Myoglobin Catalyzes Its Own Nitration

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Myoglobin is thought to facilitate oxygen transport within cells.[1] However, alternative functions such as nitric oxide scavenging have also been suggested.[2] High myoglobin concentrations (10^{-4} M) are found in cardiac cells and striated muscle,[1] both of which have been shown to use nitric oxide for intercellular signaling.[3] Likewise, a myoglobin-like neuroglobin has been found recently in brain cells.[3b] In this light, the fast reaction of oxymyoglobin with nitric oxide to afford metmyoglobin and nitrate could be a physiologically important pathway.[4] Enhanced dissipation of NO could maintain the concentration gradients needed for efficient NO signaling and mediate the respiratory inhibition of cytochrome oxidase. These considerations have prompted us to examine the reactions of metmyoglobin (metMb) with peroxynitrite (PN), since the metMb-PN adduct, Fe(III)−OO−NO, is the same species as is postulated for the reaction of oxyMb with NO.[4] We find that metMb catalyzes the decomposition of peroxynitrite to afford both nitrate *and* NO_2, and further, that metMb catalyzes its own nitration specifically at Tyr-103.

The decomposition of peroxynitrite, monitored at 302 nm at pH 7.6 as we have previously described,[5] was observed to accelerate in the presence of 5−50 μM horse heart metMb. A second-order rate constant $k_1 = 1.03 \times 10^4$ M^{-1} s^{-1} was determined for this process from the metMb concentration dependence (Figure 1). Only metMb was observed during the peroxynitrite decay, consistent with an earlier report.[6] A slow production of ferrylMb was observed after 10 s due to hydrogen peroxide[7] in the peroxynitrite solutions. No catalysis was observed in the presence of 50 μM NaCN, indicating that cyanomyoglobin,[8] which is formed under these conditions (λ_{abs} = 422, 540 nm), had no effect on peroxynitrite decay.

The stoichiometry of this myoglobin-induced decomposition of peroxynitrite was examined by directly monitoring changes in the nitrite and nitrate yields with varying [metMb](Figure 2). The nitrite yield decreased nearly 4-fold with increasing [metMb], leveling off at 11% above 50 μM Mb. That nitrite was produced even at high [metMb] suggested that NO_2 was produced during catalysis which afforded nitrite and nitrate upon hydrolysis. We can estimate from this nitrite yield that the proposed MbFe(III)−OO−NO intermediate (**1**) decomposes to afford ca. 80% nitrate and 20% NO_2 according to eq 1.

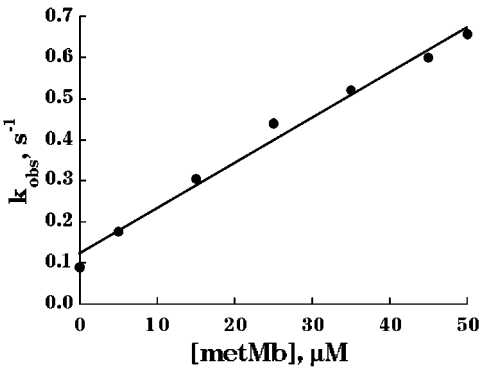


Figure 1. Pseudo-first-order rate constants for the decay of peroxynitrite vs the concentration of metMb derived from stopped-flow kinetic data monitoring peroxynitrite decay (λ_{mon} = 302 nm) upon mixing equal volumes of 1 mM $OONO^-$ in 2.5 mM NaOH and 20 mM Tris buffer, pH 7.6. Second-order rate constant, $k = 1.03 \times 10^4$ M^{-1} s^{-1}.

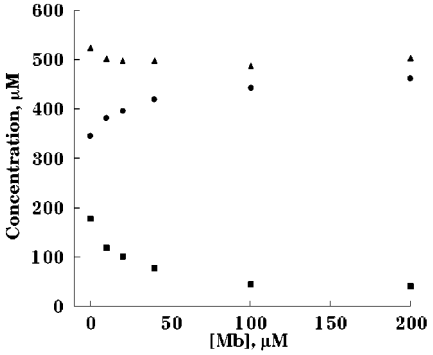
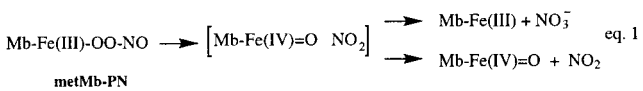


Figure 2. Effect of myoglobin on the yield of nitrate and nitrite determined directly by ion chromatography from the decomposition of 500 μM peroxynitrite in 20 mM Tris buffer, pH 7.6: (●) nitrate, (■) nitrite, and (▲) total of both. Values were corrected for nitrite levels in the peroxynitrite stock solutions.



Electrospray mass spectroscopy of metMb samples after peroxynitrite treatment showed significant *nitration* of myoglobin, consistent with the production of NO_2. After incubation of metMb with 3 mM peroxynitrite at pH 7.6, the ES/MS spectrum showed an envelope of peaks that analyzed for a species with *m*/*e* 16998 (80%) accompanied by weaker peaks corresponding to ∼20% metMb (*m*/*e* 16953) (see Supporting Information). This mass change is consistent with mono-nitration of the protein.[9] Concentrations of peroxynitrite of 0.5, 1, and 2 mM reacted with 100 μM metmyoglobin led to ∼20, 36, and 50% nitration, as estimated from ES/MS data. Likewise, repeated exposure of 100 μM oxyMb to aqueous NO in the presence of air and 5 mM ascorbate gave MS evidence for ca. 10% Mb nitration.[10]

(1) (a) Suzuki, T.; Imai, K. *Cell. Mol. Life Sci.* **1998**, *54*, 979. (b) Wittenberg, B. A.; Wittenberg, J. B. *Annu. Rev. Physiol.* **1989**, *51*, 857. (c) Takahashi, E.; Endoh H.; Doi, K. *Biophys. J.* **2000**, *78*, 3252.

(2) (a) Garry, D. J.; Ordway, G. A.; Lorenz, J. N.; Radford, N. B.; Chin, E. R.; Grange, R. W.; Bassel-Duby, R.; Williams, R. S. *Nature* **1998**, *395*, 905. (b) Garry, D. J.; Meeson, A.; Yan, Z.; Williams, R. S. *CMLS Cell. Mol. Life Sci.* **2000**, *57*, 896. (c) Frauenfelder, H.; McMahon, B. H.; Austin, R. H.; Chu, K.; Groves, J. T. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 2370−2374.

(3) (a) Conley, K. E.; Ordway, G. A.; Richardson, R. S. *Acta Physiol. Scand.* **2000**, *168* (4), 623. (b) Burmeister, T.; Weich, B.; Reinhardt, S.; Hankein, T. *Nature* **2000**, *407*, 520.

(4) (a) Doyle, M. P.; Hoekstra, J. W. *J. Inorg. Biochem.* **1981**, *14*, 351. (b) Wade, R. S.; Castro, C. E. *Chem. Res. Toxicol.* **1996**, *9*, 1382.

(5) (a) Groves, J. T.; Marla, S. *J. Am. Chem. Soc.* **1995**, *117*, 9578. (b) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 6053. (c) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 7493.

(6) (a) Exner, M.; Herold, S. *Chem. Res. Toxicol.* **2000**, *13*, 287. (b) Herold, S. *FEBS Lett.* **1999**, *443*, 81. (c) Herold, S.; Exner, M.; Nauser, T. *Biochemistry* **2001**, *40*, 3385.

(7) Yonetani, T.; Schleyer, H. *J. Biol. Chem.* **1967**, *242*, 1974.

(8) Antonini, E.; Brunori, M. *Hemoglobin and Myoglobin in Their Reactions with Ligands*; North-Holland: Amsterdam, The Netherlands, 1971.

(9) (a) Beckman, J. S.; Ischiropoulos, H.; Zhu, L.; van der Woerd, M.; Smith, C.; Chen, J.; Harrison, J.; Martin, J. C.; Tsai, M. *Arch. Biochem. Biophys.* **1992**, *298*, 438. (b) Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. *Arch Biochem. Biophys.* **1992**, *298*, 431. (c) Groves, J. T. *Curr. Opin. Chem. Biol.* **1999**, *3*, 226.

(10) Eich, R. F.; Li, T.; Lemon, D. D.; Doherty, D. H.; Curry, S. R.; Aitken, J. F.; Mathews, A. J.; Johnson, K. A.; Smith, R. D.; Phillips, G. N., Jr.; Olson, J. S. *Biochemistry* **1996**, *35*, 6976.

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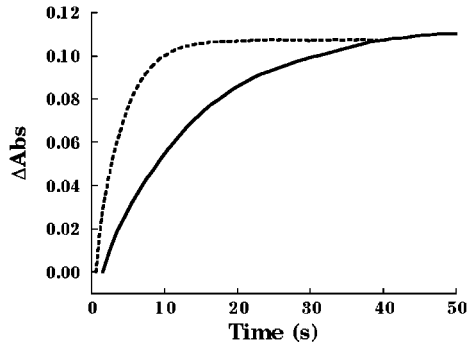


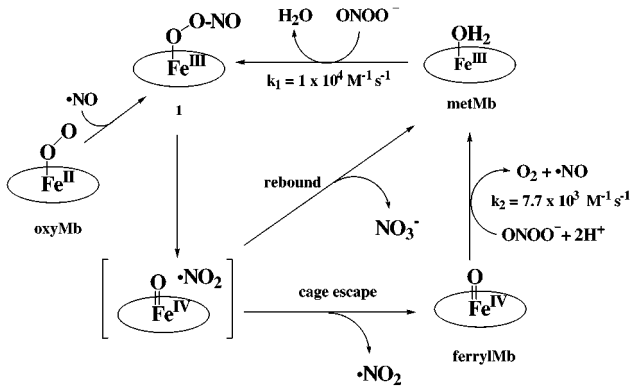
Figure 3. Nitration of fluorescein during stopped-flow spectroscopy monitored at $\lambda_{mon} = 520$ nm. Nitrofluorescein yields were 24% as determined from ΔOD. Solid line: Kinetic trace for 30 μM fluorescein in 20 mM Tris buffer (pH 7.6) + 1 mM $OONO^-$ in 2.5 mM NaOH, $k_{obs} = 0.058\ s^{-1}$. Dashed line: Same as above with 50 μM metMb (horse heart) added to fluorescein, $k_{obs} = 0.41\ s^{-1}$.

The nitration of myoglobin was also observed after the addition to metMb of H_2O_2 and NO_2^- in concentrations similar to that of PN (1−3 mM).[11] However, the addition of catalase (1 μM) to the metMb solutions proved adequate to completely eliminate this process while for the 3 mM PN case, catalase reduced the amount of myoglobin nitration observed by only 20%. Cyano-Mb showed reduced (<50%) nitration levels. Thus, the majority of the myoglobin nitration must have derived from the peroxynitrite reactivity with the heme center. Nitrated Mb catalyzed PN decomposition at an undiminished rate.

The site of metMb nitration was determined by LC/MS analysis of tryptic digests of the reaction mixture that showed all of the typical myoglobin fragments.[12] A single new peak in the digest was observed at 42 min with *m/e* 1931 that displayed a strong UV absorbance at $\lambda_{mon} = 420$ nm characteristic of nitrotyrosine.[9] There was correspondingly diminished intensity of a peak at 41 min (*m/e* 1886), the fragment corresponding to residues 103−118. This unambiguously identifies Tyr103 as the site of nitration. This tyrosine is located at the protein surface about 6 Å (edge-to-edge) from the heme. The absence of a peak in the HPLC trace in the region of authentic O_2N-Tyr-Lys (2.6 min) indicated negligible nitration at Tyr146.[13]

MetMb nitration was suppressed in a concentration-dependent manner upon addition of 10−300 μM fluorescein, a detector, and scavenger of NO_2.[14] At the higher concentrations of fluorescein, no significant metMb nitration was observed. Fluorescein was nitrated under these conditions. Figure 3 shows the time course for the appearance of nitrofluorescein observed at 520 nm upon the addition of PN. The same spectral changes, including isosbestic points at 512 nm, were observed for fluorescein nitration during PN decay in the presence of 100 μM metMb. The rate of nitration increased proportionately with the rate of peroxynitrite decomposition. Since the majority of the peroxynitrite flux passes through the metMb-catalyzed pathway under these conditions, these results indicate that the metMb-catalyzed decay of peroxynitrite produces a *freely diffusing* nitrating species, also consistent with nitrogen dioxide production.

Scheme 1



A mechanism consistent with these observations is shown in Scheme 1. The rate constants shown were determined from stopped-flow kinetic data as we have described.[5] Initial reaction of PN with metMb is proposed to afford an intermediate adduct, metMb-PN, that decomposes via O−O bond homolysis yielding ferrylMb and NO_2.[5,15] Rebound of NO_2 with ferrylMb within the heme cavity would give metMb and nitrate as the major product.[16] Cage escape of NO_2 must also occur with ca. 20% efficiency to account for the observed nitrite and metMb nitration.

The reduction of ferrylMb by peroxynitrite was observed directly. FerrylMb was produced by incubation of metMb (100 uM) for 10 min with 2 equiv of H_2O_2.[7] Stoichiometric addition of PN, as monitored by stopped-flow techniques,[5] caused the reduction of ferrylMb to metMb ($k_2 = 7.7 \times 10^3\ M^{-1}\ s^{-1}$) and a complete suppression of fluorescein nitration. This explains why ferrylMb is not seen during PN decomposition, as suggested by Herold,[6] and it shows that the reaction between ferrylMb and PN does not produce a nitrating species.[17]

In summary, metMb catalyzes the decomposition of peroxynitrite at biologically relevant concentrations. The products are nitrate (80%) and NO_2 (20%). At high peroxynitrite concentrations, this process leads to nitration of the surface tyrosine-103. Small but significant amounts of myoglobion nitration were also observed upon addition of NO to oxyMb. More detailed studies of myoglobin interactions with peroxynitrite and NO are under way.[18]

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Supporting Information Available: LC/electrospray MS data for nitrated myoglobin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) Eiserich, J. P.; Cross, C. E.; Jones, A. D.; Halliwell, B.; van der Vliet, A. *J. Biol. Chem.* **1996**, *271*, 19199.
(12) Mann, M.; Meng, C. K.; Fenn, J. B. *Anal. Chem.* **1989**, *61*, 1702.
(13) These analyses were performed on a Waters 717 HPLC using a Delta Pak C18 column with 10−50% CH_3CN in water.
(14) Jiang, Q.; Hurst, J. K. *J. Biol. Chem.* **1997**, *272*, 32767.
(15) (a) Daiber, A.; Herold, S.; Schoneich, C.; Namgaladze, D.; Peterson, J. A.; Ullrich, V. *Eur. J. Biochem.* **2000**, *267*, 6729−6739. (b) Rao, S. I.; Wilks, A.; Ortiz de Montellano, R. R. *J. Biol. Chem.* **1993**, *268*, 803.
(16) (a) Stern, M. K.; Jensen, M. P.; Kramer, K. *J. Am. Chem. Soc.* **1996**, *118*, 8735−8736. (b) Shimanovich, R.; Groves, J. T. *Arch. Biochem. Biophys.* **2001**, *387*, 307−317.
(17) Clark electrode measurements detected the production of 87 μM (87% based on ferrylMb) oxygen upon addition of peoxynitrite, cf.: McKee, M. L. *J. Am. Chem. Soc.* **1995**, *117*, 1629.
(18) We have recently shown with Y. Watanabe that double mutant metmyoglobins (H64D/V68L and F43H/H64L) decomposed peroxynitrite at accelerated rates ($k_{cat} = 7.7 \times 10^5$ and $2.3 \times 10^5\ M^{-1}\ s^{-1}$, respectively) also with extensive protein nitration. Details will be presented elsewhere.