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Iron(III) Coordination Chemistry of Alterobactin A: A Siderophore from the Marine Bacterium *Alteromonas luteoviolacea*

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Alterobactin A is a siderophore produced by the oceanic bacterium *Alteromonas luteoviolacea*. The thermodynamic stability constant of the ferric alterobactin A (Alt-A) complex was estimated from electrochemical measurements on the basis of a previously reported linear relationship between the reduction potentials and the pH-independent stability constants for known iron(III) complexes. The reduction potential of the ferric alterobactin A complex determined by square wave voltammetry is –0.972 V vs SCE and reversible, corresponding to a thermodynamic stability constant of $10^{51\pm2}$. Potentiometric titration of Fe(III)–Alt-A shows the release of six protons on complexation of Fe(III) to Alt-A. The ^{1}H NMR resonances of the Ga(III)–Alt-A complex show that the C-4, C-5, and C-6 catecholate protons and the C_α and C_β protons of both β-hydroxyaspartate moieties are shifted downfield relative to the free ligand, which along with the potentiometric titration data is consistent with a complex in which Fe(III) is coordinated by both catecholate oxygen atoms and both oxygen atoms of each β-hydroxyaspartate. The UV–vis spectrum of Fe(III)–Alt-A is invariant over the pH range 4–9, indicating the coordination does not change over a wide pH range. In addition, in the absence of a coordinated metal ion, the serine ester of Alt-A hydrolyzes forming Alt-B.

Introduction

The vast majority of bacteria require iron for growth. Under aerobic conditions, bacteria produce siderophores to solubilize and sequester iron(III).[1−4] Siderophores are low molecular weight compounds that have a high affinity for ferric ion and are produced under conditions of iron demand, in conjunction with their outer membrane receptor proteins that catalyze the transport of iron into the cell.[1−4] In contrast to the terrestrial environment in which iron is often the most abundant transition metal ion, the total soluble iron concentration in surface ocean water is only 0.02−1.0 nM.[5−9] Iron limitation of primary production by phytoplankton in the ocean has attracted much attention.[10−13] These investigations show that not only is growth of phytoplankton limited by the low levels of iron but so too is growth of heterotrophic

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(1) Raymond, K.; Dertz, E. A. In *Iron Transport in Bacteria*; Crosa, J. H., Mey, A. R., Payne, S. M., Eds.; ASM Press: Washington, DC, 2004; pp 3−18.
(2) Stintzi, A.; Raymond, K. N. Siderophore Chemistry. In *Molecular and Cellular Iron Transport*; Templeton, D. E., Ed.; Marcel Dekker: New York, 2001; pp 273−319.
(3) Boukhalfa, H.; Crumbliss, A. L. *BioMetals* **2002**, *15*, 325.
(4) Albrecht-Gary, A.-M.; Crumbliss, A. L. In *Metal Ions in Biological Systems*; Sigel, A., Sigel, H., Eds.; M. Dekker: New York, 1998; Vol. 35; pp 239−327.
(5) Martin, J. H.; Gordon, R. M.; Fitzwater, S. E. *Limnol. Oceanogr.* **1991**, *36*, 1793−1802.
(6) Johnson, K. S.; Gordon, R. M.; Coale, K. H. *Mar. Chem.* **1997**, *57*, 137−161.
(7) Bruland, K. W.; Donat, J. R.; Hutchins, D. A. *Limnol. Oceanogr.* **1991**, *36*, 1555−1577.
(8) Wu, J.; Luther, G. W., III. *Limnol. Oceanogr.* **1994**, *38*, 1119−1129.
(9) Wu, J.; Luther, G. W., III. *Geochim. Cosmochim. Acta* **1996**, *60*, 2729−2741.
(10) Martin, J. H.; Fitzwater, S. E. *Nature* **1988**, *331*, 341−343.
(11) Martin, J. H.; Coale, K. H.; Johnson, K. S.; Fitzwater, S. E.; S E.; Gordon, R M.; Tanner, S. J.; Hunter, C. N.; Elrod, V. A.; Nowicki, J. L.; Coley, T. L.; Barber, R. T.; Lindley, S.; Watson, A. J.; Watson, A. J.; Van Scoy, K.; Law, C. S.; Liddlecoat, M.; Ling, R.; Stanton, T.; Stockel, J.; Collins, C.; Anderson, A.; Bidigare, R.; Ondrusek, M.; Latasa, M.; Millero, F. J.; Lee, K.; Yao, W.; Zhang, J. Z.; Friederich, G.; Sakamoto, C.; Chavez, F.; Buck, K.; Kolber, Z.; Greene, R.; Falkowski, P.; Chisholm, S. W.; Hoge, F.; Swift, R.; Yungel, J.; Turner, S.; Nightengale, P.; Hatton, A.; Liss, P.; Tindale, N. W. *Nature* **1994** *371*, 123−129.

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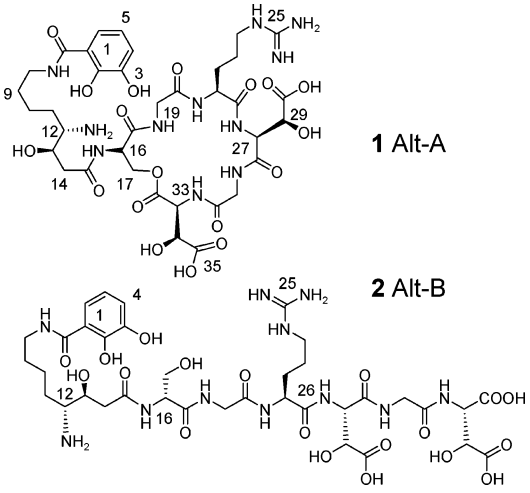
marine bacteria.[14,15] Heterotrophic bacteria compete successfully with phytoplankton for iron and in some cases contain more iron/biomass than phytoplankton.[14] Preliminary screens of oceanic bacteria show that many produce siderophores.[16,17] We have identified aerobactin,[18] a known terrestrial siderophore[19,20] from an oceanic *Vibrio* species,[18] the marinobactins and aquachelins from *Marinobacter sp.* M6 and *Halomonas aquamarina*, respectively,[21] petrobactin from *M. hydrocarbonoclasticus*,[22,23] and the amphibactins[24] and desferrioxamine G[25] from distinct marine *Vibrio* species, as well as the alterobactins A[26] (**1**; Alt-A, the subject of this report) and B (**2**; Alt-B) from *Alteromonas luteoviolacea*. Recently the total synthesis of alterobactin A was reported.[27] In addition the pseudoalterobactins from *Pseudoalteromonas* sp. KP20-4 have been reported.[28]



The stability constant for Fe(III)−alterobactin A has previously been estimated to be $10^{49}-10^{53}$ by EDTA competition.[26] This method requires knowledge of the pK_a's of the functional groups, which coordinate to the metal ion.[29] However, the pK_a of the α-hydroxyl proton of *β*-hydroxyaspartate is not known. We had previously estimated the hydroxyl pK_a to be that of an alcohol (i.e, ∼16) or higher (e.g., ∼18), as a result of the neighboring deprotonated carboxylate group. Given the uncertainty of this pK_a value, we have been interested in estimating the stability constant of the Fe(III)−alterobactin-A complex by another method.

The thermodynamic, pH-independent stability constant for the formation of Fe(III) complexes with oxygen donor ligands can be estimated from the "chelate scale" of Taylor et al.,[30] which permits measurements when micromolar concentrations of ligand are available. The Fe(III) chelate scale relates the reduction potentials of selected iron(III) complexes to known stability constants, on the basis of the equation

$$E'_p = E_p - 2.303(RT/nF)(\log K_{ox}/K_{red}) \quad (1)$$

where E_p and E'_p are the reduction potentials of the free metal ion and the complex, n is the number of electrons involved in the process ($n = 1$ for the Fe(III)/Fe(II) redox couple), and K_{ox} and K_{red} are the pH-independent stability constants of the oxidized and reduced forms of the metal−ligand complex, respectively.[31] The potential is independent of ligand concentration because excess ligand is added to ensure full complexation of the Fe(III).

Taylor et al.[30] observed a linear relationship between E'_p and log K_{ox} (over 19 log units) for seven iron(III) complexes containing only oxygen ligating atoms (primarily with catcholate binding modes) with different denticity. On the basis of the similarity between the known log K_{red} (i.e., 19) values for the iron(II) complexes of enterobactin and *trans*-1,2-cyclohexylenedinitrilotetraacetate (CDTA),[30] the K_{red} values for all the model ligand Fe(II) complexes were assumed to be similar; thus K_{red} in eq 1 could be incorporated into the intercept:

$$E'_p = [E_p + (2.303RT/nF)\log K_{red}] - (2.303RT/nF)\log K_{ox} \quad (2)$$

From the data in Taylor et al.,[30] eq 2 reduces to

$$E'_p = -0.041 \log K_{ox} + 1.10 \quad (3)$$

Using the "chelate scale"[30] (i.e., eq 3) and the reduction

(12) Coale, K. H.; Johnson, K. S.; Fitzwater, S. E.; Gordon, R. M.; Tanner, S.; Chavez, F. P.; Ferioli, L.; Sakamoto, C.; Rogers, P.; Millero, F.; Steinberg, P.; Nightingale, P.; Cooper, D.; Cochlan, W. P.; Landry, M. R.; Constantinou, J.; Rollwagen, G.; Trasvina, A.; Kudela, R. *Nature* **1996**, *383*, 495−501.
(13) Coale, K. H.; Johnson, K. S.; Chavez, F. P.; Buesseler, K. O.; Barber, R. T.; Brzezinski, M. A.; Cochlan, W. P.; Millero, F. J.; Falkowski, P. G.; Bauer, J. E.; Wanninkhof, R. H.; Kudela, R. M.; Altabet, M. A.; Hales, B. E.; Takahashi, T.; Landry, M. R.; Bidigare, R. R.; Wang, X.; Chase, Z.; Strutton, P. G.; Friederich, G. E.; Gorbunov, M. Y.; Lance, V. P.; Hilting, A. K.; Hiscock, M. R.; Demarest, M.; Hiscock, W. T.; Sullivan, K. F.; Tanner, S. J.; Gordon, R. M.; Hunter, C. N.; Elrod, V. A.; Fitzwater, S. E.; Jones, J. L.; Tozzi, S.; Koblizek, M.; Roberts, A. E.; Herndon, J.; Brewster, J.; Ladizinsky, N.; Smith, G.; Cooper, D.; Timothy, D.; Brown, S. L.; Selph, K. E.; Sheridan, C. C.; Twining, B. S.; Johnson, Z. I. *Science* **2004**, *304*, 408−414.
(14) Tortell, P. D.; Maldonado, M. T.; Price, N. M. *Nature* **1996**, *383*, 330−332.
(15) Pakulski, J. D.; Coffin, R. B.; Kelley, C. A.; Holder, S. L.; Dower, R.; Aas, P.; Lyons, M. M.; Jeffrey, W. H. *Nature* **1996**, *383*, 133−134.
(16) Trick, C. G. *Curr. Microbiol.* **1989**, *18*, 375−378.
(17) Goyne, E. R.; Carpenter, E. J. *Limnol. Oceanogr.* **1974**, *19*, 840−842.
(18) Haygood, M. G.; Holt, P. D.; Butler, A. *Limnol. Oceanogr.* **1993**, *38*, 1091−1097.
(19) Harris, W. R.; Carrano, C. J.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 2722−2727.
(20) Buyer, J. S.; Delorenzo, V.; Neilands, J. B. *Appl. Environ. Microbiol.* **1991**, *57*, 2246−2250.
(21) Martinez, J. S.; Zhang, G. P.; Holt, P. D.; Jung, H.-T.; Carrano, C. J.; Haygood, M. G. Butler, A. *Science* **2000**, *287*, 1245−1247.
(22) Barbeau, K.; Zhang, G.; Live, D. H.; Butler, A. *J. Am. Chem. Soc.* **2002**, *124*, 378−379.
(23) Bergeron, R. J.; Huang, G.; Smith, R. E.; Bharti, N.; McManis, J. S.; Butler, A. *Tetrahedron* **2003**, *59*, 2007−2014.
(24) Martinez, J. S.; Carter-Franklin,J. N.; Mann, E. L.; Martin, J. D.; Haygood, M. G. Butler, A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3754−3759.
(25) Martinez, J. S.; Haygood, M. G.; Butler, A. *Limnol. Oceanogr.* **2001**, *46*, 420−424.
(26) Reid, R. T.; Live, D. H.; Faulkner, D. J.; Butler, A. *Nature* **1993**, *366*, 455−458. (Figure 1 in this paper shows the inverted configuration of the 3-hydroxy-4-amino functional group from what it should be (although the text is correct); the correct stereochemistry is shown here in **1**).
(27) Deng, J.; Hamada, Y.; Shioiri, T. *J. Am. Chem. Soc.* **1995**, *117*, 7824−7825.

potential of Fe(III)−alterobactin A complex determined electrochemically, we report herein that the estimated stability constant for the formation of the Fe(III)−alterobactin A is consistent with the high affinity of alterobactin A for ferric ion predicted by EDTA competition.[26] Such measurements are important to begin to address the mechanism of iron uptake by marine microorganisms.

Materials and Methods

Isolation of alterobactin A. *Alteromonas luteoviolacea* strain S2 was maintained on natural seawater medium containing 5 g/L Bacto peptone (Difco), 1 g/L yeast extract (Difco), and 1.5% agar, replating the bacteria every 4−7 days.[32] For isolation of alterobactin A, *A. luteoviolacea* was grown in 2 L batch culture in 4 L flasks of natural seawater media which contained 1 g of NH_4Cl, 2 g of casamino acids, and 0.1 g of glycerophosphate L^{-1} of aged seawater. Alt-A (**1**) was isolated and purified from the supernatant of bacterial cultures as previously described,[26,32] using solid-phase extraction with Amberlite XAD-2 resin (Supelco, Inc.) followed by C-4 HPLC separation (Vydak, 10 mm × 250 mm).[32] An Ultrahydrogel 120 (Waters, 4.6 × 250 mm) HPLC column was used with isocratic elution in 10 mm acetate buffer, pH 4.8, in place of the LH20 (Pharmacia) column. The final purification was accomplished using the C-4 reverse phase HPLC column.

Stoichiometry of the Fe(III)−Alterobactin A Complex. The ferric alterobactin A complex was prepared by reaction of 3 μmol of siderophore with excess Fe(III) (i.e., 4.5 μmol of Fe(III)) in 26 mM $HClO_4$, followed by addition of phosphate to a final concentration of 0.2 M, pH 8. After equilibration for 1 h, the solution was applied to a Biogel P2 (BioRad) column (1 × 25 cm) and eluted with water adjusted to pH 8 with NaOH. Fractions of the rose-colored ferric siderophore complex were collected and pooled. The iron(III) concentration was determined spectrophotometrically after reduction with sodium dithionite as the tris(phenanthroline)iron(II) complex (λ_{max} 510 nm; ϵ 12 000 M^{-1} cm^{-1}) in 0.1 M acetate buffer, pH 5.[33] The siderophore complex concentration was determined from HPLC standard curves, monitoring the absorbance at 310 nm, using a C-4 HPLC column (4.6 × 250 mm) with a gradient from 0.1% TFA in H_2O to 20% CH_3CN and 0.1% TFA in H_2O.

Potentiometry. Potentiometric titrations were performed using a Radiometer Copenhagen pH meter (model PHM 84) and GK2321C combined glass electrode with an internal calomel reference. Solutions were kept at constant temperature using water-jacketed beakers. Samples were degassed with argon (purified by passage through a column of NaOH pellets and Drierite) and kept under an argon atmosphere by bubbling throughout the titration. The pH meter was calibrated against 0.1 M HCl (Baker Dilut-It) by titrating the acid sample with aliquots of freshly prepared 0.1 M KOH made from boiled water and standardized in triplicate against potassium hydrogen phthalate (Aldrich, primary standard grade) to a phenolphthalein end point. The carbonate level in the base solution was determined by a Gran's plot.[34,35] Sample solutions were titrated from an initial acidic pH to approximately pH 10.

Ferric alterobactin A solutions were prepared by addition of stoichiometric amounts of stock 26.5 mM $Fe(ClO_4)_3$ in 10 mM $HClO_4$. The iron(III) stock solution was standardized against ferrozine in the presence of $NH_2OH{\cdot}HCl$ at pH 5 in ammonium acetate buffer.[36] The acid content of the stock solution was measured by titration in the presence of excess EDTA.[37, 38]

Voltammetric Measurements of Fe(III)−Alt-A. The thermodynamic stability constant for Fe(III)−alterobactin A was estimated from the reduction potential of the complex, based upon the "chelate scale" developed by Taylor et al.[30] This method has been applied to other ferric siderophore complexes, including ferric alterobactin B.[39] Voltammetric measurements were made using an EG&G Princeton Applied Research (PAR) model 384-B polarographic analyzer or an Analytical Instrument Systems, Inc., DLK-100 electrochemical analyzer with a PAR model 303A mercury drop electrode and a saturated calomel reference electrode (SCE). Analyses were performed using the square wave voltammetry (SWV) mode with a hanging mercury drop electrode (large drop size) without stirring. Default instrumental parameters were used (scan rate, 200 mV/s; pulse amplitude, 20 mV; pulse frequency, 100 Hz; scan range, −0.1 to −1.5 V). Cyclic voltammograms (CV) and square wave voltammetry with the DLK-100 system (which provides the forward, reverse, and resultant current voltammograms) were utilized to assess reversibility of the electrochemical processes.[30,39,40] Cyclic voltammograms were generated using the cyclic staircase voltammetry mode of the PAR 384-B with scan rates ranging from 20 to 100 mV/s. The peak potentials in SWV and CV are different because of scan rate differences and the application of pulse amplitude in SWV but not CV. Excess ligand is added to ensure full complexation of the Fe(III), which results in a potential which is independent of ligand concentration as determined by a constant value of E'_p upon continued ligand addition.

Experimental solutions were prepared in 5 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-tris) buffer at pH 7.0 and 25 °C. Bis-tris prevents the hydrolysis and precipitation of iron by forming weak complexes with Fe(III)[41] but does not interfere with electrochemical measurement of the iron−siderophore complexes.[30,39] The ionic strength was held constant at 0.1 M with NaCl. Iron(III) solutions were prepared from a 1000 ppm commercial atomic absorption standard (Fisher). Ferric chloride solutions (3−32 μM) were prepared in 5 mL of the Bis-tris buffer, pH 7, sparged with high-purity argon to remove oxygen and titrated with siderophore until the resulting reduction peak stopped shifting, indicating excess of chelator with respect to Fe(III).

Mass Spectrometry. Electrospray mass spectrometry was performed using a VG Fisons Platform II single quadrupole mass analyzer. Samples of HPLC-purified ferric alterobactins were

(28) Kanoh, K.; Kamino, K.; Leleo, G.; Adachi, K.; Shizuri, Y. *J. Antibiot.* **2003**, *56*, 871−875.
(29) Raymond, K. N.; Muller, G.; Matzanke, B. F. *Top. Curr. Chem.* **1984**, *123*, 49−102.
(30) Taylor, S. W.; Luther, G. W., III; Waite, J. H. *Inorg. Chem.* **1994**, *33*, 5819−5824.
(31) Heyrovsky, J.; Kuta, J. *Principles of Polarography*; Academic: New York; 1966; pp 156−158.
(32) Reid, R. T.; Butler, A. *Limnol. Oceanogr.* **1991**, *36*, 1783−1792. (Note: Alterobactins A and B are referred to as S2L and S2E, respectively, in this paper.)
(33) Charlot, G.; Bezier, D. *Quantitative Inorganic Analysis*; John Wiley: New York, 1957; pp 453−454.
(34) Martell, A. E.; Motekaitis, R. J. *Determination and use of stability constants*; VCH: New York, 1992.
(35) Gran, G. *Analyst* **1952**, *77*, 661−670.
(36) Stookey, L. L. *Anal. Chem.* **1970**, *42*, 779−781.
(37) Schwarzenbach, G.; Schwarzenbach, K. *Helv. Chim. Acta*, **1963**, *46*, 1390−1400. Anderegg, G.; Eplattenier, F. L.; Schwarzenbach, G. *Helv. Chim. Acta* **1963**, *46*, 1400−1408; 1409−1422.
(38) Schwarzenbach, G. *Complexometric Titrations*; Pitman: Bath, U.K., 1957; pp 25−27.
(39) Lewis, B. L.; Holt, P. D.; Taylor, S. W.; Wilhelm, S. W.; Trick, C. G.; Butler, A.; Luther, G. W., III. *Mar. Chem.* **1995**, *50*, 179−188.
(40) Bond, A. M. *Modern Polarographic Methods in Analytical Chemistry*; Dekker: New York, 1980; Chapter 2.
(41) Taylor, S. W.; Hawkins, C. J.; Winzor, D. J. *Inorg. Chem.* **1993**, *32*, 422.

dissolved in 50% H_2O/50% CH_3CN and introduced via syringe infusion.

Preparation of Ga(III)−Alterobactin A. The gallium(III) complex of alterobactin A was prepared by reaction of 3 μmol of Alt-A with 2.85 μmol of Ga(III), from a 50 mM stock solution of Ga(III) in 1.5 M NaCl, pH 1.5 (prepared from a gallium ingot[42]). The mixture was diluted to 0.5 mL and the pH adjusted to pH 7.4 with NaOH. Samples were dissolved in 99.9% D_2O for NMR measurements. The ^1H spectra were obtained on a GN 500 MHz instrument using standard pulse sequences.

Stability of Alterobactin A. To evaluate the stability alterobactin A and to measure the rate of hydrolysis of Alt-A to Alt-B, 0.1−0.2 mM solutions of alterobactin A and Fe(III)−Alt-A in 0.2 M phosphate buffer, pH 6 and 8, were monitored by HPLC about every 30 min for ca. 24 h initially and then up to 3 weeks in the case of Fe(III)−Alt A. The hydrolysis of Alt-A to Alt-B was monitored at 310 nm with a C-4 column using a gradient from 1 to 25% CH_3CN with 0.1% TFA in H_2O. The Fe(III)−Alt complexes were kept in the dark to prevent the photooxidation of the ligand and reduction of Fe(III) as has been found to occur for α-hydroxycarboxylic acid-containing siderophores.[22,43]

Results and Interpretation

Fe(III) Stability Constant of Alterobactin A. The square wave voltammogram (SWV) of a solution of 40 μM Alt-A added to 10 μM Fe(III) (i.e., excess ligand over Fe(III)) in 5 mM Bis-tris buffer pH 7 obtained at 2 min and 1.5 h after mixing (Figure 1a,b, respectively) shows three distinct reduction reactions. The peak at −0.972 V is attributed to the reduction of the fully formed Fe(III)−Alt-A complex (see below for further characterization of the hexadentate Fe(III)−Alt-A complex). The forward and reverse peaks in both the SWV (not shown) and cyclic voltammogram (CV; Figure 1c) were well-defined indicating reversibility. The CV shows that the Fe(III)−Alt-A reduction to the Fe(II) complex is reversible with $(i_p)_a/(i_p)_c = 0.92$, $\Delta E = 72$ mV. No variation of anodic or cathodic peak potential with scan rate is observed, and both anodic and cathodic currents vary with $v^{1/2}$. For a fully reversible process measured by CV, both the cathodic and anodic peak currents are proportional to $v^{1/2}$ with $(i_p)_a/(i_p)_c = 1$. A recent voltammetric study of six Fe(III)−hydroxamate complexes showed that $(i_p)_a/(i_p)_c$ ranges from 0.92 to 0.98 and ΔE ranges from 63 to 110 mV.[44] Also, as ligand denticity increases, the thermodynamic stability constant of a complex increases and its peak potential becomes more negative.[44]

In addition to the reduction at −0.972 V, reductions are also observed at −1.45, −1.10, and −0.614 V (Figure 1). The peak at ca. −1.45 V is the reduction of Fe(II) to Fe(0); it was observed in all voltammograms of the ferric complexes.[30,39] The shoulder at −1.10 V is attributable to reduction of excess free ligand, as indicated by measurements of the ligand in the absence of added iron (data not shown).

The SWV acquired upon addition of Alt-A to the Fe(III)−Bis-tris solution varied with time. The SWV obtained within two minutes after addition of Alt-A to the Fe(III)−Bis-tris solution shows a larger current for the −0.614 V peak than at 1.5 h (Figure 1, a vs b). Over time, the peak at −0.614 V decreases (ca. 1 order of magnitude) and corresponds to a 4-fold increase of the −0.972 V peak (Figures 1b and 2); the reaction appears to be complete in ca. 15 min, indicating that full complex formation is relatively slow (see below).

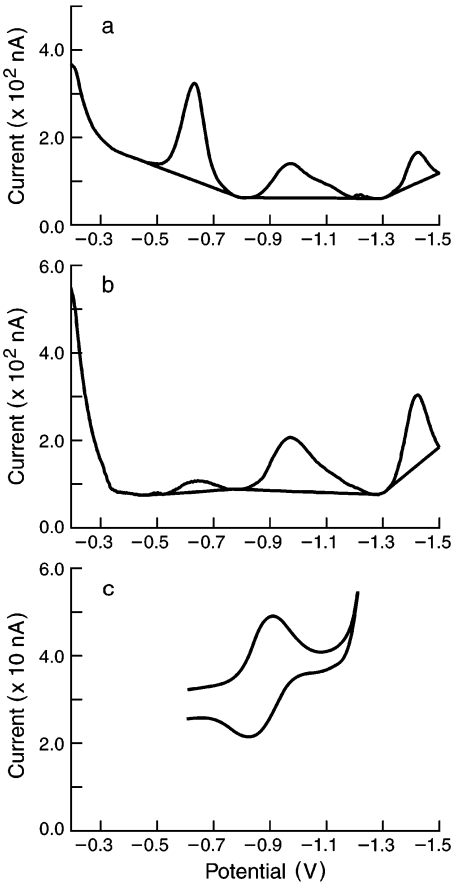


Figure 1. Square wave voltammetry (SWV) following Fe(III)−Alt-A complex formation and cyclic voltammetry (CV) of Fe(III)-Alt-A. (a, b) Square wave voltammograms within 2 min and at 1.5 h, respectively, after addition of 40 μM Alt-A to 10 μM Fe(III) in 5 mM Bis-tris buffer, pH 7 (potentials vs SCE). (c) CV of Fe(III)−Alt-A (32 μM of Fe(III) and 33 μM Alt-A after 1 h of equilibration) in 5 mM Bis-tris at pH 7.7 showing the reversibility of the reduction wave.

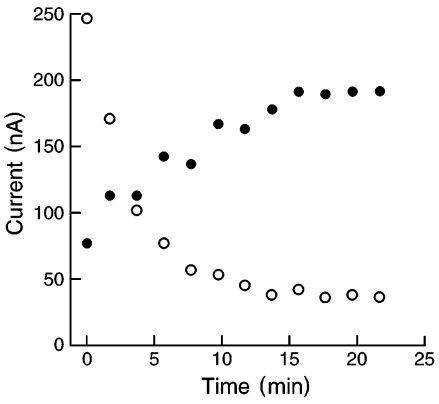


Figure 2. Time−course for the decay of the −0.614 V peak (open circles) and the appearance of the −0.972 V peak (solid circles) in the SWV upon addition of Alt-A to Fe(III) in Bis-tris buffer, pH 7. Conditions: 40 μM Alt-A added to 10 μM Fe(III) in 5 mM Bis-tris buffer, pH 7.

(42) Loomis, L. D.; Raymond, K. N. *J. Coord. Chem.* **1991**, *23*, 361−387.
(43) Barbeau, K.; Rue, E. L.; Bruland, K. W.; Butler, A. *Nature* **2001**, *413*, 409−413.
(44) Spasojeviæ, I.; Armstrong, S. K.; Brickman, T. J.; Crumbliss, A. L. *Inorg. Chem.* **1999**, *38*, 449−454.

Table 1. Masses of Alt-A and Fe(III)−Alt-A from Electrospray Mass Spectrometry in Positive and Negative Ion Modes

ionization mode	Alt-A	Fe(III)−Alt-A
+esi	928.3, 464.8[a]	981.1 (1057.1)[b]
−esi	926.2	979.2, 489.2[a]

[a] Where two values of *m*/*z* are given, the second number is for the doubly ionized species. [b] The value in parentheses represents the K_2(Fe−Alt-A) ion, which implies replacement of 5 protons from alterobactin A by K^+ and Fe^{3+}.

Spectrophotometric observation of Fe(III)−Alt-A complex formation upon addition of Alt-A to Fe(III) in Bis-tris pH 7 shows a steady increase in the absorbance at 479 nm [i.e., the λ_{max} for Fe(III)−Alt-A] consistent with the time−course for complexation observed in the SWV experiments (data not shown).

Using eq 3 which defines the Fe(III) "chelate scale", the potential at −0.972 V gives a value of log $K = 51 \pm 2$ for the thermodynamic constant of the Fe(III)−Alt-A complex. This value is in agreement with log $K = 49-53$ estimated by competition with EDTA.[26] Similarly, the reduction potential at −0.614 V corresponds to a complex with a log K of 42.2 ± 1.2, which is substantially smaller than that for the fully formed product. Because a unique UV−vis absorption maximum, distinct from the λ_{max} of the fully formed Fe(III)−Alt-A complex, was not observed immediately after addition of Alt-A to the Fe(III)−Bis-tris solution, where the current for the −0.614 V peak is highest, we do not yet have a spectrophotometric probe of the coordination environment of this species.

Stoichiometry of Complex Formation between Iron(III) and Alterobactin A. To determine the stoichiometry of iron(III) coordination to alterobactin A, the ferric complex of Alt-A was prepared and isolated by column chromatography and analyzed for iron and siderophore content (see Materials and Methods). The isolated iron(III) complex of Alt-A was found to contain 87 μM iron and 89 μM siderophore, establishing a 1:1 ratio of iron(III) to alterobactin A. A Job's plot of continuous variations[45] shows a maximum at 0.5 mol ratio corresponding to a 1:1 complex for Fe(III)−Alt-A (Figure S-1, Supporting Information). In addition the UV−vis spectrum of Fe(III)−Alt-A, i.e., λ_{max} 479 nm, is invariant between pH 4 and 9 (Figure S-2, Supporting Information).

The masses of Alt-A and Fe(III)−Alt-A, determined by electrospray mass spectrometry in both positive and negative ion modes, are summarized in Table 1. The ferric complexes show a mass increase of 53 as a result of addition of iron(III) and loss of three protons to neutralize the charge. Thus, this mass increase is consistent with a 1:1 Fe(III)−Alt-A complex, as isolated, above.

A potentiometric titration was performed to determine the number of protons released upon coordination of Fe(III) to Alt-A. The potentiometric titration of the reaction of 9.8 μmol of Alt-A and 9.8 μmol $Fe(ClO_4)_3$ in 10 mL of 0.1 M KNO_3 is shown in Figure 3. A Gran's plot[34,35] of the data indicates

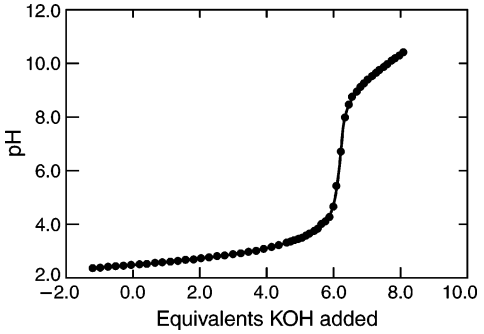


Figure 3. Potentiometric titration of Fe(III)−Alt-A. Conditions: 9.8 μmol of Fe(III)−Alt-A in 10 mL of 0.1 M KNO_3 titrated with 0.1039 M KOH. The baseline is corrected for excess acid added from the $Fe(ClO_4)_3$ solution (see Materials and Methods).

that the end point occurs at 5.9 ± 0.1 equivalents of base; therefore six protons are released from Alt-A upon complexation of iron(III). The release of six protons is consistent with coordination of iron(III) by both catecholate oxygen atoms and both oxygen atoms of each β-hydroxyaspartate.

Ga(III)−Alt-A Complex. In the ^{1}H NMR spectrum of Ga(III)−Alt-A, the resonances for C_4-C_6, i.e., catechol protons, shift from 7.04, 6.82, and 7.21 ppm to 6.57, 6.51, and 7.10 ppm (data not shown). Previous observations of gallium(III) complexes of enterobactin,[46] chrysobactin,[47] and analogues,[48] all of which contain the catechol moiety, show similar shifts. The protons of C_α and C_β of both β-hydroxyaspartate moieties are also shifted downfield (from 5.15, 5.03, 4.93, and 4.83 ppm to 4.99, 4.60, 4.42, and 4.05 ppm) in the Ga(III)−Alt-A complex relative to the free ligand. Thus, the shift in these resonances on complexation is consistent with metal coordination by the catecholate and both β-hydroxyaspartate moieties.

The 500 MHz ^{1}H NMR spectrum of Ga(III)−Alt-A in D_2O shows three resonances at 9.3, 10.9, and 11.7 ppm which are observed immediately after dissolving the complex in D_2O (Figure 4, top spectrum). These resonances arise from the glycine amide protons (i.e., the NH between C_{18} and C_{19} and the NH between C_{30} and C_{31}) and the amine proton of the 4,8-diamino-3-hydroxyoctanoic acid fragment (i.e., the amine on C_{12}). These protons slowly exchange with deuterium and become NMR silent after at least 1 day in D_2O (Figure 4, bottom spectrum). The slow exchange is likely to arise from hydrogen-bonding interactions. Hydrogen bonding between the proton of an amide (i.e., formed from a dihydroxybenzoate moiety and an amine, as in the NH between C-7 and C-8) and the *ortho* oxygen of catecholate is well established.[49,50] The structure prediction by a MM+ (Hyperchem) simulation of the of Fe(III)−Alt-A complex[26]

(45) Rossotti, F. J. C.; Rossotti, H. *The Determination of Stability Constants*; McGraw-Hill: New York, 1961; pp 47−53.

(46) Llinas, M.; Wilson, D. M.; Neilands, J. B. *Biochemistry* **1973**, *12*, 3836−3843.

(47) Persmark, M.; Neilands, J. B. *BioMetals* **1992**, *5*, 29−36.

(48) Tor, Y.; Libman, J.; Shanzer, A.; Felder, C. E.; Lifson, S. *J. Am. Chem. Soc.* **1992**, *114*, 6661−6671.

(49) Garrett, T. M.; Cass, M. E.; Raymond, K. N. *J. Coord. Chem.* **1992**, *25*, 241−253.

(50) The hydrogen bond between the NH proton and the *ortho*-catecholate oxygen in Alterobactin A using the MM+ simulation (Hyperchem) was not predicted (Figure 2 in ref 26) due to constraints on the dihedral angles in that program.

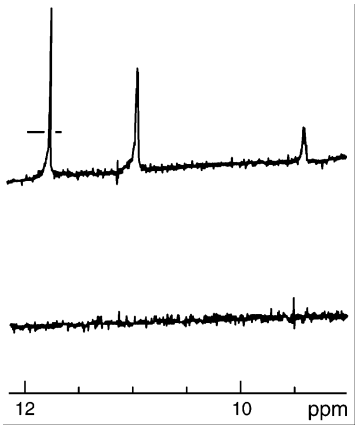


Figure 4. 500 MHz ^{1}H NMR spectra of the Ga(III)−Alt-A complex in D_2O. Top spectrum: immediately after preparation of the complex. Bottom spectrum: after 5 days. Conditions: 0.6 mM Ga(III)−Alt-A in 99.8% D_2O. The proton resonances at 9.3, 10.9, and 11.7 ppm slowly exchange with deuterium and become NMR silent.

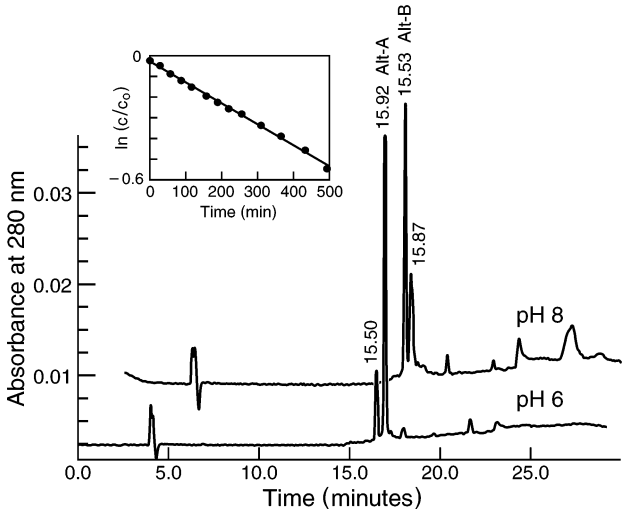


Figure 5. HPLC chromatograms after incubation of alterobactin A at pH 6 or pH 8 for 24 h. Conditions: 0.2 mM Alt-A in 0.2 M phosphate buffer at pH 6 or pH 8. The inset shows that the hydrolysis of Alt-A to Alt-B is a first-order process with $t_{1/2} = 11.6$ h at pH 8.

predicted hydrogen bonding between a glycine amide proton (NH between C_{30} and C_{31}) to the C_{20} carbonyl oxygen and between a glycine amide proton (NH between C_{18} and C_{19}) to the C_{12} amine nitrogen. In addition, the ^{1}H NMR spectrum of Ga(III)−Alt-A shows increased anisotropy of all the proton resonances, with the exception of the arginine protons, indicative of a more rigid structure in the metal complex than in the free siderophore (data not shown).

Stability of Alterobactin A. At neutral or alkaline pH, Alt-A undergoes a base-catalyzed hydrolysis of the lactone ring to form Alt-B, which was established by HPLC (see Figure 5 and Materials and Methods). At pH 8, the hydrolysis is first order in the concentration of alterobactin A with a half-life of approximately 11.6 h (Figure 5 inset). In contrast, the Fe(III)−Alt-A complex is stable in solution when stored in the dark, with little absorbance change observed over three weeks at pH 8. This stability indicates that the lactone ring does not hydrolyze in the Fe(III) complex. HPLC chromatograms of the ligand removed from the complex after 3 weeks show no evidence of conversion to alterobactin B. In addition, the Fe(III)−Alt-A complex is photoreactive, as a result of the complexation of the β-hydroxycarboxylate group to Fe(III); the identity of the photooxidized products is in progress.

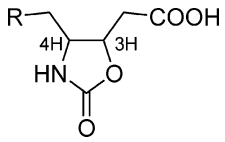


Figure 6. Oxazolidinone derivatives: R = $(CH_2)_3HN_2$ for 4,8-diamino-3-hydroxyoctanoic acid fragment from Alt-B; R = *i*-Pr for 4-amino-3-hydroxyl-6-methylheptanoic acid.

Stereocenters of Amino Acids, β-Hydroxyaspartic Acid, and 4,8-Diamino-3-hydroxyoctanoic Acid Moieties in Alterobactin A. The absolute configuration of β-hydroxyaspartic acid in Alt-A was determined by comparison of the GC retention times of the *N*-(trimethylsilyl)-*O*-(pentafluoropropionyl)-β-hydroxyaspartate dimethyl ester derivative of the siderophore-derived β-hydroxyaspartic acid to that of the derivatized standards, i.e., the *d*/*l* mixture of *threo*- and *erythro*-β-hydroxyaspartic acid.[51] The four standards were synthesized according to the method of Hedgcoth et al.[52] The hydrolyzed siderophores were found to contain *S*,*S*-β-hydroxyaspartic acid, as was also confirmed by co-injection with the 2*S*,3*S*-β-hydroxyaspartic acid standard. In addition, L-Arg and D-Ser were found to be present.[26]

To determine the absolute configuration of the 4-amino, 3-hydroxy portion of the 4,8-diamino-3-hydroxyoctanoic acid fragment of the alterobactins, the four diastereomers were analyzed for their retention times by chiral HPLC and for their chemical shifts by ^{1}H NMR. Marfey's reagent[53] derivatization of the siderophore-derived 4,8-diamino-3-hydroxyoctanoic acid fragment had the same HPLC retention time as one of the two 4*S* amino−hydroxy derivatives (i.e. 4*S*,3*R* or 4*S*,3*S*). The absolute configuration of the 4-amino 3-hydroxy portion was then determined to be 4*S*,3*R* by comparison of the ^{1}H chemical shifts and coupling constants of the oxazolidinone derivative (Figure 6) of the tryptic-digest product of alterobactin B (i.e. C_1−C_{26}) with published values of the related compound, i.e., the oxazolidinone derivatives of (3*S*,4*S* and 3*R*,4*S*)-4-amino-3-hydroxyl-6-methylheptanoic acid.[54] The chemical shift of the C-3 proton from the siderophore-derived oxazolidinone in D_2O was 5.16 ppm with a coupling constant, J_{3-4}, of 8 Hz, which compares favorably with a chemical shift of 5.1 ppm and a coupling constant of 8.8 Hz for the 4*S*-amino-3*R*-hydroxyl-6-methylheptanoic acid oxazolidinone derivative in methanol.[51] By comparison, the corresponding chemical shift and coupling constant of the 3*S*,4*S*-carbonyldiimidazole derivative of 4-amino-3-hydroxy-6-methylheptanoic acid are 4.5 ppm and 5.0 Hz,[51] respectively, which clearly differ from the siderophore-derived fragment data.

(51) Demange, P.; Abdallah, M. A.; Frank, H. *J. Chromatogr.* **1988**, *438*, 291−297.
(52) Hedgcoth, C.; Skinner, C. G. *Biochem. Prep.* **1963**, *10*, 67−72.
(53) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(54) Rich, D. H.; Sun, E. T. O.; Ulm, E. *J. Med. Chem.* **1980**, *23*, 27−33.

Table 2. Summary of the Reduction Potential Measurements in the Initial and Final Complex Formed between Fe(III) and Alt-A Obtained by Square Wave Voltammetry and Estimation of the Stability Constants Derived from the "Chelate Scale" of Taylor et al.[30]

siderophore complex	$E°$ (V vs SCE)	stability const (log K)
Fe(III)−Alt-A pH 7, initial complex[a]	−0.614	42.2 ± 2
Fe(III)−Alt-A pH 7, later complex[a]	−0.972	51 ± 2

[a] Initial and final complex refers to the peaks observed in Figure 1a,b at −0.614 and −0.972 V, respectively.

Discussion and Conclusions

The reduction potentials determined by SWV and the estimated stability constants for the ferric complex of Alt-A obtained from the chelate scale are compiled in Table 2. The reduction potential of Fe(III)−Alt-A corresponds to a proton-independent stability constant of $10^{51\pm2}$, which agrees with our original estimation of $10^{49}-10^{53}$ by EDTA competition, in which the pK_a of the α-hydroxyl proton of β-hydroxyaspartic acid was estimated as 16−18.[26] The potentiometric titration showing the release of six protons upon Fe(III) complexation to Alt-A (Figure 3), and the ^{1}H NMR shifts of the Ga(III) complex relative to the free ligand is consistent with a complex in which Fe(III) is coordinated by both catecholate oxygen atoms and both oxygen atoms of each β-hydroxyaspartate residue. The UV−vis spectrum of Fe(III)−Alt-A (Figure S-2, Supporting Information) is invariant over a wide pH range, further establishing the integrity of the hexacoordinate Fe(III)−Alt-A complex. The reaction of Alt-A with Fe(III) to form the hexacoordinate complex is complete within 15 min under the conditions of these experiments. An initial complex forms that is consistent with a four-coordinate complex on the basis of the peak potential in Table 2 and known four-coordinate complexes used to construct the Fe(III)−chelate scale.[30] We propose that catecholate coordinates Fe(III) first followed by proton loss and coordination of the β-hydroxyaspartate group.

Over 99% of Fe(III) present in surface seawater is complexed to an organic ligand.[54−57] While the identity of the ligands are not yet known,[58] in part because they are typically present at a concentration of <4 nM in seawater, the conditional stability constant of the FeL complexes has been found to range from 10^{19} to 10^{23}.[54−57] The conditional stability constants of a range of siderophores and porphyrins complexed to Fe(III) measured in seawater[43,59−61] have been found to be similar to or larger than the unknown natural ligands. Among these siderophores, alterobactins A and B have the highest conditional stability constants[59,61,62] and are thus able to complex >99% of the total iron, thus making the alterobactin-complexed iron available to the source bacterium, *A. luteoviolacea*.

Alterobactins A and B are the first natural products to contain the 4,8-diamino-3-hydroxyoctanoic acid (referred to as LySta in the reference),[63] which has been implicated in inhibition of certain peptide proteases.[54] Such fragments or other protease inhibitors may play a role in protecting the integrity of these molecules in seawater where they must function. To further elucidate the molecular mechanisms oceanic microorganisms use to acquire iron, we are continuing our investigations of marine siderophores and the iron uptake mechanisms of *A. luteoviolacea*, as well as other marine bacteria.

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Supporting Information Available: A Job's plot of continuous variations for Fe(III)−Alt-A and UV−visible spectra of Fe(III)−Alt-A vs pH. This material is available free of charge via the Internet at http://pubs.acs.org.

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(55) Gledhill, M.; van den Berg, C. M. G. *Mar. Chem.* **1994**, *47*, 41−54.
(56) Rue, E. L.; Bruland, K. W. *Mar. Chem.* **1995**, *50*, 117−138.
(57) Wu, J.; Luther, G. W. III. *Mar. Chem.* **1995**, *50*, 159−177.
(58) Macrellis, H. M.; Trick, C. G.; Rue, E. L.; Smith, G.; Bruland, K. W. *Mar. Chem.* **2001**, *76*, 175−187.
(59) Hutchins, D. A.; Witter, A. E.; Butler, A.; Luther, G. W., III. *Nature* **1999**, *400*, 858−861.
(60) Barbeau, K.; Rue, E. L.;. Trick, C. G.; Bruland, K. W.; Butler, A. *Limnol. Oceanogr.* **2003**, *48*, 1069−1078.
(61) Witter, A. E.; Hutchins, D. A.; Butler, A.; Luther, G. W., III. *Mar. Chem.* **2000**, *69*, 1−17.
(62) Previously Rue and Bruland[56] reported a calculation of the conditional stability constant of alterobactin A in which they erroneously considered our measured value of the conditional stability constant at pH 6[26] to be at pH 8. Our experimentally measured conditional stability constant was carried out by competition with EDTA at pH 6, because EDTA could not compete with Alt-A at pH 8.[26] The conditional stability constant of Fe(III)−Alt-A measured electrochemically at pH 8 is $10^{23.9}$.[61] The range of conditional stability constants of FeL (L is unknown) in seawater measured by the same method is $10^{18.6}-10^{23.7}$.[55−58] Thus, contrary to the conclusion of Rue and Bruland,[56] alterobactin A is very effective at complexing Fe(III) in seawater.
(63) Salituro, F. G.; Agarwal, N.; Hofmann, T.; Rich, D. H. *J. Med. Chem.* **1987**, *30*, 286−295.