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

Mark P. Schopfer, Biplob Mondal[†], Dong-Heon Lee[‡], Amy A. N. Sarjeant[§], and Kenneth D. Karlin^{*}

Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218 USA

Abstract

An oxy-heme complex, the heme-superoxo species (tetrahydrofuran)(F₈)Fe^{III}-(O₂^{•-}) (**2**) (F₈ = anortho-difluoro substituted tetraarylporphyrinate), reacts with nitrogen monoxide ($\cdot NO$; nitric oxide) to produce a nitrate-iron(III) compound (F₈)Fe^{III}-(NO₃⁻) (**3**) (X-ray). The chemistry mimics the action of $\cdot NO$ Dioxygenases (NODs), microbial and mammalian heme proteins which facilitate $\cdot NO$ detoxification/homeostasis. A peroxynitrite intermediate complex is implicated; if 2,4-di-*t*-butylphenol is added prior to $\cdot NO$ reaction with **2**, *o*-nitration occurs giving 2,4-di-*t*-butyl-6-nitrophenol. The iron product is (F₈)Fe^{III}-(OH) (**4**). The results suggest that heme/ $O_2/\cdot NO$ chemistry may lead to peroxynitrite leakage and/or exogenous substrate oxidative/nitrative reactivity.

The reaction of nitric oxide ($\cdot NO$; 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 $\cdot NO$ can lead to toxicological processes that include DNA damage, protein nitration leading to cell death, and formation of peroxynitrite ($\cdot OON=O$) 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.^{3cd,4} To maintain proper $\cdot NO$ levels,

karlin@jhu.edu.

[†]Current Addresses: Dept. Chemistry, IIT Guwahati, North Guwahati, Assam India

[‡]Current Addresses: Dept. Chemistry, Chonbuk National University, Jeonju Korea

[§]Current Addresses: Dept. Chemistry, Northwestern University, Evanston IL, USA

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

microbial heme protein $\cdot\text{NO}$ Dioxygenases (NODs) catalyze the reaction of O_2 and $\cdot\text{NO}$ to yield the biologically benign nitrate anion (NO_3^-).⁵

Hemoglobin (Hb) and myoglobin (Mb) are also major targets/sinks of $\cdot\text{NO}$ 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 $\cdot\text{NO}$ reacts with red blood cell oxyHb [$\text{Hb}(\text{Fe}^{\text{III}}\text{-}^{18}\text{O}_2^-)$], sperm whale oxyMb [$\text{Mb}(\text{Fe}^{\text{III}}\text{-}^{18}\text{O}_2^-)$] and *E. coli* oxyflavohemoglobin [flavoHb($\text{Fe}^{\text{III}}\text{-}^{18}\text{O}_2^-$)].⁷ The generally stated mechanism of $\cdot\text{NO}$ dioxygenase (Scheme 1) involves direct reaction of the $\text{Fe}^{\text{III}}\text{-(O}_2^-)$ oxy complex with $\cdot\text{NO}$, giving a peroxynitrite intermediate. Subsequent homolytic O-O bond cleavage produces an oxo-ferryl ($\text{Fe}^{\text{IV}}\text{=O}$) species and the free radical nitrogen dioxide ($\cdot\text{NO}_2$); the latter attacks the ferryl O-atom to produce an N-O bond giving nitrate.^{3d,8-12}

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 thermokinetic¹⁴ and theoretical^{10b} arguments, the peroxynitrite-iron intermediate would 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-)] [$\lambda_{\text{max}} = 422, 542 \text{ nm}$] reacts reversibly with dioxygen to give a diamagnetic iron(III)-superoxo species (S)(F_8) $\text{Fe}^{\text{III}}\text{-(O}_2^-)$ (**2**) [$\lambda_{\text{max}} = 416, 535 \text{ nm}$], 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 $\cdot\text{NO}$ (at -80°C) produces the five-coordinate nitrate heme complex (F_8) $\text{Fe}^{\text{III}}\text{-(NO}_3^-)$ (**3**) [$\lambda_{\text{max}} = 393, 505, 572, 634 \text{ nm}$ (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 $\cdot\text{NO}_{(\text{g})}$ 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

Thus, we sought chemical evidence which might suggest the formation of a peroxynitrite species. When $\cdot\text{NO}_{(\text{g})}$ reaction with (thf)(F_8) $\text{Fe}^{\text{III}}\text{-(O}_2^-)$ (**2**) is followed by addition of the tyrosine mimic 2,4-di-*t*-butylphenol (DTBP), (F_8) $\text{Fe}^{\text{III}}\text{-(NO}_3^-)$ (**3**) is never-the-less 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 $\cdot\text{NO}_{(\text{g})}$ to **2** (Scheme 2).¹⁸ Workup of the reaction solution¹⁸ reveals that the ferric hydroxo product (F_8) $\text{Fe}^{\text{III}}\text{-OH}$ (**4**) forms ($\sim 85\%$ yield) along with high yields ($> 82\%$) of 2,4-di-*t*-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 $\cdot\text{NO}_{(\text{g})}$ bubbled into solution had no effect on the products or their relative yields. (ii) Bubbling excess $\cdot\text{NO}_{(\text{g})}$ into a solution 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 $\cdot NO_{(g)}$ is not solely responsible for significant DTBP nitration. (iii) Warming **2** from $-80\text{ }^{\circ}C$ to RT in the presence of DTBP yielded the oxidatively coupled phenol 3,3',5,5'-tetra-*t*-butyl-(1,1'-biphenyl)-2,2'-diol ($\sim 10\%$), unreacted DTBP ($\sim 90\%$) and **4**; $\cdot NO_{(g)}$ is needed for DTBP nitration, **4** is formed from thermal decomposition of **2**.^{16a} (iv) Warming a $-80\text{ }^{\circ}C$ solution of $(F_8)Fe^{III}-(NO_3^-)$ (**3**) in the presence of DTBP gives no reaction of any kind; **3** itself won't nitrate DTBP.¹⁹

As said, these studies implicate a heme-peroxynitrite intermediate $(F_8)Fe^{III}-(^-\text{OON}=\text{O})$ (**2a**), formed from $(thf)(F_8)Fe^{III}-(O_2^{\cdot-})$ (**2**) (no excess O_2 present) plus one equiv $\cdot NO_{(g)}$. Following the literature suggestions, we can hypothesize that **2a** undergoes homolysis to give a ferryl + $\cdot NO_2$ (**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 $\cdot NO_2$ 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 $(F_8)Fe^{III}-(^-\text{OON}=\text{O})$ (**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 $\cdot NO$ 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 $\cdot NO$,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 metal ion reduced centers with O_2 followed by $\cdot NO_{(g)}$, 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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 17. For comparison of properties, a separately synthesized nitrate complex (**3**) was generated via the reaction, $(\text{F}_8)\text{Fe}^{\text{III}}(\text{Cl}) + \text{AgNO}_3 \rightarrow (\text{3})$.¹⁷
 18. See Supporting Information
 19. DTBP in a metal-complex free solution bubbled with excess $\text{O}_2(\text{g})$ and $\cdot\text{NO}(\text{g})$, yielded a 50/50 mixture of NO_2DTBP and a dinitrated product 2-*t*-butyl-4,6-dinitrophenol. Free $\cdot\text{NO}_2$ which might derive from $2 + \cdot\text{NO}(\text{g})$ chemistry is capable of effecting phenol nitration, but since production of only one $\cdot\text{NO}_2$ per heme complex is possible, the yield of NO_2DTBP would then be less than 50 %, not as observed.
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Figure 1.

UV-vis spectra (THF solvent) of $(F_8)Fe^{II}$ (**1**) (Blue), $(thf)(F_8)Fe^{III}-(O_2^{\cdot-})$ (**2**) (Red) generated from **1** + $O_2(g)$ and $(F_8)Fe^{III}-(NO_3^-)$ (**3**) (Green) generated from **2** + $\cdot NO(g)$

Figure 2. Reaction of (thf)(F₈)Fe^{III}-(O₂^{•-}) (2) (Ar^F = 2,6-difluorophenyl) + •NO_(g) produces the nitrate complex (F₈)Fe^{III}-(NO₃⁻) (3) (X-ray).¹⁷

Scheme 1.

Scheme 2.