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Manganous Phosphate Acts as a Superoxide Dismutase

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A substantial body of evidence indicates that high intracellular concentrations of inorganic manganous ions render some cells resistant to ionizing radiation and provide substantial antioxidant protection to aerobic cells lacking superoxide dismutase (SOD) enzymes.[1−4] However, the chemical mechanisms behind these antioxidant effects are far from clear.[3,5] Early reports suggested that aqueous manganous ion had SOD activity,[6] but these results were later invalidated by the discovery that manganese ions interfered with the cytochrome c SOD assay.[3] Moreover, direct studies by pulse radiolysis established that superoxide reacted rapidly but stoichiometrically with manganous ion to form a short-lived manganous-superoxide transient, MnO_2^+, and that its subsequent reactions could differ depending upon the identity of the anions present.[7]

Seeking to reconcile the results of previous studies and to delineate the possible antioxidant mechanisms of manganous ion that might occur in vivo, we have reinvestigated this system, using two different techniques to generate superoxide in aqueous solution: pulse radiolysis and gamma irradiation using a ^{60}Co source; the conditions of the experiments were designed to be more similar to those found in the cell than those that had been used previously in similar experiments.[7] In all cases, Mn^{2+} was found to react rapidly with superoxide to form the short-lived transient MnO_2^+. In the case of manganous phosphate, MnO_2^+ was formed and then was observed to disproportionate rapidly by a second-order process to give manganous phosphate, dioxygen, and hydrogen peroxide. At physiologically relevant concentrations, only manganous phosphate, not manganous chloride, sulfate, or pyrophosphate, was found to remove superoxide from solution catalytically.

^{60}Co irradiation of an aqueous solution of ethanol produces a low, continuous, relatively clean flux of superoxide, similar to what is predicted for in vivo conditions;[8] however, unlike pulse radiolysis, superoxide cannot be observed directly because of its low concentrations. Therefore, the amount of superoxide formed was quantitated by reaction with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) or (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (XTT),[9] each of which reacts with superoxide in a 2:1 ratio (O_2^-:MTS/XTT) to produce the colored monoformazan (MF) product. For each point in Figure 1, the sample was exposed to the ^{60}Co source for the indicated time (generating 0.45 μM/s O_2^-), and the resulting concentration of MF was measured spectrophotometrically.

The ability of manganous ions to inhibit reduction of MTS by superoxide was measured in the presence of different anions (Figure 1). Under our conditions, in the absence of manganous ions, 85% of the superoxide reacted with MTS to give the soluble MF independent of the nature of the anions present (Figure 1A,B,C). When phosphate (Figure 1A,C) or pyrophosphate (Figure 1B,C)

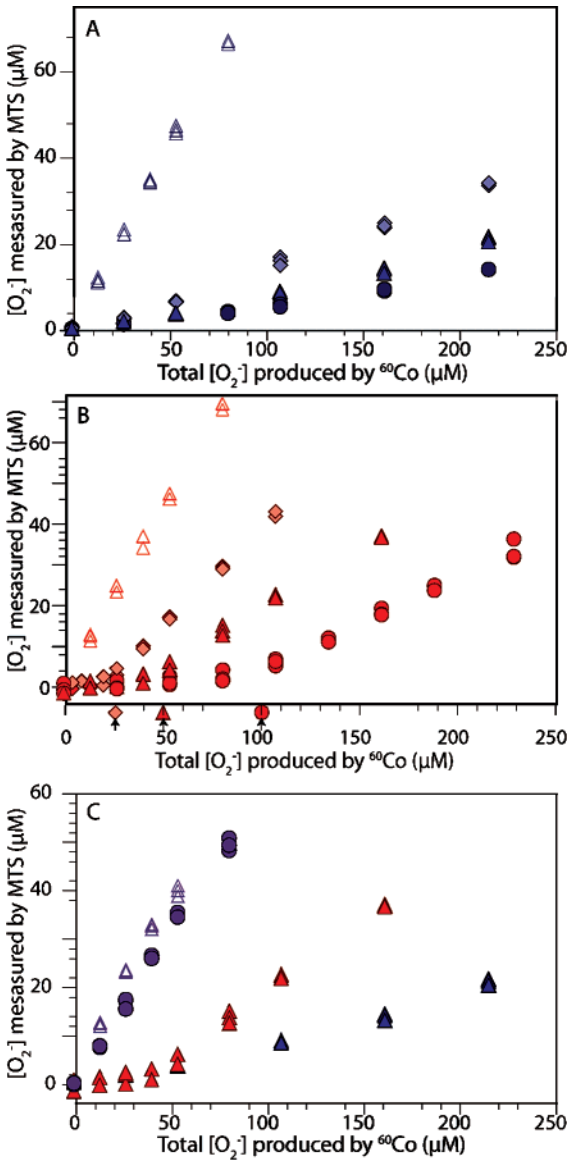

Figure 1. (A) Manganous phosphate catalyzes disappearance of superoxide. Solutions contained 50 mM phosphate and varying concentrations of manganous ion (△, 0 Mn^{2+}; ♦, 25 μM Mn^{2+}; ▲, 50 μM Mn^{2+}; ●, 100 μM Mn^{2+}). (B) Manganous pyrophosphate reacts stoichiometrically with superoxide. Solutions contained 50 mM pyrophosphate and varying concentrations of manganous ion (concentration indicated as in 1A above). Arrows indicate at which point the total superoxide generated is equal to the initial concentration of Mn^{2+}. (C) Manganous phosphate, pyrophosphate, and sulfate show different reactivities with superoxide. For each panel, the color indicates the anion present: blue, 50 mM phosphate; red, 50 mM pyrophosphate; and purple, 50 mM sulfate with 50 μM HEPES. For each panel, superoxide was generated at 0.45 μM/s by ^{60}Co gamma irradiation, and solutions were pH 7, dioxygen saturated, 1 M ethanol, and 150 μM MTS ($\epsilon_{490\ nm}$ 27 500 $M^{-1}cm^{-1}$),[11] and with other components as indicated above.

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was the anion, the presence of manganous ion caused a large decrease in the amount of MF formed, indicating that the superoxide was intercepted and removed from solution before it reacted with MTS. When sulfate was the anion, at concentrations of either 50 mM (Figure 1C) or 500 mM (data not shown), the presence of manganous ion had no effect on the MTS reduction by superoxide. On the basis of the appropriate formation constants for the manganous complex of each anion at pH 7.0, 79% of the manganous ion is bound as $MnHPO_4^-$ at 50 mM phosphate; at least 99% of the manganous ion exists as $MnHP_2O_7^-$ at 50 mM pyrophosphate; and 40% of the Mn^{2+} is bound to sulfate at 50 mM sulfate and 75% at 500 mM.[10]

Varying the concentrations of manganous phosphate and manganous pyrophosphate revealed that their reactions with superoxide were fundamentally different. In phosphate, even with only 25 μM manganous ion present, less than 10% of the 120 μM superoxide generated reacted with MTS, consistent with the catalytic removal of superoxide from the solution (Figure 1A). By contrast, in pyrophosphate, after approximately one equivalent of superoxide was consumed, the rate of reduction of MTS by superoxide increased significantly, indicating that superoxide was no longer being intercepted (Figure 1B). These results are consistent with superoxide reacting irreversibly with manganous pyrophosphate in a stoichiometric fashion at a rate faster than the reaction of superoxide with MTS. Interestingly, the rate of MTS reduction after all of the manganous pyrophosphate was oxidized was not equal to the slope where there is no manganous ion. This may be due to direct oxidation of superoxide by manganic pyrophosphate as proposed by Archibald et al.[6] or to interference by the MTS reagent, wherein the MTS^- radical reduces manganic pyrophosphate. In any case, the reaction becomes much slower after one equivalent of superoxide has reacted with the manganous pyrophosphate (Figure 1B).

To obtain information about the kinetics of these reactions and the nature of the intermediates formed, we turned to pulse radiolysis. Previous studies using this technique demonstrated that manganous pyrophosphate reacts rapidly with superoxide to give a transient that subsequently decays to give manganic pyrophosphate and hydrogen peroxide.[7] These results are entirely consistent with our results (Figure 1B), in which superoxide was not observed to oxidize MTS until all of the manganous pyrophosphate had been oxidized. Our pulse radiolysis studies confirmed the conclusions from our studies using ^{60}Co irradiation that the reactivities of the phosphate and pyrophosphate salts were fundamentally different. This difference was seen most dramatically in the observation that the final product of the reaction of manganous pyrophosphate with superoxide had the characteristic electronic absorption spectrum of manganic pyrophosphate,[7] whereas that of the manganous phosphate was unchanged after reaction with superoxide.

Catalytic disappearance of superoxide was observed in a variety of conditions, ranging from 5 to 50 mM potassium phosphate, 10 to 100 μM $MnSO_4$ and pH 6−8 (data not shown). Further study revealed that superoxide reacted rapidly with manganous phosphate complex to form a MnO_2^+ transient (Figure 2) similar to that previously reported to be formed in pyrophosphate.[7] However, rather than decaying to Mn^{3+}, as was the case when pyrophosphate was the anion present, the transient formed in the presence of phosphate was observed to disproportionate in a second-order process ($k_2 = 8.9 \times 10^6$ $M^{-1}s^{-1}$, at pH 7, 50 mM phosphate, 100 μM Mn^{2+}), regenerating the initial Mn^{2+} ion. The different reactions proposed for manganous phosphate and pyrophosphate are summarized in Scheme 1.

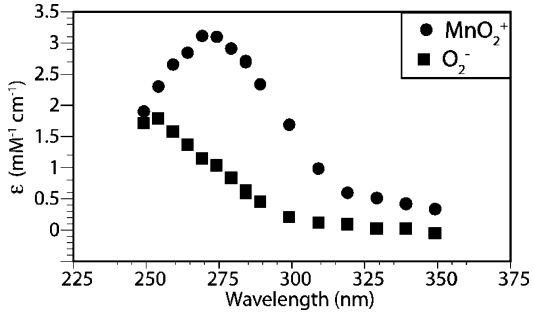


Figure 2. Spectra of MnO_2^+ and superoxide from pulse radiolysis. MnO_2^+ and O_2^- were produced in a low concentration of phosphate (100 μM Mn^{2+}, 10 mM phosphate, pH = 7.4, 10 mM formate, O_2 saturated). Both spectra were produced in a single experiment; the O_2^- spectrum was measured during the time frame where superoxide was formed but had yet to react with Mn^{2+} present, and the MnO_2^+ spectrum was measured after the superoxide had fully reacted with the O_2^-.

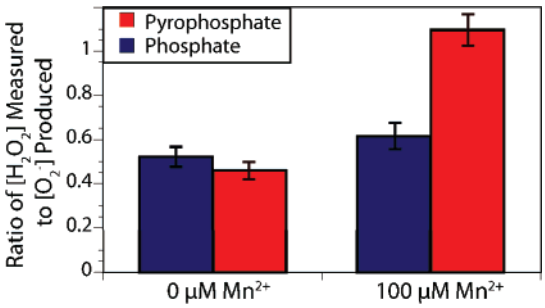
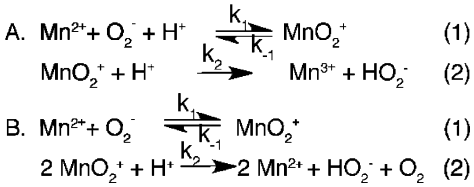


Figure 3. Differences in the yields of hydrogen peroxide formed from superoxide in the presence of manganous phosphate and manganous pyrophosphate. Solutions containing phosphate alone, pyrophosphate alone, and phosphate with Mn^{2+} yield $[H_2O_2]/[O_2^-]$ ratios that are not statistically different, $p > 0.1$; the sample containing pyrophosphate with Mn^{2+} is statistically different from the other samples $p < 0.025$ ($n = 6$). See Supporting Information for methods.

Scheme 1. Proposed Reactions of Manganous Ion with Superoxide in the Presence of (A) Pyrophosphate and (B) Phosphate



The rate of disproportionation of the transient was dependent on phosphate concentration, initial manganese concentration, and pH. Variations in phosphate concentration led to the greatest changes in k_2; low phosphate concentrations (5−50 mM) were necessary to observe catalytic dismutation of superoxide. (A detailed study of the dependence of the reaction rates on phosphate will be reported in a subsequent publication.) The requirement for low phosphate may explain why manganese-mediated disproportionation was not observed in the earlier pulse radiolytic studies, which were carried at much higher (nonphysiological) concentrations of phosphate.[7]

The reactions of superoxide with manganous pyrophosphate and manganous phosphate described in Scheme 1 predict different ratios of hydrogen peroxide produced to superoxide consumed (1:1 for pyrophosphate in Scheme 1A and 1:2 for phosphate in Scheme 1B). To test this prediction, horseradish peroxidase was used to assay H_2O_2 concentrations. Horseradish peroxidase catalyzes the reduction of H_2O_2 to water; when 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is used as the reducing agent, the

brightly colored ABTS radical is formed upon oxidation. Using the ^{60}Co source, we compared the amounts of hydrogen peroxide produced to superoxide generated (Figure 3). In the presence of 100 μM manganous ion, the ratio of peroxide produced to superoxide generated differed depending upon the anion present: in the case of manganous phosphate, the ratio was ~1:2, indicating disproportionation, and for manganous pyrophosphate, it was ~1:1, indicating stoichiometric manganous ion oxidation rather than catalysis.

What gives rise to the dramatic differences in the reactivities of superoxide with manganous sulfate, phosphate, and pyrophosphate? The first step in the reaction appears to be nearly identical in all three cases, that is, reaction of manganous ion with superoxide to form the MnO_2^+ transient (Scheme 1).[7] The forward rate constants are nearly identical, $k_1 = 5.4 \times 10^7$ $M^{-1}s^{-1}$ for sulfate; 2.8×10^7 $M^{-1}s^{-1}$ for phosphate; and 1.7×10^7 $M^{-1}s^{-1}$ for pyrophosphate. Therefore, the differences must arise in the subsequent reactions of the transient, which is differentially stabilized by interaction with the different oxyanions. In the case of sulfate, that anion apparently provides little or no stabilization to the MnO_2^+ transient under our conditions, and the equilibrium constant for its formation is consequently low. In contrast, for both pyrophosphate and phosphate, the corresponding equilibrium constants are considerably larger due to stabilization of MnO_2^+ by either of these two anions. In the case of pyrophosphate, the great stabilization of Mn^{3+} ion by that anion appears to drive the oxidation of manganous to manganic ion. In the case of phosphate, the MnO_2^+ disproportionates. The reactivity of the MnO_2^+−phosphate intermediate thus appears to be similar to the reactivity of protonated superoxide, HO_2, which disproportionates with a bimolecular rate constant of 10^6 $M^{-1}s^{-1}$, whereas no reaction occurs between two O_2^- molecules, the predominant species present at pH 7.[12]

Are manganous phosphate or manganous pyrophosphate reasonable candidates to account for the antioxidant effects of manganous ion in vivo? The typical concentration of manganese found in vivo (0.02 μg Mn/mg protein in *Escherichia coli*; where 1.0 μg Mn/mg protein corresponds to ~2 mM) compared to the high concentrations of manganous ion found in certain cells, such as *Lactobacillus plantarum*, *Deinococcus radiodurans*, and *Neisseria gonorrhoeae* (9, 1.5, and 0.5 μg Mn/mg protein, respectively)[2,3,13] makes this mechanism quite viable. Phosphate is more abundant than pyrophosphate in most cell compartments in vivo (estimates for *E. coli*[14] are 10 mM PO_4^{3-} and 2.5 mM $P_2O_7^{4-}$). But because the catalytic manganese phosphate mechanism depends upon a second-order reaction, the overall disproportionation reaction might be slow at low in vivo concentrations of superoxide. Still, the superoxide generation rate from our ^{60}Co source (0.5 μM/s) is an order of magnitude less than that estimated in aerobic systems, ~5 μM/s.[15] At that in vivo flux of superoxide, the steady-state level of MnO_2^+ was found to be ~0.5 μM by kinetic modeling.[16]

If manganous ion were bound to pyrophosphate, it would react with superoxide to form oxidized manganic pyrophosphate, but no large pool of manganic pyrophosphate has been found in these cells.[1,17] Nevertheless, such a mechanism could be made catalytic in vivo if the manganic ion formed is reduced by cellular reductants such ascorbate, with which it is known to react rapidly.[18] Such a reaction sequence would be analogous to that of the iron-containing superoxide reductase enzymes.[19] To test the feasibility of such a mechanism, we are now examining the reactivity of MnO_2^+, generated in either phosphate or pyrophosphate, with relevant reductants that are expected to be present in the cell where MnO_2^+ would be formed.

In conclusion, we find that manganous phosphate is unique among those manganous salts studied in its ability to remove superoxide rapidly and catalytically from aqueous solution via a disproportionation mechanism that is entirely different from those of the superoxide dismutase enzymes.

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Supporting Information Available: Experimental methods of MTS reduction by ^{60}Co generated superoxide, and peroxide measurements by ABTS. This information is available free of charge via the Internet at http://pubs.acs.org/.

References

(1) Daly, M. J.; Gaidamakova, E. K.; Matrosova, V. Y.; Vasilenko, A.; Zhai, M.; Leapman, R. D.; Lai, B.; Ravel, B.; Li, S. M.; Kemner, K. M.; Fredrickson, J. K. *PLoS Biol.* **2007**, *5*, e92.
(2) Daly, M. J.; Gaidamakova, E. K.; Matrosova, V. Y.; Vasilenko, A.; Zhai, M.; Venkateswaran, A.; Hess, M.; Omelchenko, M. V.; Kostandarithes, H. M.; Makarova, K. S.; Wackett, L. P.; Fredrickson, J. K.; Ghosal, D. *Science* **2004**, *306*, 1025−1028.
(3) Al-Maghrebi, M.; Fridovich, I.; Benov, L. *Arch. Biochem. Biophys.* **2002**, *402*, 104−109.
(4) (a) Chang, E. C.; Kosman, D. J. *J. Biol. Chem.* **1989**, *264*, 12172−12178. (b) Archibald, F. S.; Fridovich, I. *Arch. Biochem. Biophys.* **1982**, *215*, 589−596.
(5) (a) Sanchez, R. J.; Srinivasan, C.; Munroe, W. H.; Wallace, M. A.; Martins, J.; Kao, T. Y.; Le, K.; Gralla, E. B.; Valentine, J. S. *J. Biol. Inorg. Chem.* **2005**, *10*, 913−923. (b) Culotta, V. C.; Yang, M.; Hall, M. D. *Eukaryot Cell* **2005**, *4*, 1159−1165. (c) Lapinskas, P. J.; Cunningham, K. W.; Liu, X. F.; Fink, G. R.; Culotta, V. C. *Mol. Cell. Biol.* **1995**, *15*, 1382−1388. (d) Archibald, F. S.; Fridovich, I. *J. Bacteriol.* **1981**, *146*, 928−936.
(6) Archibald, F. S.; Fridovich, I. *Arch. Biochem. Biophys.* **1982**, *214*, 452−463.
(7) (a) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1984**, *88*, 6291−6294. (b) Cabelli, D. E.; Bielski, B. H. J. *J. Phys. Chem.* **1984**, *88*, 3111−3115.
(8) Buxton, G. In *Radiation Chemistry: Principles and Applications*; Rodgers, F. a. M., Ed.; VCH Pub., Inc.: New York, 1987; pp 321−349.
(9) Sutherland, M. W.; Learmonth, B. *Free Rad. Res.* **1997**, *27*, 283−289.
(10) Müller, B. M. *ChemEQL*; http://www.eawag.ch/research_e/surf/Researchgroups/sensors_and_analytic/chemeql.html, version 3.0, 2004; 2006.
(11) Barltrop, J. A.; Owen, T. C.; Cory, A. H.; Cory, J. G. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 611−614.
(12) Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. *J. Phys. Chem. Ref. Data* **1985**, *14*, 1041−1100.
(13) (a) Archibald, F. S.; Duong, M. N. *J. Bacteriol.* **1984**, *158*, 1−8. (b) Tseng, H.-J.; Srikhanta, Y.; McEwan, A. G.; Jennings, M. P. *Mol. Microbiol.* **2001**, *40*, 1175−1186.
(14) Wanner, B. C. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*; Neidhardt, F. C., Ed.; ASM Press: Washington D.C., 1996; Vol. 1, pp 1357−1381.
(15) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals In Biology and Medicine*, 4th ed.; Oxford University Press: Oxford, 2007.
(16) Ianni, J. C. *Kintecus*, http://www.kintecus.com/, version 3.9, 2006.
(17) Gunter, K. K.; Aschner, M.; Miller, L. M.; Eliseev, R.; Salter, J.; Anderson, K.; Gunter, T. E. *Neurobiol. Aging* **2006**, *27*, 1816−1826.
(18) Bielski, B. H. J. *Philos. Trans. R. Soc. London, B* **1985**, *311*, 473−482.
(19) Pereira, A. S.; Tavares, P.; Folgosa, F.; Almeida, R. M.; Moura, I.; Moura, J. J. G. *Eur. J. Inorg. Chem.* **2007**, 2569−2581.

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