Peroxynitrite Decomposition Catalysts

Michael K. Stern,* Michael P. Jensen, and Keith Kramer‡

Monsanto Corporate Research, Monsanto Company
St. Louis, Missouri 63167

Received April 18, 1996

Peroxynitrite ($ONOO^-$) is a highly reactive oxidant produced by the combination of nitric oxide and superoxide anion at rates approaching the diffusion limit.[1] This anion decomposes readily under acid catalysis by two distinct pathways.[2] Isomerization to nitrate is the major decay route, but a significant portion of the decomposition produces a species with reactivity akin to that of hydroxyl radical.[3] A compelling body of evidence has emerged that suggests peroxynitrite forms in significant concentrations *in vivo* and is capable of oxidizing lipid membranes[4] and sulfhydryl moieties,[5] as well as hydroxylating and nitrating aromatics.[6] In addition, $ONOO^-$ may contribute to cell death and tissue injury in a number of human diseases, including arthritis, sepsis, inflammatory bowel disease, and stroke.[7]

The objective of the work described herein was the identification of metal complexes that catalyze the isomerization of peroxynitrite to nitrate so that it becomes the exclusive decomposition pathway. Formation of oxidizing radical species thus would be preempted by production of harmless nitrate anion. Although a few examples of metal complex oxidation with $ONOO^-$ are known,[8,9] a catalytic reaction has not been documented previously. We now report the discovery that certain water-soluble iron(III) porphyrins are highly active $ONOO^-$ decomposition catalysts and that they indeed function as "peroxynitrite isomerases."

We found, by use of a stopped-flow spectrophotometric assay, that addition of micromolar concentrations of Fe^{III}(TMPyP) or Fe^{III}(TMPS) noticeably increased the rate of peroxynitrite decomposition at physiologically relevant pH and temperature.[10] Plots of k_{obs} vs [catalyst] were linear in the case of Fe^{III}(TMPS) indicating a first-order dependance on porphyrin complex.

Table 1. Catalytic Rate Constants and Shifts in NO_2^-/NO_3^- Ratios

compound	k_{cat} M^{-1} s^{-1} [a]	μmol $ONOO^-$	μmol NO_2^-	μmol NO_3^-	ratio NO_2^-/NO_3^-
peroxynitrite/buffer		0.336	0.106	0.225[b]	0.47
FeTMPyP	2.20×10^6	0.300	0.019	0.272[c]	0.07
FeTMPS	6.45×10^5	0.336	0.065	0.256[b]	0.25
H_2TMPyP	inactive	0.300	0.089	0.209[c]	0.42
ZnTMPyP	inactive	0.300	0.100	0.208[c]	0.48

[a] Determined under saturation conditions. Reaction to determine NO_2^-/NO_3^- ratios were conducted in 250 mM potassium phosphate buffer at pH = 7.4 in a total volume of 1 mL. Compounds were tested at a concentration of 20 μM using two different preparations of [$ONOO^-$]. [b] [$ONOO^-$] = 336 μM. [c] [$ONOO^-$] = 300 μM.

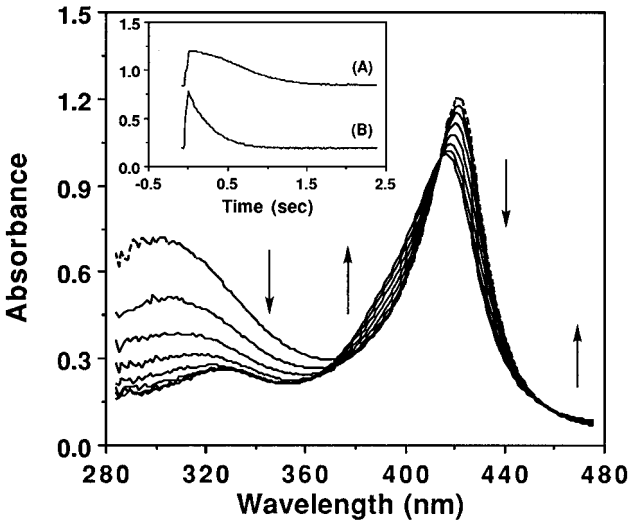


Figure 1. Time-resolved UV-vis spectra for stopped-flow reaction of Fe^{III}(TMPS) (5.1 μM) and peroxynitrite (210 μM) at pH = 7.4, 37 °C: (- - -) scan taken 36 msec after mixing. Inset: observed time-dependent relaxations of (A) O=Fe^{IV}(TMPS) at 422.0 nm and (B) $ONOO^-$ at 302.5 nm.

Moreover, analysis by ion chromatography of the reaction products from the catalyzed decomposition of $ONOO^-$ revealed that yields of NO_3^- are substantially enhanced at the expense of NO_2^- (Table 1).[12] In contrast, addition of H_2TMPS, H_2-TMPyP, Zn(TMPyP), or $FeCl_3$ produced neither increased reaction rates nor altered NO_2^-/NO_3^- product ratios when compared to the background acid-catalyzed process.

Time-resolved UV−vis spectra recorded during the catalytic reaction between $ONOO^-$ (210 μM) and Fe^{III}(TMPS) (5.1 μM) are shown in Figure 1. The porphyrin Soret band increased in intensity and shifted from 417 nm toward 422 nm immediately after mixing. The observed intermediate persisted throughout a substantial portion of the $ONOO^-$ decay and then reverted back to the starting Fe(III) spectrum with two distinct isosbestic

‡ Current address: Department of Chemistry, Harvard University, Cambridge, MA 02138.

(1) (a) 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. (b) Beckman, J. S.; Crow, J. P. *Biochem. Soc. Trans.* **1993**, *21*, 330−334. (c) Ischiropoulos, H.; Zhu, L.; Beckman, J. S. *Arch. Biochem. Biophys.* **1992**, *298*, 446−451. (d) Huie, R. E.; Padmaja, S. *Free Radical Res. Commun.* **1993**, *18*, 195−199.

(2) (a) Keith, W. G.; Powell, R. E. *J. Chem. Soc. A* **1969**, 90. (b) Hughs, M. N.; Nicklin, H. G. *J. Chem. Soc. A* **1968**, 450−451.

(3) (a) Tsai, J.-H. M.; Harrison, J. G.; Martin, J. C.; Hamilton, T. P.; van der Woerd, M.; Jablonsky, M. J.; Beckman, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 4115−4116. (b) Crow, J. P.; Spruell, C.; Chen, J.; Gunn, C.; Ischiropoulos, H.; Tsai, M.; Smith, C. D.; Radi, R.; Koppenol, W. H.; Beckman, J. S. *Free Radical Biol. Med.* **1994**, *16*, 331−338.

(4) (a) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. *Arch. Biochem. Biophys.* **1991**, *288*, 481−487. (b) Rubbo, H.; Radi, R.; Trujillo, M.; Teller, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B. A. *J. Biol. Chem.* **1994**, *269*, 26066−26075.

(5) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. *J. Biol. Chem.* **1991**, *266*, 4244−4250.

(6) (a) van der Vliet, A.; O'Neill, C. A.; Halliwell, B.; Cross, C. E.; Kaur, H. *FEBS Lett.* **1994**, *339*, 89−92. (b) van der Vliet, A.; Eiserich, J. P.; O'Neill, C. A.; Halliwell, B.; Cross, C. E. *Arch. Biochem. Biophys.* **1995**, *319*, 341−349.

(7) (a) Kaur, H.; Halliwell, B. *FEBS Lett.* **1994**, *350*, 9−12. (b) Szabo, C.; Salzman, A. L.; Ischiropoulos, H. *FEBS Lett.* **1995**, *363*, 235−238. (c) Rachmilewitz, D.; Stamler, J. S.; Karmeli, F.; Mullins, M. E.; Singel, D. J.; Loscoalzo, J.; Xavier, R. J.; Podolsky, D. K. *Gastroenterology* **1993**, *105*, 1681−1688. (d) Oury, T. D.; Piantadosi, C. L.; Crapo, J. D. *J. Biol. Chem.* **1993**, *268*, 15394−15398.

(8) (a) Hughs, M. N.; Nicklin, H. G. *J. Chem. Soc. A* **1970**, 925−928. (b) Groves, J. T.; Marla, S. S. *J. Am. Chem. Soc.* **1995**, *117*, 9578−9579. (c) Goldstein, S.; Czapski, G. *Inorg. Chem.* **1995**, *34*, 4041−4048. (d) Plumb, R. C.; Edwards, J. O.; Herman, M. A. *Analyst* **1992**, *117*, 1639.

(9) Floris, R.; Piersma, S. R.; Yang, G.; Jones, P.; Wever, R. *Eur. J. Biochem.* **1993**, *215*, 767−775.

(10) Fe^{III}(TMPyP) is 5,10,15,20-tetrakis(*N*-methyl-4′-pyridyl)porphinatoiron(III), and was used as the tetratosylate salt. Fe^{III}(TMPS) is 5,10,15,-20-tetrakis(2,4,6-trimethyl-3,5-sulfonatophenyl)porphinatoiron(III) and was synthesized with modifications of the method of Meunier *et al.*[11a] and was used as the octasodium salt. The disappearance of $ONOO^-$ was followed at 302 nm using an OLIS-RSM stopped-flow spectrophotometer. The catalyst were evaluated at 37.0 °C in 100 mM potassium phosphate buffer, pH = 7.4 prepared with double-distilled water.[11b] Carbonate levels in the buffers were measured and shown to be <10 ppm. Stock solutions of $ONOO^-$ ranged from 12 to 50 mM and were prepared according to the method of Leis.[11c] The kinetics of initial $ONOO^-$ decomposition were fitted using standard regression techniques. The extinction coefficient of peroxynitrite was taken as 1670 M^{-1} cm^{-1}.[11c]

(11) (a) Campestrini, S.; Meunier, B. *Inorg. Chem.* **1992**, *31*, 1999−2006. (b) Riley, D. P.; Rivers, W. J.; Weiss, R. H. *Anal. Biochem.* **1991**, *196*, 344−349. (c) Leis, J. R.; Pena, M. E.; Rios, A. *J. Chem. Soc., Chem. Commun.* **1993**, 1298−1299.

(12) Control reactions showed that NO_2^- and NO_3^- did not interconvert under the reaction conditions. H_2TMPS and $FeCl_3$ data are not shown.

S0002-7863(96)01279-6 CCC: $12.00

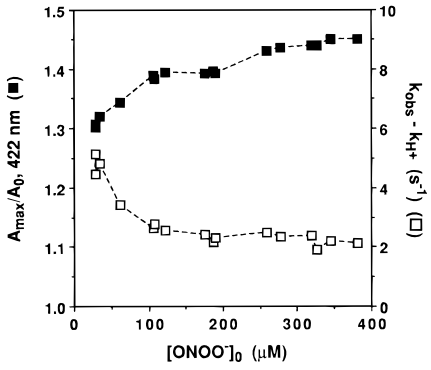


Figure 2. Maximum O═Fe^{IV}(TMPS) yields observed at 422 nm (■) and initial catalytic rates (s^{-1}) observed at 302 nm (□) *vs* peroxynitrite loading at pH = 7.4, 37.0 °C. $[Fe^{III}(TMPS)]_0 = 5.1$ μM.

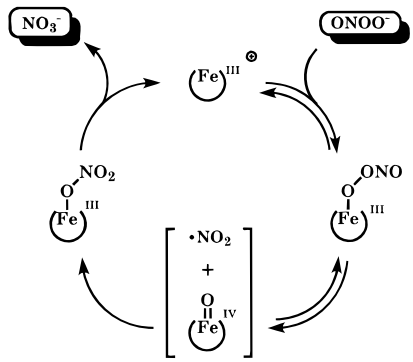
points at 415 and 459 nm. The rate of the latter process approached an apparent first-order limit after exhaustion of $ONOO^-$ (see Figure 1 inset). The spectrum of the observed intermediate species is nearly identical to those of ortho-substituted sulfonated oxo Fe(IV) porphyrins formed by oxidation of Fe(III) complexes with alkyl hydroperoxides or *m*-chloroperoxybenzoic acid (*m*-CPBA).[13] In addition, similar spectral changes have been observed from the reaction of $ONOO^-$ with the heme proteins myeloperoxidase and lactoperoxidase.[9] Therefore, we formulate the observed intermediate as O═Fe^{IV}(TMPS).

To probe the chemistry of the intermediate, Fe^{III}(TMPS) was oxidized with *m*-CPBA instead of $ONOO^-$. A shift in the Soret band from 417 nm ($\epsilon_{max} = 9.29 \times 10^4$ M^{-1} cm^{-1}) to 426 nm ($\epsilon_{max} \approx 1.2 \times 10^5$ M^{-1} cm^{-1}), with isosbestic points at 418 and 459 nm, was again observed upon addition of near-stoichiometric quantities of *m*-CPBA at pH = 7.4, 20 °C to give high yields (ca. 95%) of the O═Fe^{IV}L species. However, unlike the intermediate generated during catalysis, this complex was stable toward reduction ($t_{1/2}$ > 90 min). Most significantly, treatment of the solution with excess NO_2 gas (5.0 mL) or aqueous $NaNO_2$ (2.2 equiv) resulted in immediate quenching of the oxo species back to Fe^{III}(TMPS). Equivalent amounts of NO_3^- had no effect. Therefore, we concluded that recombination of O═Fe^{IV}(TMPS) with a species generated from $ONOO^-$, perhaps NO_2 or a hydrolysis product,[14] is responsible for turnover of the otherwise stable O═Fe^{IV}(TMPS) intermediate.

A series of stopped-flow kinetic experiments were conducted in which the Fe^{III}(TMPS) and Fe^{III}(TMPyP) catalyst concentrations were held constant (5.1 and 4.9 μM, respectively) while the initial concentration of $ONOO^-$ was varied over a wide range (27−386 μM). With decreasing initial $ONOO^-$ loading, the maximum observed absorption of the O═Fe^{IV}(TMPS) intermediate, Figure 2, fell from an asymptotic limit. At the lowest loadings, substantial loss of $ONOO^-$ occurred before peak O═Fe^{IV}(TMPS) concentration was reached. Furthermore, the apparent first-order rate of $ONOO^-$ loss (k_{cat}) increased at lower loadings of $ONOO^-$ where catalyst saturation was not approached.

The above observations suggest strongly that catalytic $ONOO^-$ turnover occurs through reversible oxidation of the metal complex to the Fe(IV) state, followed by irreversible quenching to nitrate and Fe(III). This interpretation is supported by linear Lineweaver−Burk plots[15] of inverse initial rate of peroxynitrite decomposition *vs* inverse $ONOO^-$ loading which displayed positive intercepts and slopes. Limiting turnover rates of 120 ± 75 s^{-1} and 360 ± 170 s^{-1} and Michaelis−Menten constants of 3.1×10^{-4} and 2.4×10^{-4} M were calculated for Fe^{III}(TMPS) and Fe^{III}(TMPyP), respectively. Therefore, of the two complexes, Fe^{III}(TMPyP) is the superior catalyst, $ONOO^-$ binding is tighter, and productive intermediate turnover is faster.

Scheme 1



A catalytic mechanism that is consistent with the experimental results described above is summarized in Scheme 1, where formation of an O═Fe^{IV}L intermediate is accomplished by reversible homolysis of bound peroxynitrite. Generation of 1 equiv of nitrogen dioxide is required for mass balance. Rapid recombination of the radicals reduces the metal complex back to Fe(III), with regeneration of $ONOO^-$ or production of NO_3^-. The O═Fe^{IV}L intermediate could also be generated by an outer-sphere electron transfer from Fe(III) to $ONOO^-$/ONOOH. However, oxidation of myeloperoxidase by peroxynitrite was reportedly blocked by a large excess of chloride (1.2 M), suggesting the reaction may be inhibited by competitive binding of redox-inactive ligands.[9] Furthermore, as noted by Groves and Marla for reaction of $ONOO^-$ with Mn^{III}(TMPyP),[8b] the O═Mn^{IV}L intermediate can form either from homolytic or heterolytic O−O splitting, with rapid reduction of O═Mn^{V}L by nitrate occurring in the latter case.[16] Thus, the nature of O−O bond cleavage remains uncertain. However, the products predicted for collapse of this state are certainly either nitrate or peroxynitrite,[17] in the absence of competitive NO_2 hydrolysis.

In conclusion, this study demonstrates clearly that two iron porphyrin complexes are unusually active as catalytic "peroxynitrite isomerases" under physiological conditions and at realistic dose-derived concentrations. Indeed preliminary test of these compounds in biological models of relevant disease states indicate that they have profound activity.[18,19] These observations bode well for possible development of a therapeutic strategy based on pharmacological application of these and similar complexes.

Acknowledgment. The authors thank Professors John Groves and Jack Halpern for helpful discussions and Dr. Cathleen Hanau for preparing some of the FeTMPS used in this study.

JA961279F

(13) (a) Balasubramanian, P. N.; Lee, R. W.; Bruice, T. C. *J. Am. Chem. Soc*. **1989**, *111*, 8714−8721. (b) Balasubramanian, P. N.; Lindsay-Smith,, J. R.; Davies, M. J.; Kaaret, t. W.; Bruice, T. C. *J. Am. Chem. Soc*. **1989**, *111*, 1477−1483. (c) Murata, K.; Panicucci, R.; Gopinath, E.; Bruice, T. C. *J. Am. Chem. Soc*. **1990**, *112*, 6072−6083.

(14) Schwartz, S. E.; White, W. H. *Adv. Environ. Sci. Technol*. **1983**, *12*, 1.

(15) Moore, J. W.; Pearson, R. G. *Kinetics and Mechanism*, 2nd ed.; Wiley: New York, 1981; pp 378−383.

(16) (a) Groves, J. T.; Haushalter, R. C.; Nakamura, N.; Nemo, T. E.; Evans, B. J. *J. Am. Chem. Soc*. **1981**, *103*, 2884. (b) Balch, A. L.; Latos-Grazynsky, L.; Renner, M. W. *J. Am. Chem. Soc*. **1985**, *107*, 2983.

(17) The recombination of NO_2 and $HO^\bullet$ has been studied and shown to be rapid, $k = (4.5 \pm 1.0) \times 10^9$ M^{-1} s^{-1}, suggesting that the recombination of NO_2 and O═Fe^{IV}L is not unreasonable. See: Logager, T.; Sehested, K. *J. Phys. Chem*. **1993**, *97*, 6664−6669.

(18) Stern, M. K.; Salvemini, D. PCT Int. Appl. WO95/31197.

(19) Unpublished results. Manuscript in preparation.