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Magnetic susceptibility of Mn(III) complexes of hydroxamate siderophores

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ABSTRACT: The hydroxamate siderophores putrebactin, desferrioxamine B, and desferrioxamine E bind Mn(II) and promote the air oxidation of Mn(II) to Mn(III) at pH >7.1. The magnetic susceptibility of the manganese complexes were determined by the Evans method and the stoichiometry was probed with electrospray ionization mass spectrometry (ESIMS). The room temperature magnetic moments (μ_{eff}) for the manganese complexes of desferrioxamines B and E were 4.85 BM and 4.84 BM, respectively, consistent with a high spin, d^4 , Mn(III) electronic configuration. The manganese complex of putrebactin had a magnetic moment of 4.98 BM, consistent with incomplete oxidation of Mn(II), as confirmed by X band EPR spectroscopy. Mass spectra of the Mn(III) desferrioxamine B and E complexes showed complexes at m/z 613.26 and 653.26, respectively, consistent with 1:1 complexation. Mass spectral peaks for manganese putrebactin at m/z 797.31 and 1221.41 corresponds to 1:2 and 2:3 Mn:putrebactin complexation. This study directly confirms the Mn(III) oxidation state in hydroxamate siderophore complexes.

1. Introduction

Siderophores are low molecular weight chelating agents produced by microbes in low iron environments to facilitate iron uptake [1]. In addition to Fe(III), siderophores have been shown to chelate other hard metal ions, such as Al(III) [2], Ga(III) [2], Mo(VI) [3], Ti(IV) [4], and V(V) [3, 5, 6], among others. Manganese is of particular interest because it is often found in the environment in oxide minerals. In a similar fashion to siderophore-mediated extraction of Fe(III) from iron oxide minerals, siderophores have been shown to dissolve manganite (γ -MnOOH) and hausmannite (Mn_3O_4) through pH-dependent processes [7-9]. Thus siderophores may be involved in biogenic cycling of manganese [10-12]. Mn(III) is not stable in aqueous solutions and readily disproportionates to Mn(II) and a variety of Mn(IV) oxides and hydroxides [13]. However Mn(III) can form stable complexes in aqueous solutions with oxygen donating ligands, including hydroxamates [14, 15].

Mn(III) complexes of the linear and cyclic tris-hydroxamate siderophores, desferrioxamine B (DFOB) and desferrioxamine E (DFOE), respectively, were first investigated as mimics of superoxide dismutase [16-18]. Upon complexation of Mn(II) by DFOB or DFOE, air oxidation of Mn(II) under basic conditions (pH 8.5) produced a clear green solution [19], similar to the complex formed on addition of $\text{Mn}(\text{OH})_3$ to DFOB and DFOE, as well as on addition of MnO_2 to DFOB and DFOE in the presence of a reductant [16]. Duckworth and Sposito found that the rate of oxidation of Mn(II)-DFOB by O_2 is fastest in the fully deprotonated complex [7, 19]. They also found that the proton-independent stability constant of Mn(III)-DFOB is remarkably high, at $\log K$ 29.9 ($\pm$ 0.5) [19], and approaches that of Fe(III)-DFOB at $\log K$ 30.5 [20].

To further characterize the Mn(III) complexes of hydroxamate siderophores, we have measured the magnetic susceptibility of the air-oxidized Mn(II)-siderophore complexes to directly probe the Mn(III) oxidation state in aqueous solution, using the Evans NMR technique [21-23]. EPR spectroscopy was also employed to detect if any Mn(II) remained. We selected three structural hydroxamate archetypes for this study: the linear and cyclic *tris*-hydroxamate siderophores, DFOB, DFOE, and the cyclic *bis*-hydroxamate siderophore, putrebactin (Figure 1). Comparison of the *tris*-hydroxamate to *bis*-hydroxamate siderophores is of particular interest due to differences expected in the stoichiometry of coordination, analogous to the Fe(III)-siderophore complexes [24-27]. Possible coordination complexes of Mn(III) and putrebactin could include the neutral bridged 2:3 Mn-putrebactin or 1:2 Mn-putrebactin complexes shown in Figure 2, as well as multiple 1:1 Mn-putrebactin aqua or hydroxo complexes, or possibly 2:2 Mn-putrebactin μ -oxo or μ -hydroxo dimers (Figure 2).

Figure 1.

Figure 2.

2. Experimental

2.1. General experimental procedures. All UV-visible (UV-Vis) spectrophotometry was carried out on a Varian Cary-Bio 300 UV-Vis spectrophotometer. ^1H NMR spectra were recorded on a Varian Unity Inova 400 MHz spectrometer (5 mm broad band probe) at room temperature. Mass spectra were obtained using electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) on a Micro Mass QTOF-2 mass spectrometer (Waters Corp.), using argon as the collision gas. Inductively coupled plasma atomic emission spectroscopy (ICP-AES), measurements were taken on a Thermo iCAP 6300, and calibrated

from dilutions of 1000 ppm Mn(II) or Fe(III) standard solutions (Fisher), using class A volumetric glassware. X-Band EPR spectra were obtained using a Bruker EMXplus Spectrometer with an aqueous flat cell.

2.2 Siderophores: DFOB, DFOE and putrebactin. DFOB mesylate was purchased from Sigma-Aldrich and used as obtained.

Isolation of putrebactin. Putrebactin was isolated from cultures of *Shewanella oneidensis* MR-1.[26] *S. oneidensis* MR-1 was maintained on Difco 2216 marine broth plates, and cultured in an artificial sea water medium (ASG+Fe), containing 2 L doubly deionized water, 20 g Bacto CAS amino acids, 2 g NH₄Cl, 2 g glycerol phosphate, 24.7 g MgSO₄•7H₂O, 2.9 g CaCl₂•H₂O, 33.1 g NaCl, 1.5 g KCl, 6 mL glycerol, and 2 µM (final concentration) of FeCl₃. Prior to inoculation, 20 mL of filter-sterilized 1.0 M HEPES buffer (pH 7.4), 4 mL of filter-sterilized 1.0 M NaHCO₃ buffer, and 20 mL filter-sterilized vitamin stock were added to the medium. Vitamin stock consisted of 4 mg niacin, 2 mg thiamin, 4 mg *p*-aminobenzoic acid, 2 mg calcium pantothenic acid, 20 mg pyridoxine HCl, 2 mg cyanocobalamin, 4 mg riboflavin, and 4 mg folic acid in 200 mL doubly deionized water.

Isolation of DFOE. DFOE was obtained from an uncharacterized bacterium isolated from seawater obtained from the Gulf of Mexico after the Deepwater Horizon oil spill in 2010; the bacterium was maintained on minimal nutrient seawater agar plates consisting of 0.5 g Bacto yeast extract, 5 g Bacto peptone, 15 g Bacto agar in 1 L filtered seawater. The bacterium was cultured in a minimal nutrient artificial seawater medium (ASW-Fe) composed of 30 g NaCl, 1.5g KCl, 6 g CaCl₂•2H₂O, 24 g MgSO₄•7H₂O, 2 g NH₄Cl, 0.2 g glycerol phosphate, 4 g Bacto CAS amino acids, and 6 mL glycerol in 2 L doubly deionized water (Barnstead Nanopure II).

Prior to inoculation, 20 mL of filter-sterilized 1.0 M HEPES buffer (pH 7.4), and 4 mL of filter-sterilized 1.0 M NaHCO₃ buffer were added to the medium.

For siderophore isolation, each bacterial strain was grown for 24-48 h on an orbital shaker (180 rpm) and harvested via centrifugation. Purification of putrebactin and DFOE followed the same general procedure. Amberlite XAD-2 resin (Supelco) was combined with the cell free culture supernatant (100 g/L) and shaken for 2 h (120 rpm). Supernatant was then filtered away and the XAD-2 resin was rinsed with doubly deionized water (1L/100g), prior to packing the XAD-2 resin into a glass chromatography column (2 cm internal diameter). Siderophores were eluted in 100% methanol. The eluent was lyophilized, and stored at stored at -20 °C.

In the isolation procedure for putrebactin, the resulting yellow brown powder was rinsed approximately five times with ca. 10 mL doubly deionized water until the solid became white. The white solid was re-dissolved in 300 mL MeOH with gentle heating, at which point 25 mL of H₂O was added, followed by rotary evaporation to remove MeOH. Final purification via RP-HPLC was performed on a C₄ preparatory column (10 µm particle size, 250 mm column length, x 20 mm i.d., Higgins Analytical). A gradient of 100% H₂O (doubly deionized with 0.05% trifluoroacetic acid (TFA) to 35% MeOH (0.05% TFA) over 30 min was used for putrebactin, and a 100% H₂O to 80% MeOH gradient (0.05% TFA) over 40 min was used for DFOE. The eluent was monitored continuously at 215 nm and putrebactin-containing fractions, as confirmed by mass spectrometry, were pooled, lyophilized and stored at -20 °C.

To analyze for adventitious iron contamination in the siderophore samples, the siderophore ligands (final concentration 0.1 mM) were dissolved in 1 mL of nitric acid (70%)

and diluted into 100 mL in doubly de-ionized water. The ICP-AES spectrometer was calibrated in the linear range using a blank of 0.7% nitric acid and a 0.1 ppm Fe solution in 0.7% nitric acid prepared by dilution of a 1000 ppm Fe(III) standard (Fisher). Each sample was run in triplicate with an 80 s exposure time.

2.3. Formation of Mn(III)-siderophore complexes. A stock solution of 10 mM Mn(II) was prepared from $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ in D_2O (99.9%) and standardized by ICP-AES. Mn(II) (1 mM final concentration) was reacted in air with 2 mM DFOB or DFOE and 3 mM putrebactin, in water at pH 8-10 (or up to pH 9.5 for putrebactin) for 24 hrs. To adjust the pH, ~ 1 M NaOD or DCl was added, as needed. The NaOD and DCl solutions were prepared by dissolving solid NaOH and concentrated HCl (36%) into D_2O (99.9%). Solubilization of putrebactin in water required addition of NaOD to maintain the pH above 7.1. A 6 inch micro combination electrode (Accumet) with a calomel reference was used to monitor the pH of the solutions. The oxidation of Mn(II) to Mn(III) was followed at 310 nm which is in the range of the λ_{max} for the Mn(III) siderophore complexes. Solutions were filtered for EPR spectroscopic analysis and magnetic susceptibility measurements. The solutions for EPR spectroscopy and UV-Vis analysis were buffered by 50 mM CAPSO (*N*-cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid) at pH 9.

2.4. Magnetic Susceptibility. The effective magnetic moment (μ_{eff}) of the Mn-siderophore complexes was determined at room temperature using the Evans NMR method [23, 28]. DMSO (10% v/v) was used for the reference. The initial concentration of Mn(II) (1 mM) was used for calculation purposes allowing for comparison between siderophore ligands, independent of the Mn(III)-complex stoichiometry. Calculation of the molar magnetic susceptibility (χ_{M}) was performed using a modified Evans equation [28, 29] which takes into account the geometry of superconducting magnets (Equation 1). In equation 1, χ_{D} is the diamagnetic correction for the

ligands, M is molarity, m is mass, Δf is the chemical shift, and f_o is the operating frequency [28, 29]. The μ_{eff} was calculated from equation 2.

$$\chi_M = \frac{750\Delta f}{\pi f_o M} + \chi_D \quad (1)$$

$$\mu_{eff} = 2.828\sqrt{\chi_M T} \quad (2)$$

2.5. Mass spectrometry. Samples for mass spectroscopy were prepared using stoichiometric amounts of siderophore and Mn(II). The pH was adjusted to pH 8-10 with 1 M NH_4OH . The mass spectrometer was run in positive ion mode with a flow rate of 10 $\mu\text{L}/\text{sec}$. Tandem mass spectroscopy was used to confirm the identity of the ligands used.

2.6. EPR Spectroscopy. Samples for EPR analysis were prepared in D_2O (99.9%) with 10% v/v DMSO. X-band EPR spectra (9.78 GHz) were obtained using an aqueous flat cell at room temperature to detect residual Mn(II) after air oxidation of the Mn(II)-siderophore complexes.

3. Results and Discussion

3.1. Magnetic susceptibility of Mn(III) complexes of DFOB, DFOE and putrebactin. The manganese complexes for magnetic susceptibility measurements were prepared, as described above, by reacting 2 mM DFOB or DFOE with 1 mM Mn(II) or 3 mM putrebactin with 1 mM Mn(II) in 99.9% D_2O , followed by exposure to air for 24 hr, and after adjustment to pH 8-10. Upon siderophore complexation, Mn(II) oxidation occurs within a pH range of 7.1-11.1, a range established in previous investigations on the formation of Mn(III)-DFOB.[19] The extinction coefficient of 2062 $\text{M}^{-1}\text{cm}^{-1}$ at 310 nm was measured for Mn(III)-DFOB, in agreement with previously reported values, and used to monitor the extent of the Mn(III)-DFOB formation.[16]

DFOE had a similar pH dependency and UV-Vis spectrum as DFOB (Supplementary Information Figure S1).

Effective magnetic moments of 4.85 ± 0.02 BM for Mn(III)-DFOB and 4.84 ± 0.02 BM for Mn(III)-DFOE were obtained using the Evans NMR method for these complexes in aqueous solution (Table S1). The magnetic moment values agree well with the calculated value of 4.90 BM for a d^4 high spin ion and can be used as a baseline for comparison to other hydroxamate siderophores.

The magnetic moment resulting from reaction of 1 mM Mn(II) and 3 mM putrebactin in air for 24 h and up to pH 9.5 was 4.98 ± 0.02 BM (Table S1), which is higher than the calculated magnetic moment of 4.90 BM, assuming complete oxidation of Mn(II) to Mn(III) and higher than the Mn(III)-DFOB and Mn(III)-DFOE complexes. The value of the magnetic moment in the Mn-putrebactin sample was unexpected and iron contamination was suspected. However ICP-AES analysis of the putrebactin samples showed no significant level of iron was present (Table S2).

To investigate whether the higher magnetic moment resulted from incomplete oxidation of Mn(II), the X-band EPR spectrum was examined. High spin d^5 Mn(II) has a characteristic 6 peak spectrum at room temperature [30, 31], whereas high spin d^4 Mn(III) is EPR silent. EPR analysis of the Mn-putrebactin reaction solution gave a weak 6 line signal (Figure 3), which matches the control EPR spectrum for aqueous Mn(II). Assuming that the only species contributing to the magnetic susceptibility were high spin Mn(II) and Mn(III), the observed μ_{eff} of 4.98 for the Mn-putrebactin solution is consistent with the presence of 7.8% Mn(II). The EPR spectra of the Mn(III)-DFOE and Mn(III)-DFOB did not show residual Mn(II) (Figures S2-S4).

Figure 3.

Efforts to increase the extent of Mn(II) oxidation in the putrebactin reactions at elevated pH was hampered by precipitation above pH 9.5, which differs from the stability of Mn(III)-DFOB and Mn(III)-DFOE up to pH 11.1. The second pK_a of putrebactin at 9.71 [26] is higher than the stable solution pH for the Mn(II/III)-putrebactin complexes, which may contribute to the lack of formation and stabilization of Mn(III)-putrebactin complexes, since the Mn(III) oxidation state is stabilized by complexation to hard anionic ligands.

3.2. Mass spectrometry of the Mn(III) complexes of DFOB, DFOE and putrebactin. Mass spectra for the manganese complexes of DFOB and DFOE showed masses at m/z 613.26 and 653.26, respectively, corresponding to 1:1 Mn(III)-siderophore complexes, as expected for tris-hydroxamate siderophores (Figures S5, S6). Because putrebactin is a *bis*-hydroxamate ligand, it is not expected to exhibit the 1:1 metal to ligand complexation typical of *tris*-hydroxamate siderophores. Previous studies with Fe(III) and the *bis*-hydroxamate siderophore, alcaligin (Figure 1, R=OH) [24, 25, 32, 33], revealed pH-dependent speciation with 1:1, 2:3, and 2:2 [Fe(III)_x-alcaligin_y] complexes at low (pH < 5), mid (pH 5-9), and high (pH > 9) pH values respectively [25]. The 2:2 complex formed at pH 11 is reported to be an anti-ferromagnetically coupled *bis*- μ -oxo dimer, Fe₂(alcaligin)₂O₂, [25] and not detected at lower pH by ESI-MS [33].

Mass spectrometry was used to explore the speciation present in solutions resulting from air oxidation of 1 mM Mn(II) in the presence of 1.5 mM putrebactin, pH 9.5. The peaks at m/z 797.3 and 1221.4 (Figure 4A) are consistent with 1:2 and 2:3 Mn-putrebactin complexes, [Mn-(putrebactin)₂+H⁺] and [(Mn₂-putrebactin₃)+H⁺], respectively. The peaks at m/z 425.1 and 850.2 appear to be fragmentation products of the 1:2 (m/z 797.3) and 2:3 (m/z 1221.4) complexes,

confirmed by tandem mass spectrometry (Figures 4B, 4C), and in each case corresponds to the loss of a putrebactin ligand.

Figure 4.

The 1:2 ratio of metal(III)-siderophore complexation has previously been reported for Fe(III) and alcaligin when the siderophore is present in excess [33]. Thus for the reaction of 1 mM Mn(II) and 1.5 mM putrebactin, it is not surprising to observe species of $\text{Mn}_2\text{-putrebactin}_3$ or Mn-putrebactin_2 . Evidence for a Mn(III) μ -oxo-dimer, analogous to the related Fe(III) complex, $\text{Fe}_2(\text{alcaligin})_2\text{O}_2$ [25], was not detected by negative ion mode mass spectrometry (Figure S7). Additionally, the 1:1 Mn(III)-putrebactin complex was not observed. Metal to ligand stoichiometries of 1:1 for Fe(III)-*bis*-hydroxamate complexes are normally found below pH 5 [25], however oxidation of Mn(II) does not occur below pH 7.1, possibly explaining the lack of evidence for this species.

4. Conclusions

In sum, the magnetic susceptibility determination of the reaction products of the aerobic reactions of Mn(II) and the hydroxamate siderophores, DFOB, DFOE and putrebactin, directly confirm the Mn(III) siderophore complex is formed quantitatively with DFOB and DFOE (pH 8-10). In the Mn(II) putrebactin reactions, Mn(III) formation was nearly stoichiometric however approximately 8% Mn(II) remained as detected by EPR spectroscopy and the magnetic moment. Attempts to increase the extent of aerobic oxidation of the Mn(II) putrebactin complexes was hampered by precipitation at pH > 9.5. Mass spectrometry showed the presence of two Mn-putrebactin species, consistent with $\text{Mn(III)}_2(\text{putrebactin})_3$ and $\text{Mn(III)(putrebactin)(putrebactinH)}$, which is analogous to Fe(III) complexes of alcaligin above

neutral pH [25]. These results complement previous reports on Mn(III)-DFOB and Mn(III)-DFOE [16].

Siderophores could play a role in increasing the availability of manganese to various organisms. For example, DFOB has been shown to dissolve manganite (γ -Mn(III)OOH), hausmannite (Mn_3O_4) and Mn(IV)-type oxides through both reductive and non-reductive pathways, depending on pH [7]. Thus a pool of Mn(III) chelated by siderophores could increase the availability of manganese to organisms that might not otherwise have a means to obtain it. While ligand-mediated uptake of metals other than iron has been demonstrated recently for molybdenum and vanadium with siderophores [3, 34] and for copper with chalkophores [35, 36], ligand-mediated uptake of manganese has not been reported to date. Nevertheless, siderophores may play an important role in biogenic cycling of manganese [10, 19, 37, 38] .

Supplementary Information

UV-Vis of Mn(III)-siderophore complexes, EPR spectra, Mass spectra of manganese complexes of DFOB, DFOE and putrebactin, ICP-AES data on Fe content of siderophores and magnetic susceptibility vs pH.

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Figure Legends

Figure 1. Desferrioxamine B (DFOB), desferrioxamine E (DFOE) and putrebactin.

Figure 2. Possible coordination modes of Mn(III) putrebactin complexes.

Figure 3. X-Band EPR spectrum of the Mn(III)-putrebactin complex (μ_{eff} , 5.04) in D₂O (99.9%) with 10% (v/v) DMSO in an aqueous flat cell at room temperature. Frequency 9.78 GHz.

Figure 4. (A) ESI-MS of the reaction mixture of 1 mM Mn(II) and 1.5 mM putrebactin after aqueous aerobic reaction for 24 h, pH 9.1. (B) ms-ms of m/z 1221.41 in (A) with m/z 850.24 as the only fragmentation product. (C) ms-ms of m/z 797.30 in (A) with 425.14 m/z as the only fragmentation product.

Figure 1.

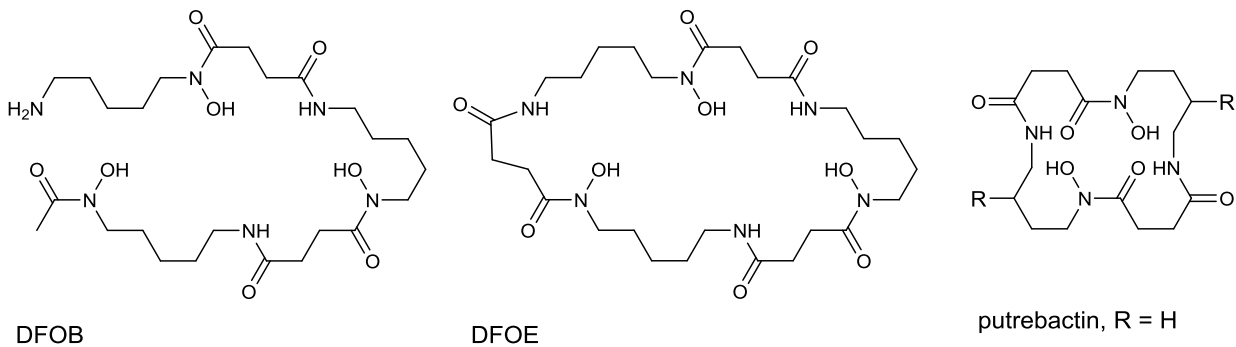


Figure 2.

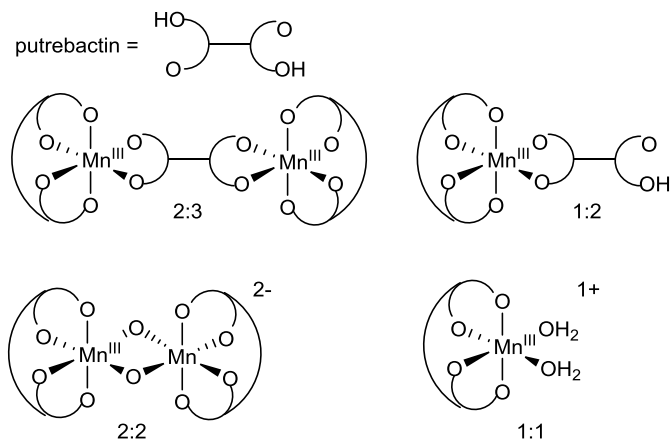


Figure 3.

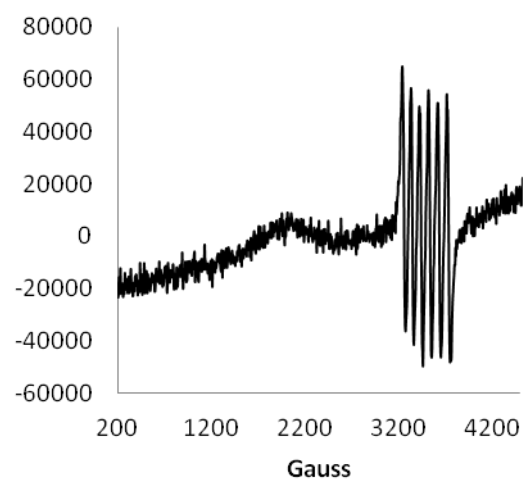


Figure 4.

