomer shift and quadrupole splitting were taken from the 53 mT data and an asymmetry parameter $\eta = 0$ was assumed. The simulations reveal that the effective magnetic field at the ^{57}Fe nucleus equals the externally applied field. Thus, the internal magnetic field is zero (i.e., the complex is diamagnetic). The measurements further reveal the sign of the quadrupole splitting to be positive. Attempts to simulate a paramagnetic center did not effectively match the observed spectra at either field. For comparison, in red we show a spectral simulation in the slow relaxation limit assuming parameters typical of ferryl heme species: $S = 1$, $\mathbf{g} = (2.1, 2.1, 2.0)$, $D = 23$ cm^{-1}, $E/D = 0$, $\mathbf{A}/g_N\beta_N = (-19, -19, -7)$ T, $\eta = 0$, with δ and ΔE_Q taken from the 53 mT spectrum.[17]

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Further evidence supporting the assignment of the P450$_{BM3}$−PN intermediate as an iron−nitrosyl comes from an examination of the UV/visible and Mössbauer spectra of P450$_{BM3}$−NO (i.e., the ferric−NO complex of P450$_{BM3}$). The UV/visible spectra for P450$_{BM3}$−NO and the P450$_{BM3}$−PN reaction mixture (300 ms after mixing) are shown in Figure 4. Both spectra exhibit absorption peaks at 432, 541, and 572 nm. Importantly, Mössbauer measurements on P450$_{BM3}$−NO yield parameters that are identical to those obtained for the P450$_{BM3}$−PN intermediate (Figure 4, right). An isomer shift of 0.15 mm/s may appear low for a ferrous complex, but it is known that the strongly π-accepting NO^+ ligand can lower the isomer shift considerably.[28,29] Calculations on an $S = 0$ {FeNO}^{6} P450$_{BM3}$ model yield $\delta = 0.09$ mm/s in good agreement with experiment.

The results of our investigations are summed up in Figure 5. It shows the reactants, reaction conditions, and observable iron-containing products for the reactions under consideration. The **top** of Figure 5 contains the reaction of P450$_{BM3}$ with peracetic acid (PA). The first spectroscopically characterizable intermediate in the P450$_{BM3}$−PA reaction is compound II.[17] We have shown that this species is best described as an iron(IV)-hydroxide. The **middle** of Figure 5 displays the reaction of P450$_{BM3}$ with peroxynitrite (PN). This reaction results in the formation of a nitrosyl complex (path **B**). We observe no ferryl species in the P450−PN reaction (path **A**). The **bottom** of Figure 5 shows the reaction of P450$_{BM3}$ with nitric oxide. The ferric P450$_{BM3}$−nitrosyl complex and the P450−PN intermediate have identical spectroscopic features.

Conclusion

Using Mössbauer spectroscopy, stopped-flow spectrophotometery, and DFT calculations, we have determined that the P450$_{BM3}$−PN intermediate is not a ferryl species. Its spin state, UV/visible spectrum, and Mössbauer parameters are consistent with an {FeNO}^{6} complex, and we assign it as such. The role (if any) that this nitrosyl complex plays in tyrosine nitration is unknown.

Our investigations underline the importance of employing multiple spectroscopic techniques to characterize transient species. PN-generated ferryls have also been reported in histidine-ligated peroxidases as well as synthetic iron-porphyrins.[14,30−31] Each of these studies relied solely on UV/visible spectroscopy for species identification. In light of our findings, it may be prudent to re-examine some of these reports. It will be interesting to see if formation of the nitrosyl complex in P450$_{BM3}$ is linked to thiolate-ligation.

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Supporting Information Available: Complete ref 23; EPR and Mössbauer spectra of P450$_{BM3}$−PN. This material is available free of charge via the Internet at http://pubs.acs.org.

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