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RAPID DECOMPOSITION OF PEROXYNITRITE BY MANGANESE PORPHYRIN-ANTIOXIDANT REDOX COUPLES

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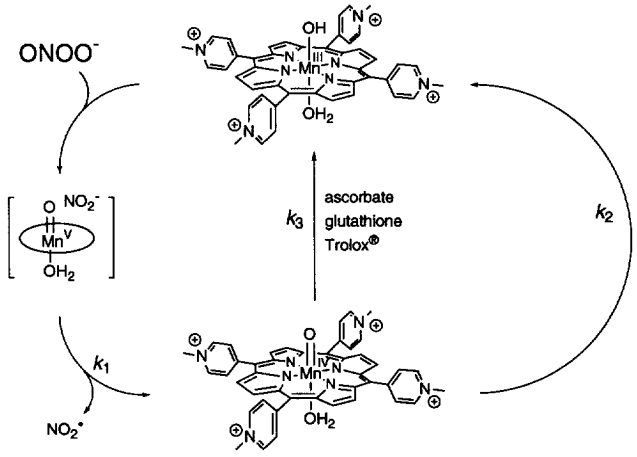
Abstract. Mn(III)TMPyP reacts rapidly with the toxic oxidant peroxynitrite ($ONOO^-$) to generate an oxoMn(IV) species, but Mn(III)TMPyP is not catalytic for $ONOO^-$ decomposition due to the slow reduction of oxoMn(IV) back to the Mn(III) oxidation state. However, when redox-coupled with biological antioxidants that efficiently reduce oxoMn(IV), Mn(III)TMPyP is transformed into an efficient "peroxynitrite reductase."

Peroxynitrite ($ONOO^-$), a potent cytotoxic agent produced by the rapid combination of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$),[1] has recently been recognized as an important member of the family of reactive oxygen species.[2] $ONOO^-$ is known to react with a broad range of biological substrates, including DNA,[3,4] lipids,[5] sulfhydryls,[6] methionine,[7] tyrosine[8] and metalloenzymes.[9,10] In light of this reactivity, $ONOO^-$ has been implicated in a host of human diseases.[11] Thus, synthetic catalysts for fast $ONOO^-$ decomposition may have important therapeutic applications. Recently, Stern et al.[12] discovered that synthetic, water-soluble iron porphyrins rapidly catalyze the isomerization of $ONOO^-$ to NO_3^-; these iron porphyrins demonstrated profound activity in $ONOO^-$ related disease models.[13] A complementary approach to alleviating the toxicity of $ONOO^-$ is to enhance the rapid dismutation of its precursor, $O_2^{\cdot-}$; for example, Riley et al.[14,15] have developed a series of manganese complexes that display significant superoxide dismutase (SOD) activity.

We have previously discovered that Mn(III)TMPyP reacts rapidly with $ONOO^-$ (see Scheme 1).[16,17]



Scheme 1. Rate constants: $k_1 = 1.8 \times 10^6\ M^{-1}s^{-1}$; $k_2 = 0.018\ s^{-1}$; $k_3 = 5.4 \times 10^7\ M^{-1}s^{-1}$ for ascorbate; $k_3 = 1.3 \times 10^5\ M^{-1}s^{-1}$ for glutathione; $k_3 = 7.0 \times 10^6\ M^{-1}s^{-1}$ for Trolox®.

This high reactivity prompted us to explore the development of novel catalysts with the potential for high $ONOO^-$ decomposition and SOD activity. Fridovich et al.[18] have shown that Mn(III)TMPyP has significant SOD activity; however, as these researchers noted, the scavenging of $O_2^{\cdot-}$ alone could not fully explain the protective effects of Mn(III)TMPyP in a SOD-null strain of *E. coli*. Here we demonstrate that Mn(III)TMPyP, in combination with physiologically significant concentrations of antioxidants such as ascorbate, glutathione, and Trolox® (a water-soluble analog of vitamin E), is a very efficient catalytic system for fast $ONOO^-$ decomposition. This catalysis involves rate-limiting oxidation of Mn(III) to an oxoMn(IV) intermediate, and subsequent reduction of oxoMn(IV) to Mn(III) by the antioxidants.

MnTMPyP is an excellent marker for $ONOO^-$ due to the diagnostic formation of oxoMn(IV)TMPyP;[16,17] this reaction is among the fastest known for $ONOO^-$.[9] Nevertheless, Mn(III)TMPyP alone is not an efficient catalyst for $ONOO^-$ decomposition. The kinetic profiles of Mn(III)TMPyP-catalyzed decomposition of $ONOO^-$ in 25 mM phosphate pH 7.4 buffer were measured using stopped-flow spectrophotometry,[19] and as can be seen in Figure 1, the presence of 10 μM Mn(III)TMPyP did not significantly accelerate the decomposition of 100 μM $ONOO^-$, as compared to its rate of self-decomposition in buffer (compare traces a and b). However, the incorporation of near stoichiometric amounts of ascorbate with the manganese porphyrin dramatically reduced the half-life ($t_{1/2}$) of $ONOO^-$ *ca.* 70-fold, from 2 s to 0.03s (trace c).

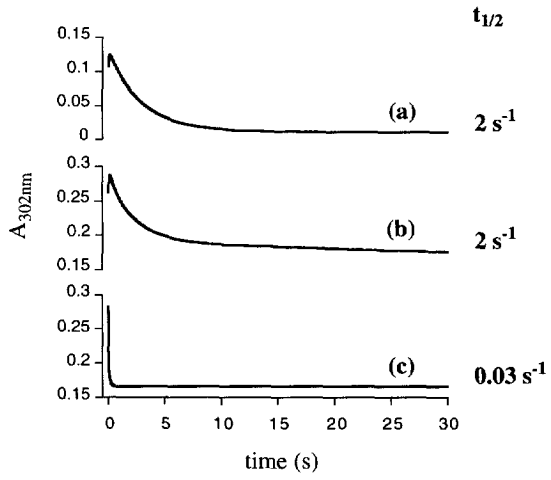


Figure 1. Decomposition of 100 μM $ONOO^-$: (**a**) in pH 7.4 buffer; (**b**) in the presence of 10 μM MnTMPyP; (**c**) in the presence of 10 μM MnTMPyP and 150 μM ascorbate.

The direct reaction of $ONOO^-$ with ascorbate is slow, first order in $ONOO^-$, and first order in ascorbate. As shown in Fig. 2a, the apparent first-order rate constant for $ONOO^-$ disappearance correlated linearly with the concentration of ascorbate under pseudo-first order conditions, yielding a second-order rate constant of 88 $M^{-1}s^{-1}$ at 25 °C in 25 mM pH 7.4 phosphate buffer. This is consistent with the results of Bartlett et al.,[20] and further supports the notion that ascorbate alone can not play a direct role in the defense against $ONOO^-$. In

fact, it has been shown that $ONOO^-$ readily oxidizes and nitrates proteins long before the endogenous antioxidants have been depleted in human plasma.[21] In marked contrast, Mn(III)TMPyP efficiently utilized ascorbate as the electron source for the reduction of $ONOO^-$, displaying "peroxynitrite reductase" activity (Figure 2b). The turnover rate of the Mn(III)TMPyP-catalyzed decomposition of $ONOO^-$ in the presence of 1.5 equiv of ascorbate was dependent on the concentration of the catalyst (Figure 2b). The catalytic rate (k_{cat}) was determined to be 2.2×10^6 $M^{-1}s^{-1}$, which is nearly equivalent to the formation rate of oxoMn(IV) by $ONOO^-$.[17] This suggests that oxoMn(IV) formation becomes the rate-limiting step in the catalytic cycle of $ONOO^-$ decomposition when ascorbate is present (see Scheme 1).

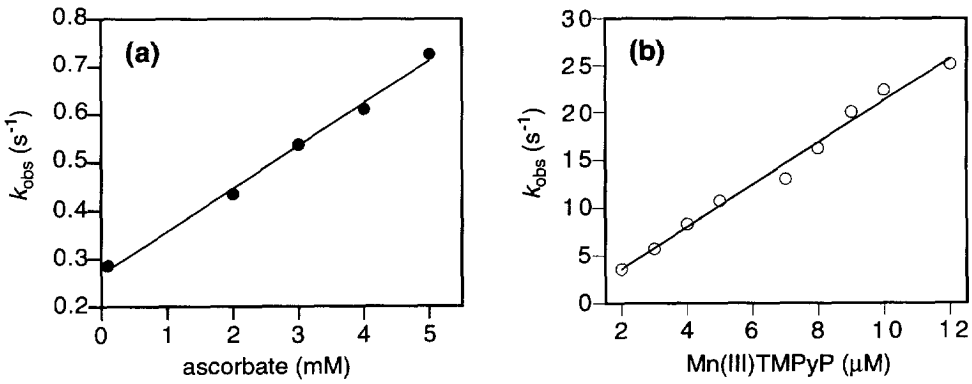


Figure 2. **(a)** Apparent first order rate (k_{obs}) of the reaction between $ONOO^-$ and ascorbate vs the concentration of ascorbate. Linear least-squares fitting of the experimental data yielded a second-order rate constant of 88 $M^{-1}s^{-1}$ ($R = 0.997$); **(b)** $ONOO^-$ decomposition catalyzed by various concentrations of MnTMPyP in the presence of 1.5 equiv of ascorbate.[22] The apparent first order rates correlated linearly with the catalyst concentrations to give $k_{cat} = 2.2 \times 10^6$ $M^{-1}s^{-1}$ ($R = 0.994$).

Mn(III)TMPyP does not catalyze $ONOO^-$ decomposition in the absence of ascorbate because the conversion of oxoMn(IV) back to Mn(III) (see Figure 3a) to complete the catalytic cycle has a first-order rate constant of only 0.018 s^{-1} (k_2 in Scheme 1), much slower than the spontaneous decay of $ONOO^-$, which is 0.35 s^{-1} under these conditions.[23] By contrast, when the oxoMn(IV) intermediate was generated by mixing Mn(III)TMPyP with 1 equiv of HSO_5^- in the first step of a double-mixing experiment, subsequent addition of 1 equiv of ascorbate greatly enhanced the reduction of oxoMn(IV) to Mn(III), as shown in Figure 3b (note the 6000-fold difference in the time axes of Figures 3a and 3b). The kinetic trace monitored at 462 nm shows the return of Mn(III), and the trace monitored at 428 nm shows the reduction of oxoMn(IV). Both nonlinear regression fitting of the trace at 462 nm and second-order kinetic analysis of the trace at 428 nm gave a second-order rate constant of 5.4×10^7 $M^{-1}s^{-1}$ for the reduction of oxoMn(IV) by ascorbate (Figure 3b inset). This rapid reduction results in the "peroxynitrite reductase" activity of MnTMPyP which is only limited by the rate of oxoMn(IV) formation (k_1) in the catalytic cycle (Scheme 1).

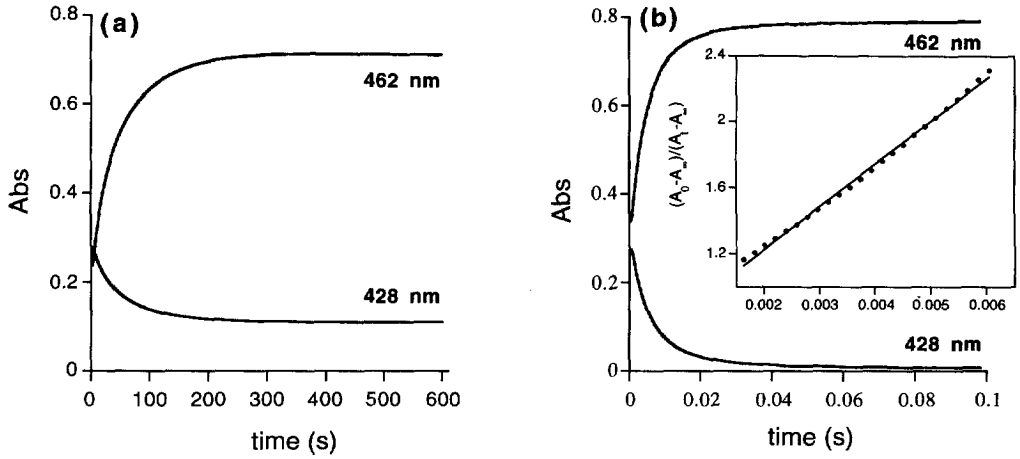


Figure 3. Double-mixing stopped-flow experiments to determine the rate of reduction of the oxoMn(IV) intermediate by ascorbate. Mn(III)TMPyP (10 μM) was first mixed with HSO_5^- (10 μM) to generate the oxoMn(IV); then **(a)** pH 7.4 phosphate buffer or **(b)** ascorbate (10 μM) was added in the second mixing step after a 5 s aging time. **(b) inset**: Second-order analysis in which $(A_0-A_\infty)/(A-A_\infty)$ was plotted against time; linear least-squares fitting of the plot gave a second-order rate constant of 5.4×10^7 $M^{-1}s^{-1}$ ($R = 0.998$).

Glutathione and Trolox® have also been examined as candidates for redox-coupling with Mn(III)TMPyP for the catalytic decomposition of $ONOO^-$. The results are summarized in Table 1. The direct reactions of $ONOO^-$ with glutathione and Trolox® are relatively slow, as in the case of ascorbate. However, both antioxidants rapidly reduced oxoMn(IV) to Mn(III)TMPyP, with second-order rate constants of 1.3×10^5 M^{-1} s^{-1} for glutathione and 7.0×10^6 $M^{-1}s^{-1}$ for Trolox® (Table 1); thus, both of these reducing agents effectively complete the catalytic cycle of $ONOO^-$ decomposition shown in Scheme 1. Interestingly, the rates of oxoMn(IV) reduction by ascorbate, Trolox® and glutathione follow the order of the reduction potentials of these biological antioxidants.[24] Mn(III)TMPyP catalyzed the rapid decomposition of $ONOO^-$ in the presence of physiologically significant concentrations of glutathione (2 mM) and Trolox® (150 μM), with k_{cat} of 3.2×10^6 $M^{-1}s^{-1}$ and 1.1×10^6 $M^{-1}s^{-1}$, respectively. Curiously, though the rate-limiting step in the catalytic decomposition of $ONOO^-$ is the oxoMn(IV) formation with a second order rate constant of 1.8×10^6 $M^{-1}s^{-1}$, k_{cat} for the MnTMPyP-glutathione redox-couple is slightly higher than expected. This may due to the acceleration of oxoMn(IV) formation by the trans-effect if the axial ligand of the manganese porphyrin is the thiolate of glutathione instead of a water molecule. The estimated k_{cat} for the MnTMPyP-Trolox® redox-couple is slower than expected because of the residual interference from the Trolox® phenoxyl radical and quinone interconversion.

Significantly, all three of these Mn(III)TMPyP-antioxidant redox-coupled systems offered complete protection of all-*trans*-retinoic acid (RA) in phospholipid vesicles from $ONOO^-$ oxidation. Addition of 250 μM $ONOO^-$ to 40 μM RA in small unilamellar vesicles resulted in a complete loss of RA chromophore, indicating efficient oxidation. At concentrations as low as 2 μM, Mn(III)TMPyP redox-coupled with 300 μM ascorbate protected 99% of the RA chromophore from $ONOO^-$ oxidation.[25] Further, the Mn(III)TMPyP-antioxidant

Table 1. Kinetic evaluation of MnTMPyP-antioxidant redox-couples for the decomposition of $ONOO^-$.[26]

antioxidant	k_{ONOO^-} [a] ($M^{-1}s^{-1}$)	k_{red} [b] ($M^{-1}s^{-1}$)	k_{cat} ($M^{-1}s^{-1}$)
none	---	$0.018\ s^{-1}$	---
ascorbate	88	5.4×10^7	2.2×10^6
glutathione	580	1.3×10^5	3.2×10^6
Trolox®	33	7.0×10^6	1.1×10^6

[a] k_{ONOO^-} = apparent first-order rate of the reaction of $ONOO^-$ with antioxidant

[b] k_{red} = rate of oxoMn(IV) reduction

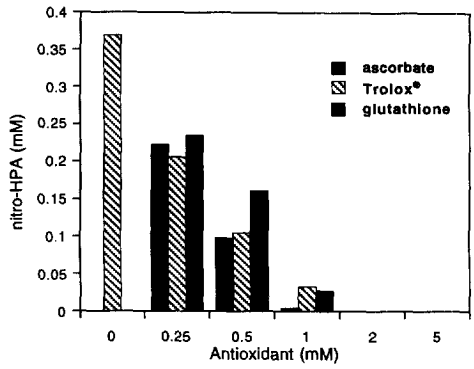


Figure 4. HPLC analysis of nitro-HPA produced in the reactions of 1 mM HPA and 1 mM $ONOO^-$ in the presence of 5 μM Mn(III)TMPyP and various concentrations of biological antioxidants.[27]

redox couples also prevented metal-catalyzed nitration of phenols by $ONOO^-$, as shown in Figure 4. Reaction of 1 mM $ONOO^-$ with 1 mM 4-hydroxyphenylacetic acid (HPA, a model for tyrosine) in the presence of 5 μM Mn(III)TMPyP yielded 37% of 4-hydroxy-3-nitrophenylacetic acid (nitro-HPA). Addition of ascorbate, Trolox®, or glutathione offered a dose-dependent protection of HPA. The presence of two equiv (2 mM) of the antioxidants completely prevented the nitration of HPA by $ONOO^-$. It should be noted that the efficacy of these biological antioxidants in preventing phenol nitration mirrors the trend in their rates of reduction of oxoMn(IV).

We have shown that a water-soluble manganese porphyrin, Mn(III)TMPyP, can become an efficient "peroxynitrite reductase" when redox-coupled with biological antioxidants, though the direct reactions of $ONOO^-$ with these antioxidants are slow. Cells exist in a reducing environment rich in antioxidants, including vitamin C (ascorbate),[28,29] glutathione,[30] and vitamin E (tocopherol);[31,32] thus, the "peroxynitrite reductase" pathway of manganese porphyrins and similar compounds could play an important role in the protection of cells from oxidative stress in $O_2^{-\cdot}$ and $ONOO^-$ related diseases.[33]

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25. Small unilamellar vesicles (SUV) containing 40 μM all-*trans*-retinoic acid (RA) were prepared following the literature procedure (Huang, C. *Biochemistry* **1969**, *8*, 344). The membrane-bound substrate was mixed with 250 μM $ONOO^-$, and the extent of oxidation was evaluated as the decrease in absorbance at 340 nm of the RA chromophore. Protection of RA oxidation was attempted by adding Mn(III)TMPyP (2, 5, and 10 μM) in conjunction with ascorbate (300 μM).
26. The apparent first-order rates of the $ONOO^-$ reaction (k_{ONOO^-}) with glutathione or Trolox® in 25 mM phosphate, pH 7.4, are linearly dependent on the concentrations of glutathione or Trolox® ($R = 0.997$ for glutathione; $R = 0.994$ for Trolox®). In the Trolox® reactions, $ONOO^-$ decay was monitored at 289 nm to avoid the interference from the Trolox® phenoxyl radical and quinone interconversion (289 nm is at the isosbestic point region). The rate of oxoMn(IV) reduction by glutathione was determined by a double-mixing stopped-flow experiment similar to that described for ascorbate. The second-order rate constant was obtained by a linear plot of k_{obs} (s^{-1}) vs glutathione concentrations ($R = 0.999$). The rate of oxoMn(IV) reduction by Trolox® was obtained by a second-order kinetic analysis of the experimental data of a stoichiometric reaction between oxoMn(IV) and Trolox® in a double-mixing stopped-flow experiment analogous to that described for ascorbate (plot of $(A_0-A_\infty)/(A-A_\infty)$ vs time, $R = 0.996$). In the presence of 2 mM glutathione or 150 μM Trolox®, the rate of $ONOO^-$ decomposition is dose-dependent in Mn(III)TMPyP. Linear least-squares fitting of the k_{obs} (s^{-1}) vs MnTMPyP concentrations gave the k_{cat} shown in Table 1 ($R = 0.989$ for glutathione; $R = 0.998$ for Trolox®).
27. Reaction of 1 mM $ONOO^-$ with 1 mM 4-hydroxyphenyl acetic acid (HPA) in the present of 5 μM Mn(III)TMPyP produced 4-hydroxy-3-nitrophenyl acetic acid (nitro-HPA), which was quantitated by reverse phase HPLC analysis (Waters Delta PAK 5 μ C18 300 Å column; gradient of methanol and 5 mM pH 7.4 phosphate buffer (v/v): 10:95 at 0 min, 40:60 at 10 min). Ascorbate, Trolox® or glutathione (0.25, 0.5, 1, 2, 5 mM) was added to the reaction mixtures to prevent the nitration of HPA.
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