

Multicopper oxidase involvement in both Mn(II) and Mn(III) oxidation during bacterial formation of MnO₂

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Abstract Global cycling of environmental manganese requires catalysis by bacteria and fungi for MnO₂ formation, since abiotic Mn(II) oxidation is slow under ambient conditions. Genetic evidence from several bacteria indicates that multicopper oxidases (MCOs) are required for MnO₂ formation. However, MCOs catalyze one-electron oxidations, whereas the conversion of Mn(II) to MnO₂ is a two-electron process. Trapping experiments with pyrophosphate (PP), a Mn(III) chelator, have demonstrated that Mn(III) is an intermediate in Mn(II) oxidation when mediated by exosporium from the Mn-oxidizing bacterium *Bacillus* SG-1. The reaction of Mn(II) depends on O₂ and is inhibited by azide, consistent with MCO catalysis. We show that the subsequent conversion of Mn(III) to MnO₂ also depends on O₂ and is inhibited by azide. Thus, both oxidation steps appear to be MCO-mediated, likely by the same enzyme, which is indicated by genetic evidence to be the MnxG gene product. We propose a model of how the manganese oxidase active site may be organized to couple

successive electron transfers to the formation of polynuclear Mn(IV) complexes as precursors to MnO₂ formation.

Keywords Manganese oxidation · Biogenic Mn oxides · Multicopper oxidase · *Bacillus* sp. SG-1

Abbreviations

| | |
|-------|--|
| HEPES | 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid |
| MCO | Multicopper oxidase |
| PP | Pyrophosphate |
| TEM | Transmission electron microscopy |

Introduction

The redox cycling of manganese is a key chemical process in the natural environment. MnO₂ is widespread in soils and sediments, and serves as an electron sink for microbial metabolism when O₂ and nitrate are absent. In addition, MnO₂ adsorbs other metal ions avidly [1, 2], including pollutants like Pb²⁺, and thereby plays a role in controlling their mobility in the environment.

MnO₂ and Mn(II) are the thermodynamically stable forms of manganese under aerobic and anaerobic conditions, respectively, but the uncatalyzed oxidation of Mn(II) is very slow [3, 4]. The stability of its half-filled (*d*⁵) electronic shell makes the extraction of an electron from Mn(II) a highly endothermic process ($E_{\text{Mn}^{3+}/\text{Mn}^{2+}}^0 \sim 1.5 \text{ V}$) unless strong chelating agents are available to stabilize the Mn(III). The lattice energy of MnO₂ drives the two-electron oxidation, but the high initial barrier to electron transfer greatly slows the process.

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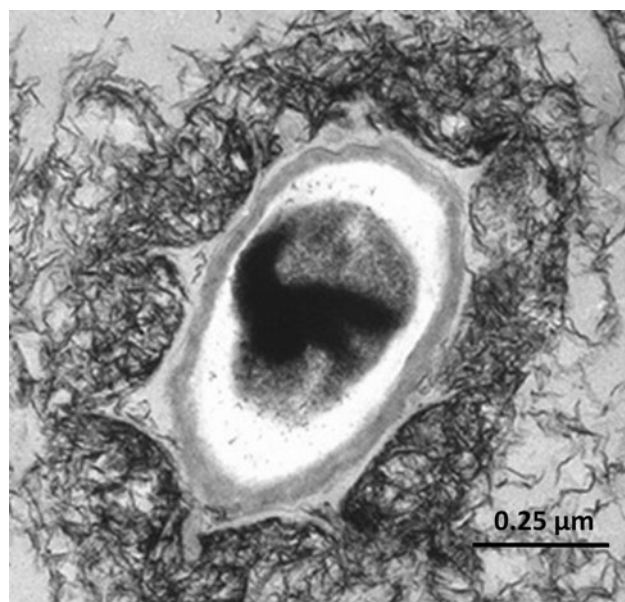


Fig. 1 TEM of MnO₂-coated spores from *Bacillus* sp. SG-1 [9]

However, a variety of bacteria and fungi can catalyze the conversion of Mn(II) to MnO₂, increasing the rate by three to five orders of magnitude [2, 5]. Thus, most of the MnO₂ present in the environment has been processed by these organisms. Why they do so is uncertain. A number of functions have been suggested [2, 6], including protection from toxic metals, or from UV light, or from predation or viruses. MnO₂ can also break down natural organic matter into metabolizable substrates, thereby enhancing the bacterial food source [7]. An intriguing possibility is that the MnO₂ deposits that bacteria coat themselves in (Fig. 1) may serve as a reservoir of Mn(II), which can protect them from oxidative damage [2, 6]. A combination of benefits may have driven the evolution of Mn-oxidizing ability in these organisms.

Of considerable bioinorganic interest is the finding that the enzymes responsible for bacterial MnO₂ formation are likely to be multicopper oxidases (MCOs). In three well-studied bacteria, Mn-oxidizing ability is lost upon the disruption of a gene whose sequence identifies it as a MCO [8–11]. Cell homogenates of these and related organisms display Mn-oxidizing protein bands on acrylamide gels [12–14]; in one case, the sequence analysis of peptides derived from the band confirmed that the protein is a MCO gene product [15]. In another bacterium, antibodies raised to the protein in a Mn-oxidizing band were found to cross-react with a MCO gene product from an expression library [16].

However, the finding that bacterial Mn oxidases are likely to be MCOs raises a mechanistic conundrum. MCOs are one-electron catalysts. How can they catalyze a two-electron oxidation? In MCOs, single electrons are

transferred from substrates to the type 1 Cu, from which they are relayed to O₂ at the type 3 Cu site [17]. Thus, Mn(III), not MnO₂, should be the product of MCO-mediated Mn(II) oxidation. Indeed, fungal laccase has been shown to convert Mn(II) to Mn(III) in the presence of the strong Mn(III) chelator pyrophosphate (PP) [18, 19].

Mn oxidases have yet to be purified in amounts sufficient to permit mechanistic studies. However, Webb et al. [20] utilized a Mn-oxidizing preparation of exosporium (the loose outer part of the spore coat) from *Bacillus* sp. SG-1 [9, 13] to show, via PP trapping, that Mn(III) is an intermediate in the production of MnO₂. In this bacterium, disruption of the MCO gene *MnxG* abolishes Mn-oxidizing activity [9]. Following the addition of Mn(II) and PP to the exosporium, the concentration of Mn(III)PP rose to a peak and then fell again, while MnO₂ particles were observed to form. In addition, exogenous Mn(III)PP was shown to be a substrate for the exosporium, the Mn(III)PP concentration falling at about the same rate when Mn(III) instead of Mn(II) was added to the exosporium in the presence of PP. Thus, MnO₂ formation occurred in two steps, both of which required exosporium. Mn(II) oxidation required O₂, as expected, and was abolished by azide, a known inhibitor of MCOs [21, 22]. However, since the protein organization of exosporium is unknown, it was unclear whether both oxidation steps were MCO mediated or whether only the initial oxidation of Mn(II) was. Conceivably, Mn(III) oxidation to MnO₂ took place at a different site, either through further reaction with O₂ or via a disproportionation mechanism. The present study was undertaken to answer this question and to consider how the second step of the two-step oxidation might proceed.

Methods

UV–vis absorption spectra were monitored with an Agilent (Santa Clara, CA, USA) 8453 UV–vis spectrophotometer using a thermostatable multicell transport configuration with an automated kinetic scan capability. Spectra were recorded at room temperature every 15 min during the 24–36 h, using a 10 mm path length cuvette with a magnetic stirrer. The samples were stirred continuously during the UV–vis time courses using a Spinette cell stirrer (Starna Cells, Atascadero, CA, USA). Typically, several reaction mixtures and a control were monitored in a parallel configuration.

Bacillus sp. SG-1 spores were harvested [23] and exosporium was prepared as previously described [13]. All experiments were carried out at pH 7.5 in a 10 mM HEPES/50 mM NaCl buffer. For the UV–vis absorption time courses, 1 mL samples contained 50 μL aliquots of exosporium suspension, 0.1 mM Mn(II) chloride or

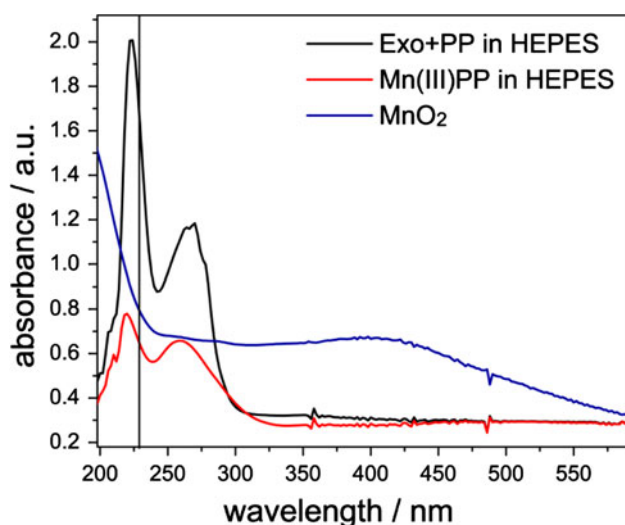


Fig. 2 UV-vis absorption spectra of exosporium (50 μL in 1 mL of HEPES buffer) (*black*), Mn(III)-pyrophosphate complex (100 μM in HEPES buffer) (*red*), and MnO_2 suspension in water (*blue*), all of which were used as basis spectra for a linear squares fit analysis of the UV-vis absorption spectra taken over 30 h of experiments investigating Mn oxidation catalyzed by exosporium

Mn(III) acetate and 500 μM NaPP, added as a trapping agent for the Mn(III) ions. For experiments performed under anaerobic conditions, O_2 was eliminated from the reaction mixture by continuously blowing Ar over the solution for 1 h; after that, the cuvette was sealed and transferred to the spectrophotometer. For inhibition experiments, sodium azide was added to the reaction mixture at a concentration of 10 mM.

Time-dependent absorption spectra were modeled with pure component spectra using a linear least squares fit. Three basis spectra were used (Fig. 2): (1) exosporium (50 μL in 1 mL of HEPES), (2) Mn(III)-PP (0.1 mM in HEPES), and (3) MnO_2 in water (obtained as the supernatant after centrifuging a “Mn(II) + PP + exosporium” mixture that had reacted for 26 h). The 231–650 nm spectral region was used for fitting. The exosporium component was kept fixed during the fitting.

Results

The experiments were much the same as those reported by Webb et al. [20], with the crucial addition of experiments to test the effects of oxygen exclusion and azide inhibition on Mn(III) oxidation. Also, instead of simply monitoring the Mn(III)PP absorption intensity at 258 nm, we performed a full analysis of the absorption spectra by fitting them to three-component spectra (Fig. 2): (1) exosporium in HEPES buffer plus PP, (2) Mn(III)PP, and (3) MnO_2 . A fourth component, Mn(II)PP, was present in the reaction

mixtures, but contributed negligibly to the spectra at the concentrations in use. Since exosporium + PP is unaltered throughout the course of the reaction, the first component was held constant when fitting the Mn(III)PP and MnO_2 contributions.

Exosporium and Mn(III)PP both have absorption bands at 220 and 255–260 nm, but their shapes and relative intensities differ, allowing for discrimination between them. The spectrum of MnO_2 is variable; it depends on particle size. We took the product spectrum to be that of the MnO_2 colloid in the reaction mixture supernatant after 26 h of incubation. Beyond this time, the absorption spectrum of the reaction mixture did not change significantly, except for alterations due to aging of the MnO_2 , which formed larger particles. The MnO_2 colloid displays broad UV-vis absorption, with a weak maximum at ~ 420 nm (Fig. 2).

Exosporium converts Mn(II) to Mn(III) and then MnO_2

When Mn(II) was added to exosporium in the presence of excess PP, the absorption spectra evolved with time (Fig. 3a), showing increased absorption in both the UV and visible regions. Component fitting revealed a rise and fall in the Mn(III)PP fraction, with a maximum at around 10 h (Fig. 3b). In addition, we see that MnO_2 production commenced after only 10 h, when Mn(III)PP had reached its peak. The MnO_2 fraction then rose steadily as the Mn(III)PP fraction fell. Thus, there is a clear two-stage reaction sequence, with Mn(II)PP first being converted to Mn(III)PP and then to MnO_2 .

When Mn(III) was added to exosporium instead of Mn(II), the absorption spectra showed little change in the UV region and a rise in the visible region (Fig. 4a). Component fitting (Fig. 4b) revealed a steady decline in the Mn(III)PP fraction, while the MnO_2 fraction rose from the beginning. Thus, the second stage in the reaction sequence proceeds independently of the first.

Eliminating O_2 or adding azide blocks the oxidation of both Mn(II) and Mn(III)

When O_2 was eliminated from the reaction mixture (see the “Methods” section), no reaction with Mn(II) was observed. Likewise, there was no reaction when 10 mM sodium azide was added in the presence of O_2 (data not shown). In both cases, the Mn(II) + exosporium UV-vis absorption spectrum remained essentially unchanged. Crucially, there was likewise no reaction of Mn(III) if the reaction mixture was de-aerated (Fig. 5a) or if 10 mM sodium azide was added (Fig. 5b). Again, the initial UV-vis absorption spectrum remained essentially unchanged (Fig. 5) over the time course of the reaction with O_2 (Fig. 4).

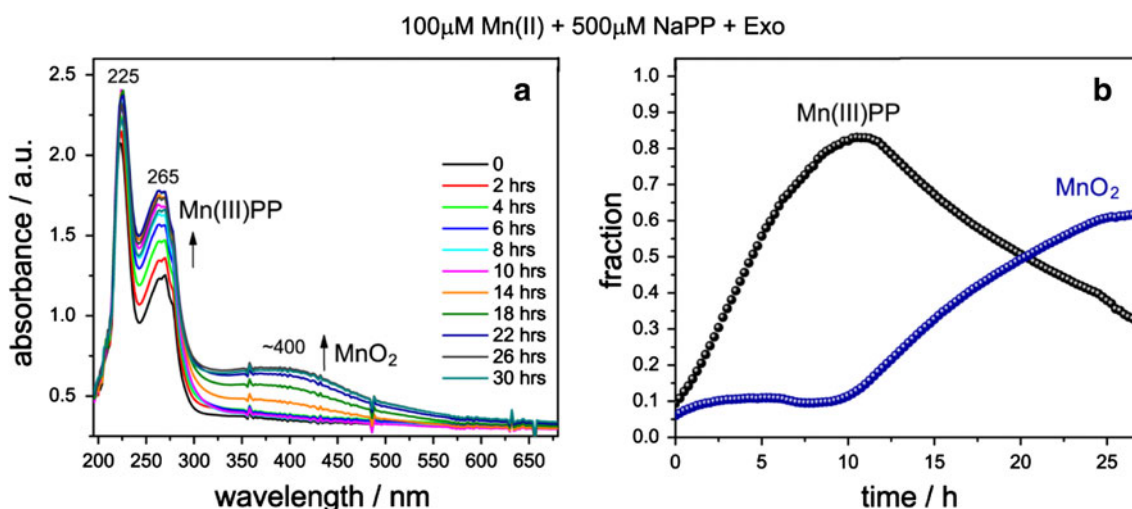


Fig. 3 Time-resolved UV–vis absorption spectral measurements of Mn(II) oxidation by exosporium at room temperature. **a** Selected UV–vis absorption spectra of the reaction mixture taken at the indicated time points during the course of Mn(II) oxidation. The growth of the 265 nm band indicates the formation of the Mn(III) intermediate trapped by pyrophosphate; the 400 nm band is due to the formation of

particulate Mn oxides. **b** Time courses followed by Mn(III)–pyrophosphate and MnO₂ species during the 30 h UV–vis absorption experiment and obtained from the fit of the absorption spectrum at each time point to a linear combination of the component spectra depicted in Fig. 2

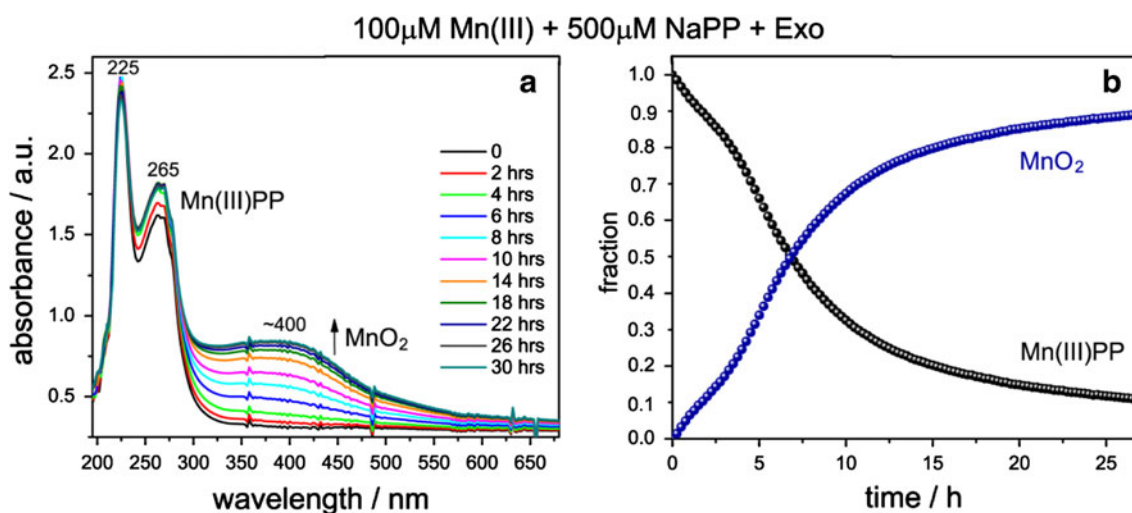


Fig. 4 Time-resolved UV–vis absorption spectral measurements of Mn(III) oxidation by exosporium at room temperature. **a** Selected UV–vis absorption spectra of the reaction mixture taken at the indicated time points during Mn(III) oxidation. The growth of the 400 nm band indicates the formation of the particulate Mn oxides.

b Time courses followed by Mn(III)–pyrophosphate and MnO₂ species during the 30 h UV–vis absorption experiment and obtained from the fit of the absorption spectrum at each time point to a linear combination of the component spectra depicted in Fig. 2

Discussion

Our results corroborate those of Webb et al. [20], showing that the oxidation of Mn(II) by SG-1 exosporium produces chelatable Mn(III), and that this reaction requires O₂ and is blocked by azide. These results are consistent with Mn(II) oxidation by a MCO, since MCOs couple O₂ reduction to the one-electron oxidation of substrates, and are inhibited by azide binding. These findings corroborate the genetic

and biochemical evidence that the manganese oxidase in SG-1 exosporium, MnxG, is an MCO.

We also confirm the observation [20] that in the presence of exosporium, chelated Mn(III) is further converted to MnO₂. However, it was uncertain whether this step was also catalyzed by an MCO, since purified enzyme is unavailable. The exosporium might have harbored separate enzymes for Mn(II) oxidation and MnO₂ production. Moreover, it was uncertain whether MnO₂ production

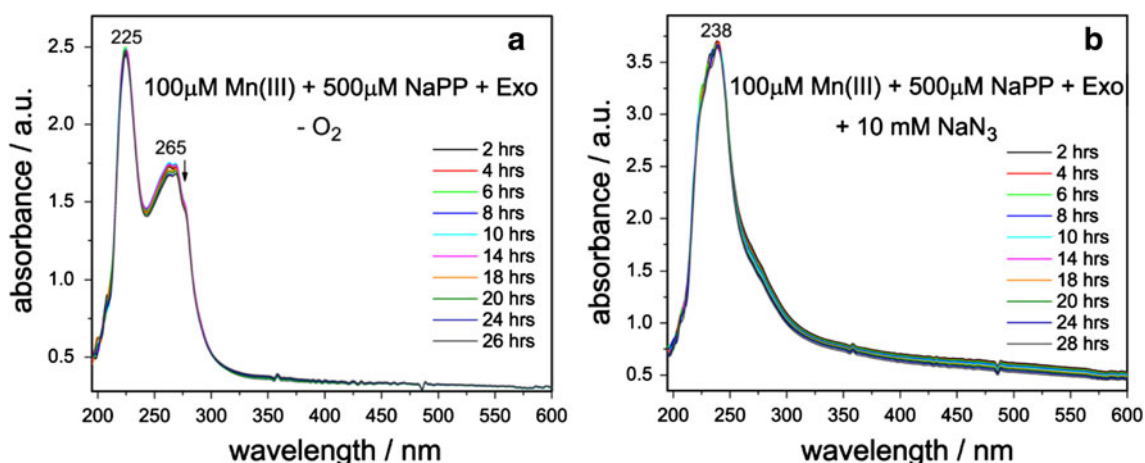


Fig. 5 UV-vis absorption spectra of the reaction mixture taken at the indicated time points during Mn(III) oxidation by exosporium **a** under anaerobic conditions and **b** with 10 mM azide present. Both

conditions—the exclusion of oxygen and the addition of azide—resulted in the inhibition of Mn(III) oxidation

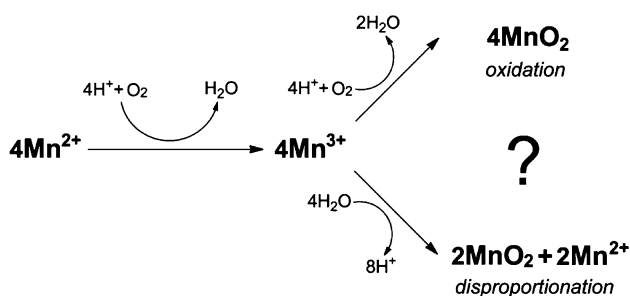


Fig. 6 Possible pathways for bacterial MnO₂ formation by MCO-catalyzed Mn(II) to Mn(III) oxidation followed by further oxidation (*top*) or by disproportionation (*bottom*) of complexed Mn(III)

involved a one-electron oxidation of Mn(III). It might instead have resulted from enzyme-catalyzed disproportionation of Mn(III) followed by re-oxidation of the resulting Mn(II). The possible pathways are illustrated in Fig. 6.

The present experiments were designed to address these issues. If MnO₂ production resulted from disproportionation, it should have proceeded in the absence of O₂, although its production would have been cut in half because the Mn(II) produced in equal amounts by disproportionation would not have been reoxidized. However, de-aeration completely blocked MnO₂ production from Mn(III) as well as Mn(II). Thus, Mn(III) conversion to MnO₂ requires O₂.

There remained the question of whether the oxidations of Mn(II) and of Mn(III) are catalyzed by the same or different enzymes. The observed inhibition by azide is consistent with an MCO being involved with Mn(III) as well as with Mn(II) oxidation. It is possible that azide acts at other enzyme sites than the MCO trinuclear Cu site (known to be the site of MCO inhibition [22, 24–26]).

Azide is an inhibitor of manganese catalase [27], whose active site is a dinuclear Mn(III) complex; such a complex is a likely intermediate in Mn(III) oxidation (see below). Azide could bind this intermediate and thereby inhibit oxidation. However, this intermediate is likely formed within the same protein as the MCO Cu centers. The genetic evidence points to MnxG as a single protein associated with MnO₂ formation, and a single native gel protein band converts Mn(II) to MnO₂ [9, 15].

If a single MCO is responsible for both steps of the reaction, how might its active site be organized? The initial reaction, Mn(II) conversion to Mn(III), is straightforward enough. MCO-catalyzed Mn(II) oxidation has previously been observed in Mn(III) trapping experiments with a laccase enzyme [18, 19], and the mechanism is likely similar to MCO-catalyzed Fe(II) oxidation by human ceruloplasmin [28–30] or yeast Fet3p [21, 30–32], or Cu(I) oxidation by CueO from *E. coli* [33, 34]. Crystallographic studies [28] of the ferroxidase MCO, ceruloplasmin, indicate that Fe(II) binds to a site adjacent to the type 1 Cu, and that, after electron transfer, the product Fe(III) moves several angstroms toward the solvent interface, to a “holding site” (Fig. 7). It seems likely that a similar translocation of Mn(III) in MnxG would provide ready access to chelating agents, accounting for the facile production of Mn(III)PP in solution.

However, MnO₂ production from Mn(III) has no precedent in MCO chemistry. Oxidation to mononuclear Mn(IV) is improbable, since Mn(IV) is a powerful oxidant, and it cannot be stabilized by the relatively weak donor ligands available from protein side chains; these are likely to be carboxylates, as seen in ceruloplasmin [28]. In the absence of strong donor ligands (e.g., porphyrin [35, 36]), known Mn(IV) complexes are polynuclear, and are

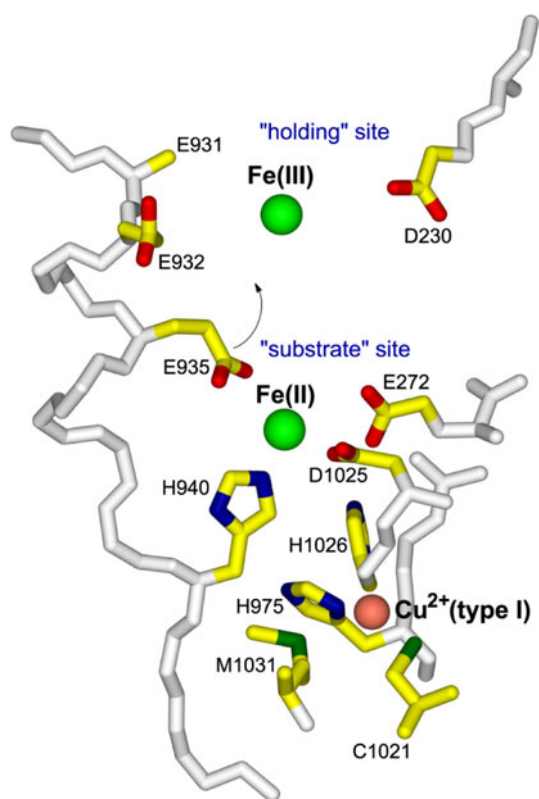


Fig. 7 The structure of human ceruloplasmin (pdb #:1KCW), showing a mononuclear type I copper site and “substrate” and “holding” cation-binding sites in domain 6. *Arrow* indicates a movement of sidechain E935 from the substrate site to the holding site upon iron translocation after oxidation. Adapted from [28]

stabilized by oxide bridges [37–39]. We speculate that the MnxG holding site for Mn(III) might accommodate multiple Mn(III) ions, which could then form polynuclear Mn(IV) complexes upon electron transfer. Binuclear binding sites are common in metalloproteins. A pertinent example is Mn catalase [27, 38, 40], in which a pair of Mn ions cycle between Mn(II) and Mn(III) oxidation states during enzymatic turnover. (A “superoxidized” form of the enzyme contains Mn(III) and Mn(IV), but is not in the catalytic cycle [40, 41]).

However, if the trivalent ion holding site is displaced from the type I Cu, as in ceruloplasmin, then direct electron transfer from Mn(III) to Cu is unlikely. A possible pathway that circumvents this difficulty is sketched in Fig. 8. In this model, a binuclear site is envisioned adjacent to the holding site. If two Mn(III) ions fill the binuclear site, then a third Mn(III) in the holding site could serve as an electron shuttle. It could abstract an electron from the binuclear site then migrate back to the Mn(II) site and deliver its electron to the type I Cu, permitting successive oxidations by O₂. The driving force for electron abstraction from the binuclear Mn(III) complex would be the formation of oxide bridges from coordinated water or hydroxide

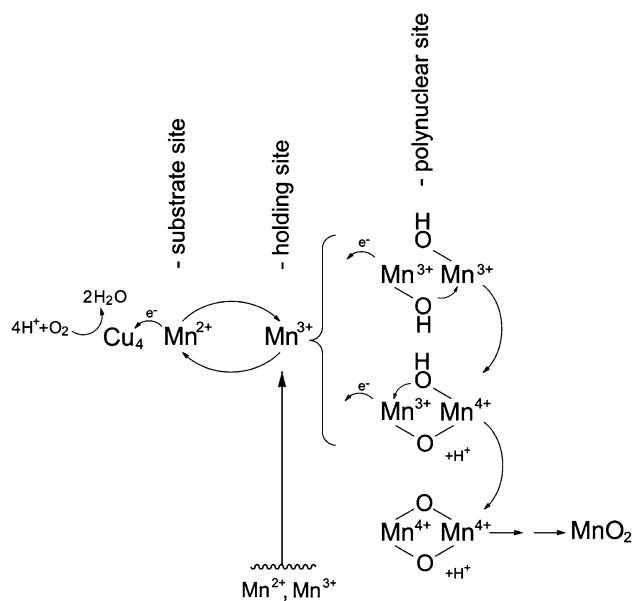


Fig. 8 Proposed mechanism of Mn(II) oxidation and MnO₂ formation catalyzed by manganese oxidase, MnxG (see text for details)

ions, concerted with Mn(IV) formation. Two electron shuttle rounds would produce a [Mn(IV)₂O₂]⁴⁺ unit, likely bound to carboxylate side chains, and primed to nucleate MnO₂ formation. (A binuclear complex is the minimum structure for oxide bridge formation. Higher-nuclearity complexes are possible. There are many synthetic examples of tri- and tetranuclear Mn complexes [37, 42–44], and the water oxidation complex of photosystem II contains four Mn ions [37, 43, 45, 46]).

A precedent for how oxide mineral nucleation may proceed is offered by ferritin, the iron storage protein [47, 48]. In ferritin, a hollow protein shell is filled with Fe₂O₃ after the oxidation of Fe(II) at a binuclear ferroxidase site. Recent NMR experiments have revealed details of how the binuclear Fe(III) intermediates that are formed at the ferroxidase site migrate along protein channels to nucleation sites within the core [49]. While bacterially produced MnO₂ is not confined to a protein core, it is likely that manganese oxidases likewise offer pathways for polynuclear Mn(IV) complexes to migrate toward nucleation sites.

In this proposed mechanism, Mn can be delivered to the enzymes as either Mn(II) or as Mn(III). A conceptual issue is that the Mn(III) oxidation step is actually a disproportionation, with conversion to Mn(IV) being accompanied by Mn(II) production. Why then is MnO₂ not produced from exogenous Mn(III) in the absence of oxygen? The simplest explanation would be that access to the substrate site is restricted to the channel connecting it to the holding site. Mn(II) could enter through this channel, but would be unable to migrate back to solution once the holding site contains Mn(III). Thus, the disproportionation reaction

would be blocked unless the resulting Mn(II) is reoxidized by O₂.

An alternative possibility is that Mn(III) oxidation involves direct electron transfer to O₂. If O₂ could react directly with the putative binuclear Mn(III) intermediate, the products would be [Mn(IV)₂O₂]⁴⁺ and peroxide; the peroxide would additionally oxidize Mn(III). This scenario seems unlikely, since Mn(III) is normally unreactive with O₂. Literature reports of Mn(III) oxidation by O₂ have involved complexes with electron-rich organic ligands [50–53], unlike the potential ligands available in the protein. On the other hand, a mass-spectrometric oxygen isotope study [54] has indicated that about half the O atoms in bacterially produced MnO₂ are derived from molecular O₂. This observation is inconsistent with a purely MCO mechanism, since MCOs reduce O₂ to water without releasing intermediates. The present results do not rule out a direct reaction of Mn(III) with O₂, since, as noted above, azide inhibition may result from complexation at the Mn(III) site, as well as at the MCO trinuclear Cu center. Resolution of this important issue will require further experiments to distinguish between the reactivities at the Cu and putative Mn(III) sites. Efforts are underway to produce purified MnxG in order to clarify the features of this intriguing enzyme.

Conclusions

The bacterial oxidation of Mn(II), a process central to the global manganese cycle, appears to be controlled by manganese oxidases that are MCOs. Since MCOs are one-electron oxidants, this finding raises the issue of how they can catalyze the two-electron transformation of Mn(II) to MnO₂. Trapping experiments with PP and exosporium from the Mn-oxidizing *Bacillus* sp. SG-1 have shown that Mn(III) is the initial oxidation product, consistent with its oxidase being an MCO. However, it has not been clear how the subsequent conversion of Mn(III) to MnO₂ is catalyzed.

The present experiments show that this conversion requires O₂ and is inhibited by azide. This finding is consistent with the MCO catalysis of Mn(III) as well as Mn(II) oxidation. We present a model of how the MnxG active site might be organized to couple multiple electron transfers to the formation of MnO₂-precursor polynuclear Mn(IV) complexes via a Mn(II)/Mn(III) shuttle.

However, our results do not rule out an alternative possibility: that O₂ might react directly with a putative polynuclear Mn(III) complex, a process that might also be inhibited by azide. While such a reaction seems chemically unlikely, it would explain the otherwise puzzling observation that O₂ is incorporated into bacterially produced

MnO₂. Further experiments, hopefully with purified protein, will be needed to distinguish these possibilities.

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