J|A|C|S COMMUNICATIONS

Published on Web 07/23/2009

Heme/O_2/•NO Nitric Oxide Dioxygenase (NOD) Reactivity: Phenolic Nitration via a Putative Heme-Peroxynitrite Intermediate

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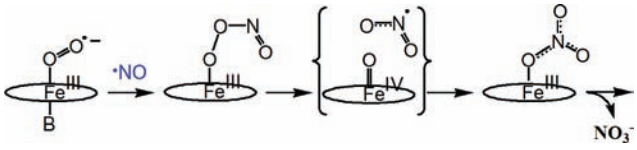
The reaction of nitric oxide (•NO; nitrogen monoxide) with oxygenated heme proteins is of considerable biological interest. Nitric oxide is generated in vivo by the oxidation of L-arginine to L-citrulline mediated by the enzyme Nitric Oxide Synthase (NOS).[1] Nitric oxide itself plays an important role in a number of physiological processes that include as a signaling agent leading to smooth muscle vasodilation, platelet disaggregation, neurotransmission, and immune response to bacterial infection.[2]

Overproduction of •NO can lead to toxicological processes that include DNA damage, protein nitration leading to cell death, and formation of peroxynitrite ($^-$OON=O) with the latter's further chemistry leading to highly reactive free radicals.[3] It effects oxidation/nitration of biomolecules; most evident is the nitration of tyrosine.[3c,d,4] To maintain proper •NO levels, microbial heme protein •NO Dioxygenases (NODs) catalyze the reaction of O_2 and •NO to yield the biologically benign nitrate anion (NO_3^-).[5]

$$\bullet NO + O_2 + e^- \xrightarrow{NOD} NO_3^-$$

Hemoglobin (Hb) and myoglobin (Mb) are also major targets/sinks of •NO in mammals, and they exhibit NOD activity.[6] High fidelity incorporation of both ^{18}O-labeled oxygen atoms into the nitrate (NO_3^-) product occurs when •NO reacts with red blood cell oxyHb [Hb(Fe^{III}-$^{18}O_2^{\bullet-}$)], sperm whale oxyMb [Mb(Fe^{III}-$^{18}O_2^{\bullet-}$)], and *E. coli* oxyflavohemoglobin [flavoHb(Fe^{III}-$^{18}O_2^{\bullet-}$)].[7] The generally stated mechanism of •NO dioxygenase (Scheme 1) involves direct reaction of the Fe^{III}-($O_2^{\bullet-}$) oxy complex with •NO, giving a peroxynitrite intermediate. Subsequent homolytic O−O bond cleavage produces an oxo-ferryl (Fe^{IV}=O) species and the free radical nitrogen dioxide (•NO_2); the latter attacks the ferryl O-atom to produce a N−O bond giving nitrate.[3d,8−12]

Scheme 1



There have been some reports on the detection of a peroxynitrite intermediate: (i) Under alkaline conditions, Herold and co-workers obtained UV−vis spectroscopic evidence for a high-spin Fe^{III} intermediate for both the Hb and Mb systems.[13] (ii) Olson and co-workers suggested EPR evidence for a high-spin Fe^{III} species ($g = 6$) using rapid-freezing techniques.[8] However, based on thermokinetic[14] and theoretical[10b] arguments, the peroxynitrite-iron intermediate should be too short-lived to detect. This is consistent with a very recent report employing rapid-freeze quench resonance Raman spectroscopy using Mb. This shows that the high-spin Fe^{III} species detected was not the proposed peroxynitrite intermediate, but rather an iron-bound nitrate complex formed prior to its decay to metMb.[9]

We report here the chemistry of a synthetic oxy-heme which exhibits NOD reactivity, where the intermediacy of a peroxynitrite species is implicated.[15] As previously described,[16] (F_8)Fe^{II} (**1**) {F_8 = tetrakis(2,6-difluoro-phenyl)porphyrinate(2-)} {λ_{max} = 422, 542 nm} reacts reversibly with dioxygen to give a diamagnetic iron(III)-superoxo species (S)(F_8)Fe^{III}-($O_2^{\bullet-}$) (**2**) {λ_{max} = 416, 535 nm}, which is stable in solution below −40 °C in coordinating solvents (S) such as tetrahydrofuran (THF), acetone, or propionitrile (Figure 1).[16] Subsequent addition of 1 equiv of •NO (at −80 °C) produces the five-coordinate nitrato heme complex (F_8)Fe^{III}-(NO_3^-) (**3**) {λ_{max} = 393, 505, 572, 634 nm (Figures 1, 2)} in near quantitative yield;[17,18] its structure (X-ray) is shown in Figure 2. In these benchtop experiments, where 10−20 s are required to add •$NO_{(g)}$ in THF by syringe and record a spectrum, **3** has already fully formed. No hint of an intermediate is observed, even if the chemistry is carried out in 2-methyl-THF at −120 °C.[18]

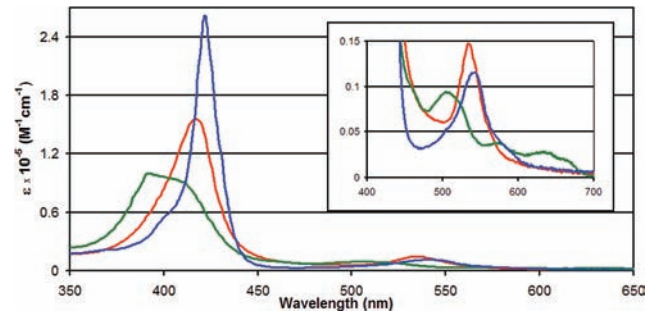


Figure 1. UV−vis spectra (THF solvent) of (F_8)Fe^{II} (**1**) (blue), (thf)(F_8)Fe^{III}-($O_2^{\bullet-}$) (**2**) (red) generated from **1** + $O_{2\ (g)}$, and (F_8)Fe^{III}-(NO_3^-) (**3**) (green) generated from **2** + •$NO_{(g)}$.

Thus, we sought chemical evidence which might suggest the formation of a peroxynitrite species. When the reaction of •$NO_{(g)}$ with (thf)(F_8)Fe^{III}-($O_2^{\bullet-}$) (**2**) is followed by addition of the tyrosine mimic 2,4-di-*tert*-butylphenol (DTBP), (F_8)Fe^{III}-(NO_3^-) (**3**) is nevertheless formed and unreacted DTBP is recovered.[18] However, we do observe effective nitration chemistry when DTBP (≥1 equiv) is added *prior* to addition of 1 equiv of •$NO_{(g)}$ to **2** (Scheme 2).[18] Workup of the reaction solution[18] reveals that the ferric hydroxo product (F_8)Fe^{III}-OH (**4**) forms (∼85% yield) along with high yields (>82%) of 2,4-di-*tert*-butyl-6-nitrophenol (NO_2DTBP).

The following control experiments indicate that a new heme-NO_x intermediate forms and is able to effect a phenol nitration reaction faster than its own isomerization to the nitrate complex **3**: (i) Use of excess •$NO_{(g)}$ bubbled into solution had no effect on the products or their relative yields. (ii) Bubbling excess •$NO_{(g)}$ into a solution

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10.1021/ja904832j CCC: $40.75

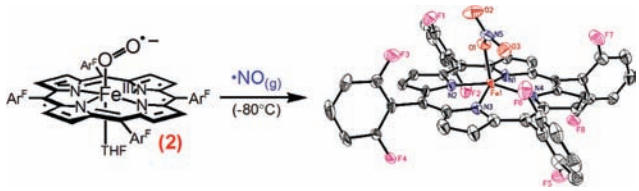
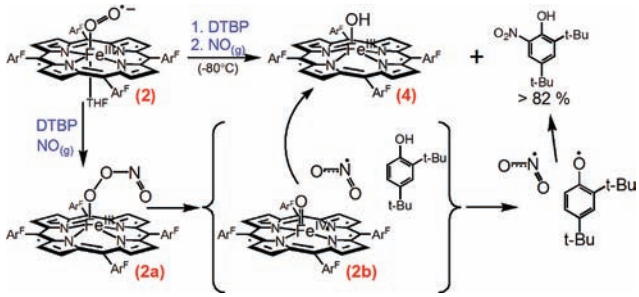


Figure 2. Reaction of (thf)$(F_8)Fe^{III}$-$(O_2^{\bullet-})$ (**2**) (Ar^F = 2,6-difluorophenyl) + $\bullet NO_{(g)}$ produces the nitrato complex $(F_8)Fe^{III}$-(NO_3^-) (**3**) (X-ray).[17]

containing **1** at −80 °C in the presence of DTBP and subsequent warming yielded less than 2% of the NO_2DTBP and no other products besides the starting DTBP and the iron-nitrosyl $(F_8)Fe^{II}$-(NO) (**5**); excess $\bullet NO_{(g)}$ is not solely responsible for significant DTBP nitration. (iii) Warming **2** from −80 °C to RT in the presence of DTBP yielded the oxidatively coupled phenol 3,3′,5,5′-tetra-*tert*-butyl-(1,1′-biphenyl)-2,2′-diol (∼10%), unreacted DTBP (∼90%), and **4**; $\bullet NO_{(g)}$ is needed for DTBP nitration. **4** is formed from thermal decomposition of **2**.[16a] (iv) Warming a −80 °C solution of $(F_8)Fe^{III}$-(NO_3^-) (**3**) in the presence of DTBP gives no reaction of any kind; **3** itself will not nitrate DTBP.[19]

Scheme 2



As previously mentioned, these studies implicate a heme-peroxynitrite intermediate $(F_8)Fe^{III}$-$(^-OON{=}O)$ (**2a**), formed from (thf)$(F_8)Fe^{III}$-$(O_2^{\bullet-})$ (**2**) (no excess O_2 present) plus 1 equiv of $\bullet NO_{(g)}$. Following the literature suggestions, we can hypothesize that **2a** undergoes homolysis to give a ferryl + $\bullet NO_2$ (**2b**) (caged?), which however can be captured by a phenol which is already present in solution (Scheme 2). The ferryl would oxidize the phenol to a phenoxyl radical which will react with $\bullet NO_2$ to give the nitrophenol. The very stable heme-Fe^{III}-hydroxo complex **4** is formed by the wayside, as we observe.

Another reaction mechanism that should be considered is that $(F_8)Fe^{III}$-$(^-OON{=}O)$ (**2a**) undergoes heterolytic O—O bond cleavage, producing nitronium (NO_2^+) ion which is an effective phenol nitrating agent.[20] Generation of NO_2^+ in reactions of copper−zinc superoxide dismutase (CuZnSOD) with peroxynitrite was in fact suggested by Beckman,[21] and other literature reports suggest this chemistry may be preferred when metal ions are present.[4c,20] However, for metalloporphyrins, O—O heterolytic cleavage to give NO_2^+ seems to have been ruled out or not considered.[22]

In summary, we have here described a heme complex that acts as a nitrogen monoxide dioxygenase, facilitating the reaction of O_2 and •NO to yield the nitrate anion NO_3^-. Generation of a heme-peroxynitrite species is implicated; it can be trapped by a phenolic substrate, leading to *o*-nitration. The results lead to the suggestion that in heme proteins peroxynitrite may leak and effect nitration of nearby residues or exogenous substrates. While Herold observed essentially no peroxynitrite leakage in oxyMb reactions with •NO,[13] other studies show that addition of peroxynitrite to various metal complexes and metalloproteins leads to protein tyrosine (including for Mb) or exogenous phenol nitration.[23] Other examples include MnSOD and CuZnSOD.[3d,24] Perhaps there are subtle differences in reactions of reduced metal ion centers with O_2 followed by $\bullet NO_{(g)}$, compared to conditions involving addition of peroxynitrite reagent to oxidized metal ion centers. Further studies which address these issues and the mechanism of the reaction observed here are in progress.

Acknowledgment. This work was supported by NIH Grant GM 60353 (K.D.K.).

Supporting Information Available: Details concerning synthesis, spectroscopy, reactivity and CIF file. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Marletta, M. A.; Hurshman, A. R.; Rusche, K. R. *Curr. Opin. Chem. Biol.* **1998**, *2*, 656–663. (b) Crane, B. R. *Biochem. Soc. Trans.* **2008**, *36*, 1149–1154.

(2) (a) Ignarro, J. L.E. *Nitric Oxide, Biology and Pathobiology*; Academic Press: San Diego, CA, 2000. (b) Moncada, S.; Palmer, R. M. J.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109–142.

(3) (a) Denicola, A.; Radi, R. *Toxicology* **2005**, *208*, 273–288. (b) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620–1624. (c) Groves, J. T. *Curr. Opin. Chem. Biol.* **1999**, *3*, 226–235. (d) Pacher, P.; Beckman, J. S.; Liaudet, L. *Physiol. Rev.* **2007**, *87*, 315–424.

(4) (a) Radi, R. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4003–4008. (b) Szabó, C.; Ischiropoulos, H.; Radi, R. *Nature* **2007**, *6*, 662–680. (c) Gunaydin, H.; Houk, K. N. *Chem. Res. Toxicol.* **2009**, *22*, 894–898. (d) Beckman, J. S. *Chem. Res. Toxicol.* **1996**, *9*, 836–844.

(5) Gardner, P. R.; Gardner, A. M.; Martin, L. A.; Salzman, A. L. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 10378–10383.

(6) (a) Ouellet, H.; Ouellet, Y.; Richard, C.; Labarre, M.; Wittenberg, B.; Wittenberg, J.; Guertin, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5902–5907. (b) Gardner, P. R. *J. Inorg. Biochem.* **2005**, *99*, 247–266.

(7) Gardner, P. R.; Gardner, A. M.; Brashear, W. T.; Suzuki, T.; Hvitved, A. N.; Setchell, K. D. R.; Olson, J. S. *J. Inorg. Biochem.* **2006**, *100*, 542–550.

(8) Olson, J. S.; Foley, E. W.; Rogge, C.; Tsai, A.-H.; Doyle, M. P.; Lemon, D. D. *Free Radical Biol. Med.* **2004**, *36*, 685–697.

(9) Yukl, E. T.; de Vries, S.; Moënne-Loccoz, P. *J. Am. Chem. Soc.* **2009**, *131*, 7234–7235.

(10) (a) Herold, S.; Koppenol, W. H. *Coord. Chem. Rev.* **2005**, *249*, 499–506. (b) Blomberg, L. M.; Blomberg, M. R. A.; Siegbahn, P. E. M. *J. Biol. Inorg. Chem.* **2004**, *9*, 923–935. (c) Ford, P. C.; Lorkovic, I. M. *Chem. Rev.* **2002**, *102*, 993–1017.

(11) Computational chemistry indicates that peroxynitrous acid (HOON=O) decomposition to nitrate involves the "caged radical pair" (HO•) (•NO_2). See: Gunaydin, H.; Houk, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 10036.

(12) Protein heme-NO species also react with O_2, leading to nitrate. The mechanism is said to require NO dissociation, ligation of O_2 to form the oxy species, and its subsequent reaction with NO as in Scheme 1. See: Herold, S.; Röck, G. *Biochemistry* **2005**, *44*, 6223–6231, and refs 10a,c. From such a reaction involving Mb(NO), a visible spectrum of a putative peroxynitrite intermediate was extracted; see: Møller, J. K. S.; Skibsted, L. H. *Chem. Eur. J.* **2004**, *10*, 2291–2300.

(13) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385–3395.

(14) Goldstein, S.; Merenyi, G.; Samuni, A. *J. Am. Chem. Soc.* **2004**, *126*, 15694–15701.

(15) Heme-NO complexes may react with O_2 to give (heme)Fe^{III}-nitrates; for example, see:Munro, O. Q.; Scheidt, W. R. *Inorg. Chem.* **1998**, *37*, 2308–2316.

(16) (a) Ghiladi, R. A.; Kretzer, R. M.; Guzei, I.; Rheingold, A. L.; Neuhold, Y.-M.; Hatwell, K. R.; Zuberbühler, A. D.; Karlin, K. D. *Inorg. Chem.* **2001**, *40*, 5754–5767. (b) Kim, E.; Helton, M. E.; Wasser, I. M.; Karlin, K. D.; Lu, S.; Huang, H.-W.; Moënne-Loccoz, P.; Incarvito, C. D.; Rheingold, A. L.; Honecker, M.; Kaderli, S.; Zuberbühler, A. D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3623–3628.

(17) For comprison of properties, a separately synthesized nitrate complex (**3**) was generated via the reaction, $(F_8)Fe^{III}$-(Cl) + $AgNO_3$ → (**3**).[18]

(18) See Supporting Information.

(19) DTBP in a metal-complex free solution bubbled with excess $O_{2(g)}$ and $\bullet NO_{(g)}$ yielded a 50/50 mixture of NO_2DTBP and a dinitrated product, 2-*tert*-butyl-4,6-dinitrophenol. Free $\bullet NO_2$ which might derive from **2** + $\bullet NO_{(g)}$ chemistry is cabable of effecting phenol nitration, but since production of only one $\bullet NO_2$ per heme complex is possible, the yield of NO_2DTBP would then be less than 50%, not as observed.

(20) (a) Reynolds, M. R.; Berry, R. W.; Binder, L. I. *Biochemistry* **2007**, *46*, 7325–7336. (b) van der Vliet, A.; Eiserich, J. P.; O'Neill, C. A.; Halliwell, B.; Cross, C. E. *Arch. Biochem. Biophys.* **1995**, *319*, 341–349.

(21) Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. *Arch. Biochem. Biophys.* **1992**, *298*, 431–437.

(22) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 6053–6061.

(23) (a) Bourassa, J. L.; Ives, E. P.; Marqueling, A. L.; Shimanovich, R.; Groves, J. T. *J. Am. Chem. Soc.* **2001**, *123*, 5142–5143. (b) Crow, J. P. *Arch. Biochem. Biophys.* **1999**, *371*, 41–52.

(24) Demicheli, V.; Quijano, C.; Alvarez, B.; Radi, R. *Free Radical Biol. Med.* **2007**, *42*, 1359–1368. (b) Quint, P.; Reutzel, R.; Mikulski, R.; McKenna, R.; Silverman, D. N. *Free Radical Biol. Med.* **2006**, *40*, 453–458.

JA904832J