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Iron(III) Coordination Chemistry of Linear Dihydroxyserine Compounds Derived from Enterobactin[1]

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The solution thermodynamic stability and iron transport properties of the hydrolysis products of enterobactin are reported. Enterobactin, a tricatechol siderophore, is a cyclic trilactone that undergoes three stepwise hydrolysis reactions to form a linear trimer, then dimer, and finally the monomeric *N*-(2,3-dihydroxybenzoyl)serine. The protonation and ferric complex formation constants of the linear trimer and linear dimer have been determined. The enthalpies of formation of the linear trimer complex and ferric enterobactin have been measured by calorimetric titrations. The greater stability of the ferric enterobactin complex, compared with tricatechol ligand analogues, is about one-third enthalpic and two-thirds entropic in origin. The Δ chirality of the ferric enterobactin is also maintained by the linear trimer complex. Assays of the mediation of iron uptake in *Escherichia coli* RW193 show that both the linear dimer and linear trimer are nearly as effective as enterobactin in mediating iron uptake in this organism.

Introduction

Of all the natural[2-4] and synthetic[5] iron(III) chelating agents, enterobactin (**1** in Figure 1) remains the most effective at physiological pH. This natural product can be viewed as a cyclic trilactone formed from the amide monomer, *N*-(2,3-dihydroxybenzoyl)serine (DHBS). The result is a trigonally symmetric ligand that contains three catechol groups and is a hexadentate ligand with an unequaled affinity for Fe(III). We and others have described the coordination properties of this ligand, which are significant because of its role as a microbial iron transport agent (siderophore).[6,7] Siderophores are low molecular weight compounds produced and excreted by microorganisms for the purpose of solubilizing environmental iron and facilitating its transport into the microbial cell. The soluble ferric siderophore complex typically is recognized by specific cell surface receptors. In the case of *Escherichia coli* and other enteric bacteria, the most effective siderophore is enterobactin, which has a metal-ligand stability constant with log $K_f \approx 49$. Particularly since the complex is so stable, an early question regarding the role of enterobactin-mediated iron transport into a bacterium was how the iron is released once the ferric enterobactin complex is absorbed by the cell. Since hydrolysis of a ligand has been found to accompany iron uptake, one plausible mechanism would be hydrolysis of the ligand portion of the iron complex, leading to a less stable complex and easier iron removal. This mechanism was supported by the observation that the extremely low reduction potential of ferric enterobactin (-1.0 V above pH 10) is raised in a stepwise manner as the ligand is hydrolyzed: first to the linear trimer complex (Figure 1), then to the dimer, and finally to the monomeric DHBS complex.[2] However, the redox potentials of these complexes only give information about the relative affinity of the ligands for Fe(III) versus Fe(II) under the conditions studied. They do not provide specific equilibrium constants for the metal complexation reaction or the protonation constants of the ligands. That determination was one purpose of this study.

The second purpose was to examine those features of ligand design that make for effective Fe(III) complexing agents. A number of synthetic analogues of enterobactin have been prepared that are intended to be highly specific iron(III) sequestering agents, potentially of use as therapeutic agents in human iron overload.[5] These compounds have formation constants (log K_f) with iron in the range of 40-43, which, although highly stable, are substantially lower than that of the ferric enterobactin complex. Is this due to the fact that the amide groups of enterobactin are all exocyclic to the ring, compared to the synthetic analogues, most of which have nitrogen incorporated as part of the backbone of the ligand? Alternatively, is this added stability due to a conformational feature of the trimeric ring (which is thought to predispose the ligand for binding and which is lost upon hydrolysis)? The answers to these questions are a second purpose to this study.

The hydrolysis products of desferrienterobactin, first the linear trimer, then the linear dimer, and finally DHBS (Figure 1), were first isolated from a microbial fermentation and structurally characterized by O'Brien and Gibson.[8] However, the coordination chemistry properties, including the question of whether chirality is maintained in the metal complexes of these ligands, have not been examined. Evaluation of the possible role that such complexes might themselves play as iron uptake agents in *E. coli*, and their relative effectiveness with respect to their chemical and structural properties, is a third purpose of the present study.

Direct calorimetric measurements of the heats of reaction have been utilized to determine the enthalpies for both the metal complexation and protonation reactions. These results establish the relative importance of the enthalpy and entropy components of the solution free-energy changes.

Experimental Section

Microbial Fermentation and Ligand Isolation. *E. coli* AN102 was kindly provided by Professor Dick van der Helm. Enterobactin and linear DHBS compounds were isolated from the culture supernatant of *E. coli* AN102 as previously described[2,6] (ϵ_{316} = 9500 and ϵ_{315} = 9700) except that DEAE Sephadex was used instead of DEAE cellulose. Chemical analyses and fast atom bombardment (FAB) mass spectrometry were performed by the Analytical Service, Department of Chemistry, University of California, Berkeley, CA. Enterobactin and the DHBS linear trimer were isolated in sufficient quantities so that they could be accurately weighed and analyzed. Anal. Calcd (found) for enterobactin, $C_{30}H_{27}N_3O_{15}\cdot H_2O$ (mol wt 687): C, 52.45 (52.01); H, 4.20 (4.08); N, 6.11 (6.05). Calcd (found) for DHBS linear trimer, $C_{30}H_{29}N_3O_{16}\cdot H_2O$ (mol wt 705): C, 51.06 (51.20); H, 4.42 (4.32); N, 5.96 (5.82). The ligands all gave sharp parent ion peaks (m + 1) by FAB mass spectrometry, with no evidence for contaminating molecular species. The extinction coefficients (M^{-1} cm^{-1}) at the wavelength maxima for each of these compounds in methanol are ϵ_{316} = 9500 for enterobactin and ϵ_{315} = 9700 for the DHBS linear trimer. Only a small amount of the DHBS

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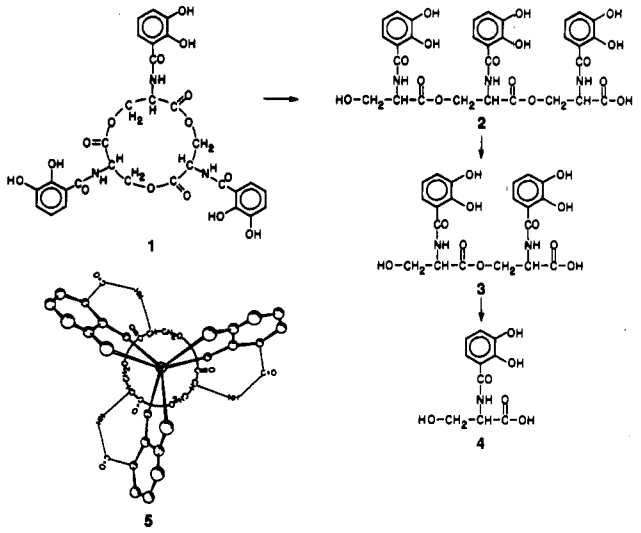

Figure 1. Structures of enterobactin and its hydrolysis products: enterobactin (**1**); *N*-(dihydroxybenzoyl)serine (DHBS) linear trimer (**2**); DHBS linear dimer (**3**); DHBS (**4**); hexacoordinate ferric enterobactin (**5**).

linear dimer was isolated, so this ligand was assayed by measuring the absorbance in methanol at 315 nm and using two-thirds the extinction coefficient for the DHBS linear trimer. [This estimate is justified because in methanol the catechol and carboxyl groups are fully protonated; the absorption spectra for all three compounds are nearly identical, and there appears to be little hypochromic effect.]

Chromatography and Electrophoresis. Electrophoresis of enterobactin and DHBS compounds was done on a Savant TLE 20 electrophoresis apparatus with pyridine/acetic acid/water (14:10:930) buffer pH 5, on Whatman 3-mm paper at 1000 V and 60 mA. At this pH enterobactin is uncharged and remains at the origin. The DHBS linear trimer, dimer, and monomer are all monoanions due to their free carboxyl groups and migrated toward the anode with the following relative mobilities: DHBS, 1.0 (5.4 cm); DHBS linear dimer, 0.6; DHBS linear trimer, 0.4. This is similar to the mobilities previously reported in a different solvent system.[9] Thin-layer chromatography was performed on Baker C_{18} reversed-phase plates with methanol/25 mM formic acid (65:35) solvent. Ligands were visualized by either fluorescence under UV light or by spraying the chromatogram with $FeCl_3$ in methanol. Isolated DHBS compounds were found to be pure by both electrophoresis and thin-layer chromatography.

HPLC purification of MECAMS employed a C_{18} reverse-phase semipreparative column (Altex) on a Beckman-Altex Model 340 system connected to a Hewlett-Packard 1040a UV/vis chromatographic detector. A Gilson model fraction collector was used in manual mode with a programmed delay time. A ca. pH 3.5 buffer of 0.1 M triethylamine and 0.2 M formic acid was used in methanol/water mixtures. A 0.11-g sample of crude K_3MECAMS was dissolved in 3 mL of 20% (by volume) methanolic buffer and the solution passed down a short (2-mL bed) column of Bio-Rad AG 50W-X8 cation-exchange resin preequilibrated with the same triethylammonium formate buffer. Injections of 0.45 mL of the ion-exchanged MECAMS solution were used followed by a ramp from 20 to 70% methanol over 10 min. At least nine peaks could be identified when absorbance at 310 nm was monitored; the collected peak (at 7.3 min) was 10-fold higher than any of the other peaks (which probably arise from incomplete sulfonation of the ligand). When the fractions corresponding to this peak were combined from nine injections and an aliquot was reinjected, a single peak was observed.

Spectroscopy. Ultraviolet and visible spectra were measured on a Hewlett-Packard 8450 A spectrophotometer equipped with an 82901 M flexible disk drive. Circular dichroism spectra were recorded on a Jasco J-500C spectropolarimeter equipped with a Jasco DP-500N data processor. The CD spectrometer was calibrated at 33.6 mdeg at 290.5 nm with a 1 mg/mL sample of *d*-10-camphorsulfonic acid in a 1-mm cell.

Spectrophotometric pH Titrations. The visible spectra of the Fe(III)–DHBS linear trimer (1:1) and Fe(III)–DHBS linear dimer (1:1) complexes were recorded as a function of pH in a custom flow cell that fit in the spectrometer and allowed titration of the solution without disturbing the position of the quartz cell in the spectrometer. Water-saturated argon gas pressure was used to push the sample out of the quartz window into a reservoir (8-mL capacity) that had a stirrer, pH electrode, and buret protected from oxygen and temperature controlled at 25 °C. The pH was adjusted in small increments by the addition of nitric acid from the buret, and after the pH stabilized, the solution was allowed to flow back into the quartz window and its visible spectrum was recorded.

Potentiometric Studies. All chemicals were reagent grade. The computer-controlled titration apparatus has been described elsewhere.[10] The procedures used in analyzing these data were also the same as those used earlier.

Linear trimer (10 mg) was dissolved in doubly distilled water in a water-jacketed cell kept at 25 ± 0.5 °C via a constant-temperature bath. Ionic strength of the solution was adjusted to 0.100 M with KNO_3.

To minimize excess ligand hydrolysis, the pH of the ligand solution was adjusted to 8.6 with carbonate-free KOH and the solution was titrated with standardized HNO_3. After titration, the ligand solution was acidified to pH 2 and extracted with ethyl acetate. Electrophoresis revealed that less than 5% of the ligand was hydrolyzed in the course of titration.

Titrations of the 1:1 Fe(III)/ligand solutions were similarly performed and were uncomplicated (plots of the titration curves are available as supplementary material).

EDTA Competition Reactions. The proton-dependent formation constant of ferric linear trimer was determined spectrophotometrically by competition with EDTA as previously described.[11,12] Spectra were recorded 8–12 h after mixing to ensure equilibrium condition. The proton-dependent formation constant of ferric linear dimer was also determined by spectrophotometric competition with EDTA over a pH range 5.5–7.5.

Calorimetry. A Tronac Model 450 isoperibol calorimeter employing a Northstar Horizon microcomputer was used with locally developed software written in BASIC. The digital microvoltmeter output of the thermistor/Wheatstone bridge was converted to ΔT (the temperature within the 25-mL reaction vessel relative to the surrounding 25 °C constant-temperature bath) by using the experimentally determined relation (for $|\Delta T| \leq 2°$ C) $\Delta T = (26.9\ °C\ V^{-1})E + (13.1\ °C\ V^{-2})E^2$. The heat capacity of the solution pulse reaction vessel was measured by using a calibrated resistance heater both before and after the addition of titrant and was assumed to follow $C_p = a + b\Delta V$, where a and b are constants and ΔV is the volume of titrant added.[13] The heating of the solution from stirring and thermistor and reaction vessel heat leak were approximated by the equation $q_{bckgd} = (c_1 + c_2\Delta V)(1 - c_3\Delta T)$. The constants c_1, c_2, and c_3 were determined from 100-s determinations of baseline $d(\Delta T)/dt$ values immediately before and 20–60 s after titrant delivery, as well as after the final heat capacity determination.

ΔT was monitored at every second before, during, and after titrant delivery and the rate of chemical heat generation determined as $q_{chem} = C_p(\Delta T/dt) - q_{bckgd}$. The quantity $\Delta H_{reacn} = \int q_{chem}\, dt$ was set to average zero over each 100 s prior to titrant delivery, and the value of $\Delta H°$ was based on the average of ΔH_{reacn} values over the 100-s baseline period starting 20 to 60 s after the titrant delivery. The longer delay following titrant delivery was generally used for the iron exchange reactions, although ΔH_{reacn} became essentially constant within a few seconds after the completion of the titration.

The $Fe(ClO_4)_3$ stock solution (A) was prepared from G. F. Smith nonyellow ferric perchlorate reagent and standardized with EDTA by using Variamine Blue indicator.[14] Nitrilotriacetic acid (NTA, Eastman Chemicals) and tris(hydroxymethyl)aminomethane (Tris, Sigma Chemicals) were oven-dried before weighing. The supporting electrolytes and $HClO_4$ and KOH were reagent grade. The synthesis of MECAMS has been described elsewhere.[15] The triethylammonium formate buffer was partially removed by successive rotary evaporations, and the pH was adjusted to 9 before addition of Tris and HCl to give solution H. The concentration of MECAMS was determined spectrophotometrically on the basis of an extinction coefficient (ϵ_{330} = 12 000) determined from a batch of K_3MECAMS, whose purity was determined potentiometrically.

Biological Transport Assays. In vivo biological transport assays in *E. coli* RW193 were performed by following the uptake of radioactively

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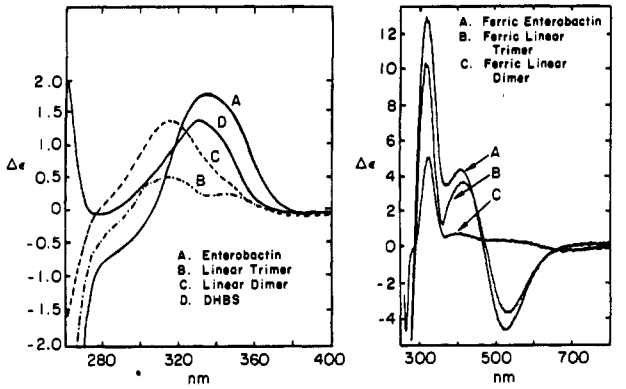

Figure 2. Circular dichroism spectra of the free ligands and ferric complexes of enterobactin and its hydrolysis products in aqueous solution at pH 7.5, 0.1 M HEPES buffer.

labeled iron complexes to enterobactin and DHBS compounds as previously described.[6]

Results and Discussion

CD Spectroscopy and Structure. Examination of CPK molecular models shows that, although the DHBS trimer backbone is more flexible than the cyclic enterobactin backbone, it is difficult to make molecular models of the Fe(III)–DHBS linear trimer in both cis and trans configurations and each of these in both Δ (right-handed propeller) and Λ (left-handed propeller) absolute configurations, for a total of four possible isomers. With ferric enterobactin, only cis isomers are sterically possible. Although both Δ and Λ isomers can be made in models, ferric enterobactin apparently exists in solution exclusively as the Δ isomer.[2]

The visible region CD spectra, which are due to ligand-to-metal charge-transfer transitions of ferric enterobactin and ferric DHBS linear trimer are very similar (Figure 2), suggesting the same (Δ) absolute configuration for both complexes. Molar elipticities are slightly greater for ferric enterobactin than ferric DHBS linear trimer. In contrast, the CD spectrum of the ferric DHBS linear dimer (1:1) is essentially featureless in the visible region, consistent with the Δ ↔ Λ equilibrium just described.

The CD spectra in the near-UV region for the free ligand forms of enterobactin and the trimer and dimer hydrolysis products are characterized by a weak positive feature from ligand-based n → π* transitions at approximately 320 nm (Figure 2) that increase in intensity upon metal binding.

Potentiometric Titrations. The potentiometric titrations (supplementary material Figure S1) of the linear trimer in the absence of metal ion are consistent with the expected seven dissociable protons in the ligand. No attempt was made to determine the three highest protonation constants of the catechol groups, since significant deprotonation of these phenolic oxygens occurs only at pH > 12. At that pH, rapid ligand degradation takes place through the hydrolysis of the ester linkages. Therefore, only the lowest four protonation constants were determined. These were refined by standard procedures,[16] to give log K values of 8.23 (4), 7.39 (4), 6.43 (6), and 2.5 (1).[17]

The potentiometric titration curve of the linear trimer in the presence of equal molar Fe(III) ion (supplementary material Figure S1) displayed the expected strong complexation of ferric ion by the ligand in the pronounced lowering of the Fe(III) curve; an inflection observed at $a = 7$ is assigned to formation of the totally deprotonated complex FeL. It was not possible to calculate the standard formation constant, K_{ML}, directly from the potentiometric data, since complete complexation occurs even at very low pH. However, a buffer region between $a = 4$ and $a = 7$ allowed the determination of the metal chelate protonation constants. Refinement of the potentiometric data[11] yielded log K_{MHL} = 5.75 (5), log K_{MH_2L} = 4.7 (1), and log K_{MH_3L} = 4.2 (1), where the chelate protonation constants are defined as

$$MH_{n-1}L + H \leftrightarrow MH_nL \qquad K_{MH_nL} = \frac{[MH_nL]}{[MH_{n-1}L][H^+]} \quad (1)$$

EDTA Competition Reactions. The formation constant of the ferric linear trimer was determined by competition with EDTA from pH 4 to 6 by using its known stability constant.[18] The competition equilibria were expressed by the following equations:

$$Fe(EDTA) + H_3L \leftrightarrow FeL + EDTA + 3H^+$$

$$K_1^c = \frac{[FeL][H^+]^3[EDTA]}{[Fe(EDTA)][H_3L]} = \frac{K_1^*}{K_{Fe(EDTA)}} \quad (2)$$

where

$$K_1^* = \frac{[FeL][H^+]^3}{[Fe][H_3L]} \quad (3)$$

for the reaction $Fe^{3+} + H_3L = FeL + 3H^+$. The proton-dependent formation constant of the ferric linear trimer (log K_1^*) thus determined is 6.5 (2).[17] If the three highest protonation constants of the linear trimer are assumed to have an average value very close to that of enterobactin,[22] the proton-independent formation constant of the ferric linear trimer, log K_{ML}, can be estimated as 43.0.

The proton-dependent formation constant of the ferric linear dimer was also determined by competition with EDTA. The equilibria were described by the following equations:

$$Fe(EDTA) + H_5L \leftrightarrow FeL + 5H^+ + EDTA$$

$$K_2^c = \frac{[FeL][H^+]^5[EDTA]}{[FeEDTA][H_5L]} = \frac{K_2^*}{K_{Fe(EDTA)}} \quad (4)$$

where

$$K_2^* = \frac{[FeL][H^+]^5}{[Fe][H_5L]} \quad (5)$$

The resultant ferric linear dimer formation constant, log K_1^* is 6.7 (1). If the protonation constants of the catechol phenolic oxygens of the linear dimer are assumed to be on average the same as that of enterobactin, and the protonation constant of the carboxylate group is assumed to be the same as that of the linear trimer, the conventional formation constant of the ferric dimer, log K_{ML}, can be estimated as 36.

Spectrophotometric Titration of Ferric DHBS Linear Trimer. The visible spectra of ferric enterobactin and ferric DHBS linear trimer are identical and do not change above pH 7.5, confirming that the ferric DHBS linear trimer is a hexacoordinate complex, $[Fe(\text{linear trimer})]^{4-}$, similar to $[Fe(ent)]^{3-}$ or synthetic tricatechol complexes; the extra negative charge of the linear trimer is due to the deprotonated carboxyl group (Figure 1). As the pH is lowered, the same spectral shift is observed with ferric DHBS linear trimer as was previously observed with ferric enterobactin, with an isosbestic point at 450 nm (Figure 3). The isosbestic spectral changes indicate an equilibrium between only two metal complexes with different visible spectra in this pH region. A Schwarzenbach plot[11] of the data was linear only when fit to a single-step protonation

$$[Fe(\text{linear trimer})]^{4-} + H^+ \leftrightarrow [HFe(\text{linear trimer})]^{3-}$$

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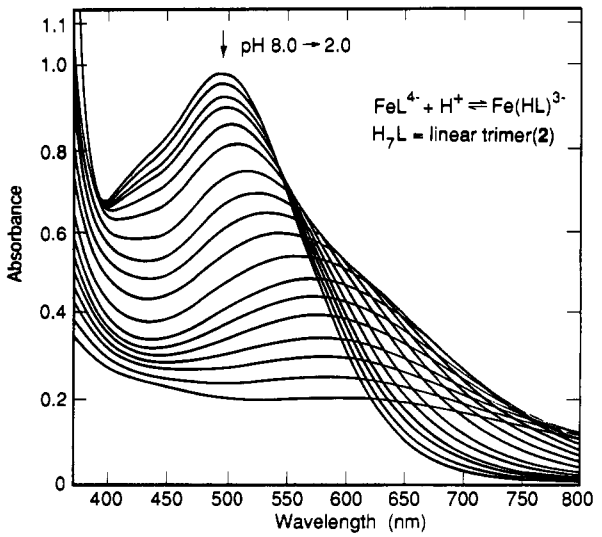

Figure 3. Spectrophotometric pH titration of 1:1 ferric complex of **2** (ferric linear trimer), in absorbance units corrected for dilution by multiplication by $V_{total}/V_{initial}$. The uppermost curve is the spectrum of the fully formed (tris(catecholate)) complex at pH 8.0. This is identical with the spectrum of ferric enterobactin. The lower curves result from stepwise decrease of the pH (lowermost curve is at pH 2.0).

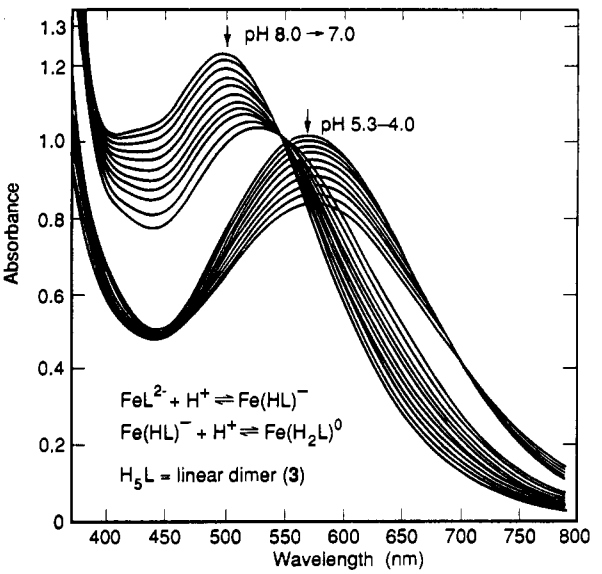

Figure 4. Spectrophotometric titration of the 1:1 ferric complex of **3** (ferric linear dimer), in dilution-corrected absorbance units (see Figure 3). The uppermost set of spectra are from pH 8.0 (uppermost curve) to 7.0. The lower set of spectra are from the pH range 5.3–4.0. The spectra recorded in between these two pH ranges are not plotted, since they obscure the two isosbestic points.

This result is qualitatively identical with what was previously observed with ferric enterobactin. However, log K (obtained from the slope of the Schwarzenbach plot) for the single-step protonation of the ferric DHBS linear trimer was 5.7, substantially higher than the value for ferric enterobactin (4.9). This parallels the increased stability of ferric enterobactin relative to the linear trimer complex.

Linear relations of values of the absorbance at a given wavelength and pH (i.e. a Schwarzenbach plot) utilize only a small fraction of the absorbance spectra data. They also can be used only where there is isosbestic behavior (i.e. only two absorbing species in equilibrium). Full utilization of all data can be accomplished by using factor analysis techniques and a least-squares fit of the equilibrium constants for the characterized species. Since the absorbance data for the linear trimer were very good but showed overlapping equilibria, factor analysis refinement was carried out with the program REFSPEC.[10] Five species, $Fe(H_nL)$, were identified for n = 0–4 and L^{4-}, the fully deprotonated linear trimer. The spectra of these species (resulting from the compo-

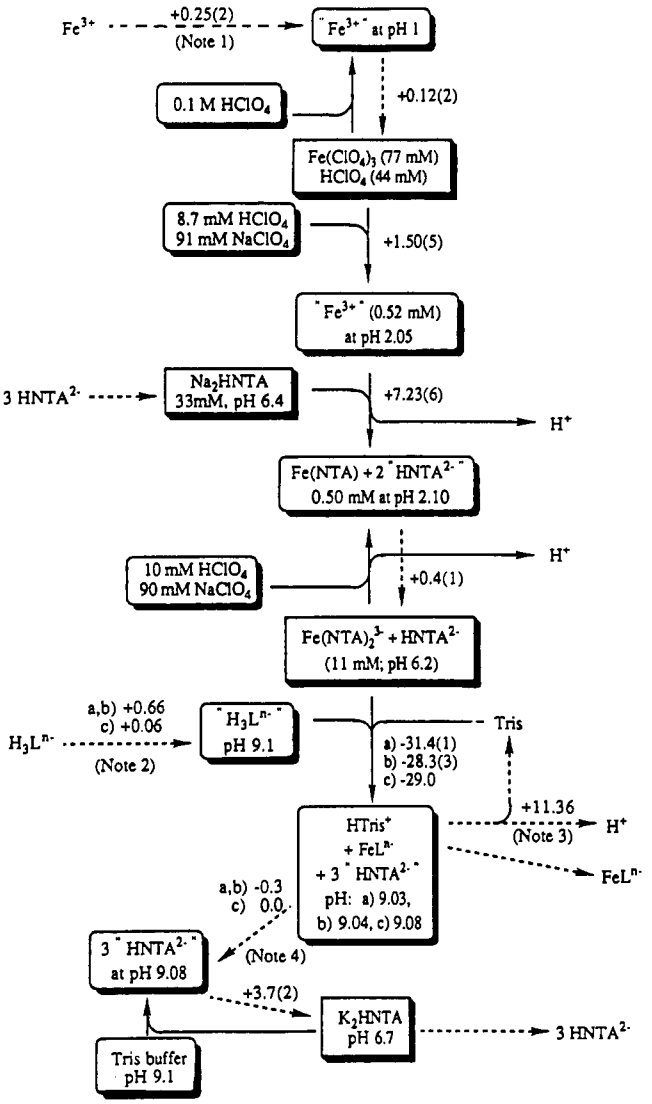

Figure 5. Schematic diagram to illustrate the procedures and values used in the calorimetric determination of the enthalpies of complex formation for $Fe^{3+}(aq) + H_3L^{n-} \leftrightarrow FeL^{n-} + 3H^+$, where L is as follows: (a) enterobactin (n = 3); (b) its linear trimer (n = 4); (c) MECAMS (n = 6). Rectangular and rounded boxes enclose titrant and titrand solutions, respectively. Solid arrows indicate reactions for which heats were measured; dashed arrows indicate heats for reverse reactions or from literature values (see notes). All heats are in kcal/mol, and numbers in parentheses represent the estimated standard deviations in the least significant digits, based on 3–4 replications of each measurement. Key for notes: (note 1) heat calculated for 2.4% hydrolysis of Fe^{3+} by using the thermodynamic constants (μ = 0.1, 25 °C) of ref 18; (note 2) calculated heat for the introduction of H_3L^{n-} into pH 9.1 Tris/HTris buffer, assuming 21% (a, b) and 2% (c)[21] protonation of H_3L^{n-}, as determined from their protonation constants (ΔH = −8.24 kcal/mol for $Hcat^- + H^+ = H_2cat$);[20] (note 3) value from ref 20; (note 4) titration of $HNTA^{2-}$ into pH 9.1 Tris buffer results in 20% proton transfer to Tris; the measured $\Delta H°$ is −3.7 kcal/mol for 3 mol of $HNTA^{2-}$ in the case when the final pH is 9.08; small correction (0.3 kcal/mol) is necessary for ligands a and b, since the pH was 9.03 instead of 9.08.

nentization of the spectra shown in Figure 3) are shown in Figure 6. The corresponding protonation constants (log K for the equilibria described by eq 1) are 5.81 (3), 4.82 (3), 4.32 (3), and 2.38 (2). (The first of these values corresponds to the one reaction characterized by the Schwarzenbach plot, with log K = 5.7.)

From the spectra of Figure 6 and the values of the protonation constants, we can characterize the first three protonation reactions as the formation of $[FeH_nL]$ complexes (n = 1–3) in which the phenolate m-oxygens of the 2,3-dihydroxybenzoyl (DHB) rings are protonated and there is a rearrangement of the structure to a "salicylate" mode of coordination (in which bidentate coordination occurs through the amide carbonyl oxygen and the o-O

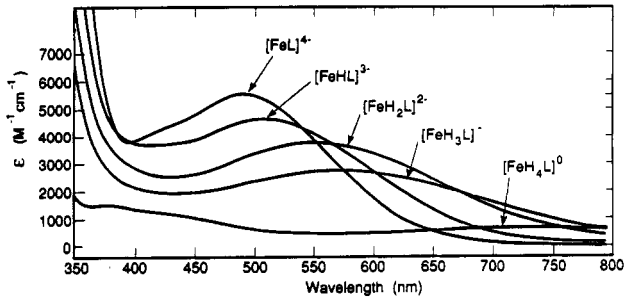

Figure 6. Extinction coefficient spectra from the factor analysis and refinement of the spectrophotometric pH titration of the 1:1 ferric complex of the linear trimer. For L^{7-} representing the fully deprotonated linear trimer (**2**), the species and their maximum absorbances (M^{-1} cm^{-1}) are as follows: FeL^{4-} (ϵ_{494} = 5400); $Fe(HL)^{3-}$ (ϵ_{508} = 4500); $Fe(H_2L)^{2-}$ (ϵ_{554} = 3700); FeH_3L^- (ϵ_{574} = 2700) and FeH_4L^0 (ϵ_{732} = 700). Also refined were log K_{FeH_4L} values of 5.81, 4.82, 4.38, and 2.38. Refinement was on 36 spectra (350–800 nm) of a 0.18 mM FeL^{4-} solution initially at pH 8.0 and acidified to pH 2.0.

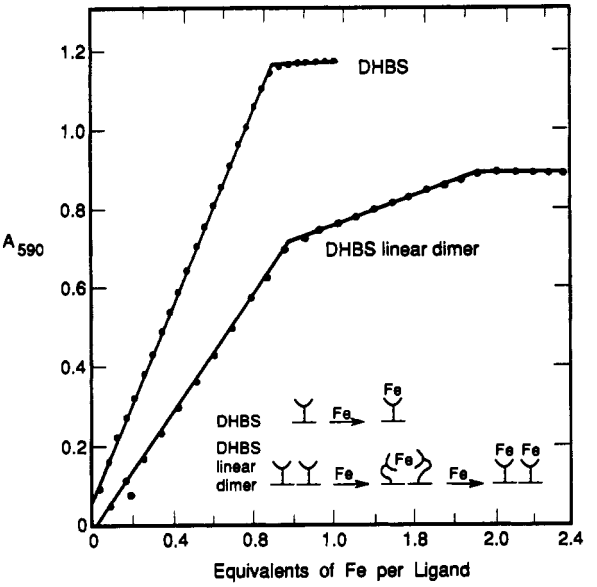

Figure 7. Plot of the absorbance at 590 nm (conditions the same as those in Figure 3) for the titration of solutions of the DHBS monomer (**3**) and linear dimer (**2**) with Fe(III). Solutions were buffered at pH 9, and ferric ion was added as the NTA complex.

from catecholate). This parallels the protonation behavior of ferric enterobactin,[7] except that the neutral enterobactin complex Fe(H_3ent) precipitates from solution, while the charged $[FeH_3L]^-$ linear trimer complex is soluble. The last protonation reaction (log K = 2.38) is accompanied by a sharp decrease in absorbance and a shift of λ_{max} to 732 nm. We interpret this as protonation and decomplexation of two of the (monoprotonated, salicylate-coordinated) catechol groups to give a ferric mono(catecholate) complex. Protonation of the terminal carboxylate group probably also occurs in this region (that protonation constant for the free ligand is 2.5), but this would not generate a significant change in the absorbance spectra.

Spectrophotometric Titration of Ferric DHBS Linear Dimer. Both enterobactin and the DHBS linear trimer have three catechoylamide chelating groups, such that one ligand molecule can satisfy all six coordination sites of ferric ion to form a typical red tris(catecholate)iron(III) complex. The DHBS linear dimer (Figure 1) has only two catechoylamide groups and binds to iron in a 1:1 stoichemistry to form a blue complex more typical of a ferric bis(catecholate). The spectrum of the ferric DHBS linear dimer of pH 5.3 is essentially the same as the spectrum reported for the bis(catecholate) $Fe(DMB)_2$,[11] where DMB (*N*,*N*-dimethyl-2,3-dihydroxybenzamide) is a monomeric analogue of the chelating groups in enterobactin and the DHBS compounds. In both these bis(catecholate) complexes, the remaining two iron coordination sites presumably are occupied by water molecules. Proof of the stoichiometry of these reactions of both DHBS monomer (**4**) and the linear dimer (**3**) is found in spectrophotometric titrations of the ligands with Fe(III) (supplementary material Figures S2 and S3). Plots of the absorbance at 590 nm versus equivalents of Fe(III) (Figure 7) show linear changes of absorbance with sharp breaks in the curves at 1.0 (iron mono(catechol) complex of DHBS and dicatechol complex of the linear dimer) and at 2.0 (iron mono(catechol) complex of the linear dimer). Increasing the pH of the ferric DHBS linear dimer solution between pH 7 and 8 results in a spectral shift to shorter wavelength, with a sharp isosbestic point. A Schwarzenbach plot of the data is linear if fit to a one-step protonation; the log K is 7.4. We interpret this shift as due to the hydrolysis of one of the two coordinating waters.

On lowering of the pH below 5.3, the formation of a new species was observed with about the same wavelength maximum but lower intensity. A poorly resolved isosbestic point is observed for this equilibrium at 700 nm. Schwarzenbach analysis of these data gives a linear plot for a one-step protonation to form an uncharacterized Fe(H^+ linear dimer) species with a protonation constant (log K) of 4.4.

Calorimetry. The $\Delta H°$ values for iron complexation at pH 9 by enterobactin and the linear trimer of DHBS were determined by solution calorimetry in order to determine if the greater iron-binding ability of enterobactin in aqueous solution is due primarily to entropic or enthalpic effects. To simplify the analysis of the calorimetric data, the measurement of ΔH should be made at a pH where a single protonation state of the ligand and of the ferric complex would be predominant. Unfortunately, enterobactin is insoluble below pH 7 because of precipitation of neutral H_6L and rapidly hydrolyzes at pH 10 and above, so pH 9 was chosen for this study. At pH 9, small amounts of H_4L^{2-} are present in addition to the triprotonated species H_3L^{3-}, and correction was made for this.

The buffer employed was 0.100 M Tris, 0.010 M Tris HCl, and 0.090 M KCl, which has μ = 0.1 M and pH (concentration scale[18]) 9.08.[19] Incubation of enterobactin and the DHBS linear trimer in this buffer at ca. 23 °C for 2 h results in hydrolysis of approximately one-third of the enterobactin to linear trimer and approximately 10% of the linear trimer to DHBS, on the basis of the visual inspection of the resulting iron-stained electrophoretogram (electrophoresis run in pH 5 pyridine acetate buffer, as described in the Experimental Section). In contrast, enterobactin in pH 10.0 $KHCO_3$ (0.025 M)/K_2CO_3 (0.025 M) buffer was completely hydrolyzed in 2 h and roughly one-third of the linear trimer was hydrolyzed to DHBS; therefore, this buffer was not used.

A 1:3 ferric NTA solution at pH 6 with $[Fe^{3+}]$ = 0.0111 M was used as the titrant to deliver iron to the catecholate ligands. This titrant rapidly and completely delivers iron to tricatecholate ligands at pH 9–10. Calculations using known and estimated equilibrium constants (for μ = 0.1 M)[18] indicate that the significant species in solution are $HNTA^{2-}$, $Fe(NTA)_2^{3-}$, Fe(OH)NTA$^-$, and $Fe_2(OH)2(NTA)_2^{2-}$, with the last three species accounting for 85, 11, and 4% of the iron in solution. These percentages are estimates due to uncertainty in the dimerization constant for Fe(OH)NTA$^-$.[18] The exact molecular composition of this "$Fe(NTA)_2^{3-}$/$HNTA^{2-}$" titrant is not important as long as the $\Delta H°$ for formation of this solution from Fe^{3+} and NTA can be determined. The determination of this $\Delta H°$ was accomplished indirectly from four experimentally determined reaction heats and with a small correction required for the ideal standard state of a pure $Fe(H_2O)_6^{3+}$ in a μ = 0.100 M ionic strength, 25 °C solution.

The heats of reaction for iron reacting with the triprotonated catecholate ligands[20,21] were similarly determined as sums of experimentally determined $\Delta H°$ values. At the pH of the ligand-exchange reaction, the average protonation number of the DHBS linear trimer is 3.2, which can be assumed to be approximately correct for enterobactin as well. The source and combination of the reactions and their enthalpies used for determi-

Table I. Summary of Solution Thermodynamic Data

log K for	enterobactin[a]	linear trimer[b]	MECAMS[c]
$H_3L + H = H_4L$	8.55 (9)	8.23 (4)[d]	7.26[d]
$H_4L + H = H_5L$	7.5 (2)	7.39 (4)[d]	6.44[d]
$H_5L + H = H_6L$	6.0 (5)	6.43 (6)[d]	5.88[d]
$H_6L + H = H_7L$		2.5 (1)[d]	
$FeL + H = FeHL$	4.95	5.81 (3)[e]	5.74[d]
$FeHL + H = FeH_2L$	3.52	4.82 (3)[e]	4.10[d]
$FeH_2L + H = FeH_3L$		4.32 (3)[e]	3.46[d]
$FeH_3L + H = FeH_4L$		2.38 (2)[e]	
$H_6L + Fe = FeL + 6H$	−9.7 (3)	−15.6	−13.0
$H_3L + Fe = FeL + 3H$	12.4 (4)	6.5 (2)[f]	6.57 (10)[f]
ΔG, kcal/mol	−16.8 (5)	−8.9 (3)	−9.0 (2)
ΔH, kcal/mol	−6.5 (3)[g]	−3.4 (4)[g]	−4.4 (3)[g]
ΔS, cal/(mol K)	35 (2)	18 (2)	15 (1)

[a] The ligand protonation constants are those recently determined by spectrophotometric titration in the course of a separate study.[22] Other data, with the exception of the calorimetry, are from ref 11. [b] This study. [c] Protonation and metal complexation constants are from ref 21. [d] Values from potentiometric titration. [e] Values from spectrophotometric titration. [f] Values from spectrophotometric competition with EDTA for iron binding. [g] Values from solution calorimetry (this work).

nation of the ferric complex enthalpies are presented schematically in Figure 5. The net reaction for all processes illustrated in Figure 5 is as follows:

$$H_3L^{q-} + Fe^{3+} = FeL^{q-} + 3H^+ \quad (6)$$

The values of $\Delta H°$ for loss of three protons and iron binding were determined for the synthetic sulfonated tris(catecholate) ligand MECAMS as well as for enterobactin and the linear trimer of DHBS. Table I summarizes the thermodynamic constants[11,21,22] associated with these reactions. The results indicate that the greater stability of the ferric enterobactin complex, relative to the ferric complexes of MECAMS or of the linear trimer, is dominated by entropic factors (two-thirds) relative to enthalpic factors (one-third).

The complexation of iron by enterobactin is enthalpically 2–3 kcal/mol more favorable than complexation by MECAMS or the linear trimer. Shanzer et al. performed force field calculations to compare the complexation of iron by fully deprotonated enterobactin and complexation by fully deprotonated MECAM.[23] Their results suggested that an unfavorable enthalpic contribution arises from the ring strain induced upon complexation. Although not predicted by these calculations, it was suggested that the hydrogen-bonding network of the triester ring of enterobactin stabilizes a conformation of the free ligand which is sterically quite similar to the iron complex, so that the ring strain for enterobactin upon complexation is 2.0 kcal/mol less than for the synthetic analogue MECAM. This difference in ΔH happens to be the same as the 2 kcal/mol difference we observe in comparing complexation by MECAMS and enterobactin. This identity is certainly coincidental, since the heats we have measured involve reaction with triprotonated ligands, and sulfonation of the catechol groups of MECAM to give MECAMS will have an effect. However, the calculations of Shanzer et al. suggest that the observed enthalpic difference probably can be attributed to differences in ring strain upon complexation.

Our results show that complexation by the linear trimer is enthalpically even less favored than complexation by MECAMS, suggesting that iron complexation by the linear trimer results in unfavorable interaction involving the carboxylate and hydroxyl, which in turn result from hydrolysis of the ester bond. It is likely that the additional ring strain found in the iron complexes of MECAMS and the linear trimer can be partially relieved by a shift of one of the ligand arms from catecholate to the salicylate mode of bonding, which occurs upon protonation of the iron–ligand complexes.[7] As can be seen from Table I, these changes occur about 1 pH unit higher for the complexes of MECAMS and linear

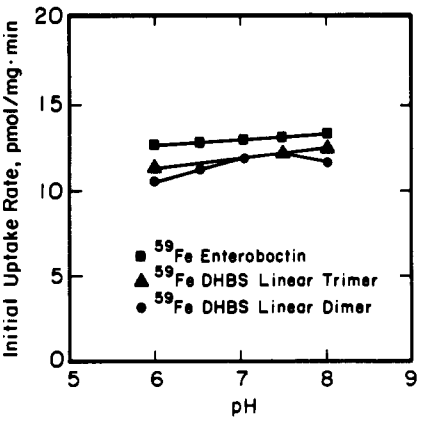

Figure 8. Biological transport activity of ^{59}Fe complexes of enterobactin and its hydrolysis products in *E. coli* RW193. The transport substrate concentration was 1 μM, and the cell concentration was 1 mg/mL. The initial rate of uptake of radiolabeled iron was observed over 15 min in cell cultures buffered at the pH values shown. All metal complexes were performed before adding to the cell cultures. A detailed description of the protocol used is in ref 6.

trimer than they do for enterobactin, consistent with the latter having less ring strain to relieve.

The entropic ($T\Delta S$) contribution to the difference in $\Delta G°$ values shown in Table I creates a 5–6 kcal/mol advantage for enterobactin in complexing iron compared to either MECAMS or linear trimer. We have chosen reaction 6 for our comparison because the charge on the uncomplexed H_3L and the iron complex (FeL) are the same for any given ligand. Therefore, differences in $T\Delta S$ for reaction 6 cannot be attributed to simple ion–dipole solvent ordering but must reflect the different conformations available to the three ligands.

For reaction 6, regardless of the identity of L, a large decrease in solvent ordering occurs with the delocalization of the positive charge (from Fe^{3+} to three H^+ ions), which gives rise to the positive ΔS values observed. However, partly offsetting this is the loss of conformational mobility of the ligand when it complexes the iron. As mentioned above, Shanzer et al. found a well-defined minimum energy conformation for free enterobactin that does not differ much from the conformation found in the ferric complex.[23] This would indicate that the decrease in conformational entropy is small when enterobactin complexes iron.

However, the structures of catechol amide ligands analogous to enterobactin have shown that there is a major difference in structure of the free ligand and the ligand anion or its metal complex:[24] there is a rotation of 180° around the amide bond due to interchanging C=O by N—H as the hydrogen-bond partner of the ortho phenol/phenolate group. Presumably, MECAMS and the linear trimer have much more conformational flexibility as free ligands (which is lost upon complexing iron) and this causes the entropy change associated with the complexation reaction to be less positive for these ligands compared with enterobactin. Hence, enterobactin is a better iron chelator than either its hydrolysis product (the linear trimer) or the synthetic mimic MECAM because it is able to complex iron with a minimum of added ring-strain energy (an enthalpic effect) and because the conformational mobility of iron-free enterobactin is small (an entropic effect). This may also be important in increasing the rate of iron incorporation by enterobactin. These features of enterobactin should be kept in mind in future attempts to synthesize ligands with very high affinities for metal ions.

The iron complex of the linear trimer is sufficiently less stable than that of enterobactin so that hydrolysis of one of the ester bonds is a plausible effective prerequisite to iron removal from enterobactin by microbes. In contrast, the stability of the linear trimer complex with iron is comparable with the complexes of the highest affinity synthetic iron chelators (such as MECAMS). Hence the ability of microbes to remove iron from MECAM and other synthetic ligands lacking ester bonds should not be taken

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as evidence against the requirement of ester hydrolysis before iron removal from enterobactin.

Biological Activity. Both the DHBS linear trimer and DHBS linear dimer are active as transport agents for radioactively labeled iron in whole *E. coli* RW193 cells. The initial rate of iron uptake from these compounds was essentially identical with the rate of enterobactin-mediated iron uptake when the pH of the uptake buffer was from pH 6 to 8 (Figure 8). The data in Figure 8 were derived from a series of uptake kinetics experiments by following the incorporation of ^{59}Fe–ligand complex into the cells and determining the initial rate. The lack of pH dependence on the uptake ratio indicates that the cell surface receptor is not sensitive to either bis(catecholate coordination (as in the Fe–DHBS linear dimer complex) or partially protonated tris(catecholate) coordination (as in the Fe linear trimer complex).

Summary

The stabilities of the ferric complexes of the stepwise hydrolysis products of enterobactin have been investigated. By comparison of the linear trimer (the first hydrolysis product of the cyclic trilactone enterobactin) with the parent compound, it is found that the exceptional stability of ferric enterobactin versus ferric complexes of tris(catechol) analogues is one-third enthalpic and two-thirds entropic in origin. Both the linear trimer and linear dimer hydrolysis products are effective iron uptake mediators when compared with enterobactin.

Acknowledgment. This research was supported by NIH Grant AI 11744. An American Cancer Society fellowship (to D.J.E.) is gratefully acknowledged.

Registry No. MECAMS, 71353-06-5; Fe, 7439-89-6; linear trimer, 30414-16-5; ferric linear trimer, 131685-07-9; linear dimer, 30414-15-4; ferric linear dimer, 131685-08-0; enterobactin, 28384-96-5; ferric enterobactin, 61481-53-6.

Supplementary Material Available: Figures S1–S3, showing a pH titration profile for the free linear trimer (**2**) and 1:1 mixtures of linear trimer and Fe^{3+}, UV/vis spectra of the linear dimer (**3**) titrated with ferric NTA, and UV/vis spectra of DHBS (**4**) titrated with ferric NTA (3 pages). Ordering information is given on any current masthead page.

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Solution Equilibria of Enterobactin and Metal–Enterobactin Complexes[1]

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The solution thermodynamics of the siderophore enterobactin (ent), a powerful iron chelator, and of the synthetic analogue MECAM (*N,N',N''*-tris(2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene) have been studied by UV spectrophotometry. Spectra were taken at high dilution by using a fully automatic, microcomputer-controlled spectrophotometer. The protonation constants for the *o*-hydroxyl groups of the ligand catechol moieties have been determined to be identical within experimental error: for L^{6-} to $LH_n^{(6-n)-}$, $\log K_6 = 6.0$ (5), $\log K_5 = 7.5$ (2), and $\log K_4 = 8.5$ (1). On the basis of these values, the overall protonation constant β_{016} for ent can be estimated as $10^{58.5}$, yielding a revised estimate for the formal stability constant of $[Fe(ent)^{3-}]$ of 10^{49}. The thermodynamic constants for protonation of the ferric complexes of ent and MECAM have been measured, for $Fe(L)^{3-}$ to $Fe(H_nL)^{(3-n)-} + nH$: for ent, $\log K_1 = 4.95$, $\log K_2 = 3.52$, and $\log K_3 = 2.5$; for MECAM, $\log K_1 = 7.2$, $\log K_2 = 6.03$, $\log K_3 = 4.5$, and $\log K_4 = 3.8$. Protonation takes place by discrete one-proton steps, consistent with a shift from catecholate binding to salicylate binding geometry upon protonation of the ligand *m*-hydroxyl groups. Protonation constants were also obtained for the complexes between ent and other trivalent metals: Sc^{3+}, Al^{3+}, Ga^{3+}, and In^{3+}. The order of protonation is (from highest pK_a to lowest) Al > Fe > Sc > Ga ≫ In. While $[Sc(ent)]^{3-}$ and $[Ga(ent)]^{3-}$ are similar to the ferric complexes and protonate in single proton steps, $Al(ent)^{3-}$ and $In(ent)^{3-}$ have mechanisms involving multiproton steps, possibly involving catecholate dissociation. This behavior was monitored by circular dichroism spectra.

Introduction

Iron is the most abundant transition metal found in the biosphere; it is required for almost all living organisms from hominids to bacteria.[2] In humans iron is of medical concern because of its effects on infectious organisms[3,4] and in diseases of iron deficiency and iron overload.[5,6] Current applications for iron-binding drugs include treatment of iron poisoning and of chronic iron overload associated with certain kinds of anemias.[5-7] Because iron in its most common form is not a readily available nutrient (the solubility of ferric hydroxide is 10^{-38},[8] strategies have been evolved by living organisms to acquire sufficient quantities of this vital metal. Many microorganisms, for example, produce siderophores—low molecular weight chelating agents that bind and solubilize ferric iron.[9-13] In part driven by clinical concerns, considerable research has taken place on the binding of iron by siderophores and by synthetic siderophore analogues. Of all the natural and man-made compounds so far investigated, the one that binds iron the most strongly under physiological conditions is a siderophore produced by *Escherichia coli*, enterobactin (ent) (Figure 1).[13-16]

Enterobactin employs three catechol (1,2-dihydroxybenzene) moieties to tightly encapsulate ferric iron in a hexadentate coordination sphere.[15-17] The overall reaction that defines the formal stability constant between enterobactin and trivalent metal ions, such as Fe^{3+}, is

$$M^{3+} + ent^{6-} = [M(ent)]^{3-} \quad \beta_{110} \qquad (1)$$

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