Coordination Chemistry of Microbial Iron Transport Compounds. 16. Isolation, Characterization, and Formation Constants of Ferric Aerobactin[1a]

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Abstract: Aerobactin, a dihydroxamate derivative of citric acid, is a siderophore produced by *Aerobacter aerogenes*. The iron complex has been isolated from neutral aqueous solutions as the trisodium salt. The high-spin octahedral complex is formed using the two bidentate hydroxamate groups and the central carboxylate and hydroxyl moieties of the citrate backbone. Ferric aerobactin exists predominantly as the Λ optical isomer in aqueous solution. The stability constants ($\log \beta_{113} = 31.74$, $\log \beta_{112} = 29.70$, $\log \beta_{111} = 26.68$, $\log \beta_{110} = 23.06$, $\log \beta_{11\bar{1}} = 18.48$) and redox potential have also been determined from spectroscopic, potentiometric titration, and electrochemical techniques. The implication of these results to the mechanisms of iron uptake and release by *A. aerogenes* is discussed.

Introduction

Iron is an essential element for almost all living organisms, the lactic acid bacteria being the only possible exception.[1b] In an aerobic environment, iron exists exclusively in the ferric state and as such forms quantitatively insoluble ferric hydroxides at neutral pH.[2] To combat the biological unavailability of this vital element, microbes have developed powerful low-molecular-weight chelating agents known as siderophores.[3,4] These molecules generally utilize three secondary hydroxamate or catecholate groups to provide an octahedral environment that is extremely stable and highly specific for ferric ion.

The ferrichromes and ferrioxamines form two classes of these microbial iron transport agents that have been well studied, and their iron complexes have been characterized by X-ray crystallographic analysis.[5,6] They both utilize three hydroxamate groups as the iron binding centers, the former having the hydroxamic acid linkages provided by a cyclic tripeptide of N^{δ}-acyl-N^{δ}-hydroxy-l-ornithine and the latter via linear repeating units of 1-amino-ω-hydroxyaminoalkane and succinic acid.

A far less well understood class of siderophores uses only two hydroxamate groups, the third ligating portion probably being a citrate moiety. The salient example of this class is aerobactin (Figure 1). Aerobactin, first isolated and described by Gibson and Magrath in 1969,[7] is a conjugate of 6-(*N*-acetyl-*N*-hydroxyamino)-2-aminohexanoic acid and citric acid. It is produced by *Aerobacter aerogenes* 62-1 under conditions of low iron stress and has been found to function as an iron transporting agent for this organism.[8] Apart from a brief account of spectral changes of a mixture of aerobactin and iron over a narrow range of pH, nothing is known of the chemistry of this compound. As part of our program to elucidate the coordination chemistry of the siderophores, we report here the isolation and characterization of the ferric complex of aerobactin and the determination of its formation constants.

Experimental Section

Ultraviolet-visible spectra were obtained with a Cary 118 spectrophotometer, and circular dichroism (CD) spectra were measured with a Jasco J-20 automatic recording spectropolarimeter. The NMR spectra were recorded in D_2O on a Varian T-60 spectrometer using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as a standard. Chemical analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley, Calif.

Materials. Reagent grade chemicals were used throughout. Chromium trichloride-tris(tetrahydrofuran) adduct was prepared by literature procedures.[9] Aerobactin was isolated, as a light yellow hygroscopic solid, from low iron cultures of *A. aerogenes* 62-1 as described by Gibson and Magrath.[7] It was found to be pure via chromatography and had the expected NMR pattern. Ion exchange chromatography was performed on AG-1 X8 (100–200 mesh, chloride form) anion exchange resin, and AG-50 W-X2 (200–400 mesh, hydrogen form) and Bio-Rex 70 (200–400 mesh, sodium form) cation exchange resins.

Paper Chromatography. Paper chromatography on Whatman No. 1 was performed on both the free ligand and metal complexes. The solvent systems used were (I) ethanol/water/NH_3 (16:3:1), (II) butanol/acetic acid/water (60:15:25), and (III) methanol/water (1:1). Spots were detected visually, stained with iodine vapor or $FeCl_3$ in methanol.

Titrations. Standard carbonate-free KOH solutions were prepared from Baker Dilut-It ampules using recently boiled distilled water and were stored under argon, which had been passed over ascarite to remove any traces of CO_2. Base solutions were standardized by titration of potassium hydrogen phthalate to a phenolphthalein end point.

Stock iron solutions were prepared by dissolving $Fe(NO_3)_3 \cdot 9H_2O$ in 2 mM HNO_3 to prevent hydrolysis of the iron. The HNO_3 solution was prepared by quantitative dilution of a more concentrated solution which had been standarized by titration with KOH. The exact iron concentration was determined by adding excess EDTA and back titrating with Zn(II) using eriochrome black T as an indicator.[10] A sample of ferric ion was also titrated with KOH in the presence of an excess of Na_2EDTA. The millimoles of base required to reach the first break in the titration curve is equal to the sum of the millimoles of HNO_3 and twice the millimoles of ferric ion. This result serves to double-check the internal consistency of the acid and ferric concentrations.

Potentiometric equilibrium measurements were carried out in a capped, jacketed titration vessel connected directly to a constant temperature circulating water bath. The temperature was maintained at 25.00 ± 0.05 °C, and the ionic strength of each solution was initially adjusted to 0.100 M by the addition of the appropriate amount of 1.0 M KNO_3. Typical samples consisted of 40-mL solutions which were roughly 2 mM in each reagent. Solutions were thoroughly degassed with argon while still acidic to remove dissolved oxygen and carbon dioxide and were kept under an argon atmosphere throughout the titration. Gran's plots confirmed the effective exclusion of CO_2 during calibration titrations of HNO_3 solutions with KOH.

Hydrogen ion concentrations were measured with a Corning Model 130 digital pH meter equipped with Corning glass and saturated calomel electrodes and standardized with dilute HNO_3 solution to read the negative log of the hydrogen ion concentration, rather than hydrogen ion activity.

Electrochemistry. Electrochemical measurements were performed as previously described.[11]

Ferric Aerobactin. To 134 mg (0.25 mmol) of aerobactin was added an excess of freshly prepared and carefully washed ferric hydroxide. The mixture was heated to 80 °C and stirred for 2 h, during which

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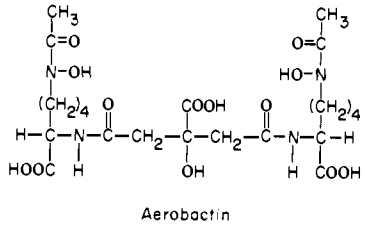

Aerobactin

Figure 1. Structural formula of aerobactin.

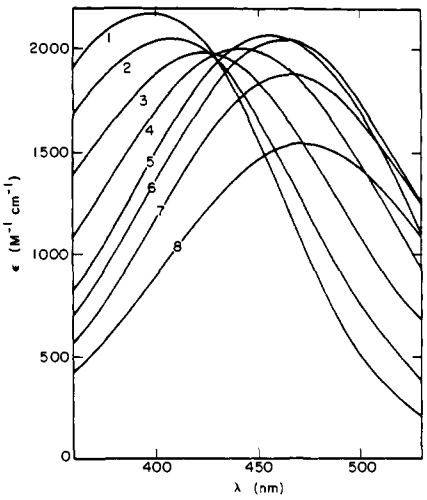

Figure 2. Visible spectrum of ferric aerobactin as a function of pH. μ = 0.10, t = 25 °C. Curve 1, pH 7.47; 2, 4.70; 3, 4.01; 4, 3.28; 5, 2.51; 6, 1.93; 7, 1.40; 8, 1.00.

time the characteristic red coloration of the ferric aerobactin complex appeared. The solution was allowed to stir overnight without heating and was then filtered through paper and finally through a Unipore filter (1.0 μm, Bio-Rad Laboratories). The red solution was then concentrated via rotary evaporation and applied to a Bio-Rex 70 column. The orange band of the desired product was eluted with water and taken to dryness to give a 77% yield of trisodium ferric aerobactin. The complex was taken up in wet methanol and precipitated several times with 2-propanol. The resulting bright orange solid was dried under vacuum for 24 h: mp 270 °C dec; R_f (III) 0.73. Anal. Calcd for $Na_3C_{22}H_{30}N_4O_{13}Fe{\cdot}CH_3OH$: C, 38.61; H, 4.79; N, 7.83; O,[12] 31.31; Fe, 7.81; Na, 9.64. Found: C, 38.84; H, 5.24, N, 7.54; O,[12] 31.60; Fe, 6.97; Na, 9.81.

Chromic Aerobactin. To 0.16 g (0.29 mmol) of aerobactin in 50 mL of methanol was added a slight excess of $CrCl_3{\cdot}3THF$ (0.12 g). The solution was refluxed for 1 h and stirred overnight. The solution was evaporated to dryness and taken up in a small amount of H_2O. It was applied to the top of a Bio-Rex 70 column and eluted with H_2O. A bright green band separated from some immobile grey material and was collected. The material was then chromatographed on Bio-Gel P-2 and finally on silica gel G using 30% MeOH/H_2O as an eluant. The chromium content of the solution was measured by oxidation of an aliquot with alkaline peroxide. The resulting $[CrO_4]^{2-}$ was analyzed spectrophotometrically (ϵ_{372}^{max} 4815 L mol^{-1} cm^{-1}).[13] Isolation of analytically pure solids was precluded by the low yield of the reaction and the small quantity of starting material available.

Results

Electronic Absorption Spectra. At neutral pH and higher, ferric aerobactin forms a characteristic orange-colored complex with λ_{max} 399 nm, ϵ 2170 L mol^{-1} cm^{-1}. The magnetic moment of the ferric complex in solution, as determined by the Evans method,[14] was found to be μ_B = 5.7 BM. Since high-spin ferric ion has no spin-allowed d-d transitions, the visible absorption bands must be due to charge-transfer transitions. The pH dependence of ferric aerobactin (Figure 2) differs from that

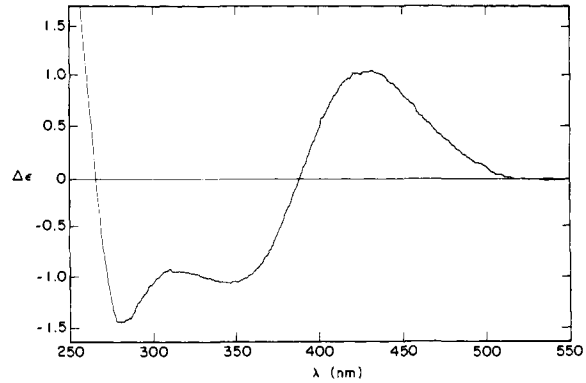

Figure 3. Circular dichroism spectrum of ferric aerobactin at neutral pH.

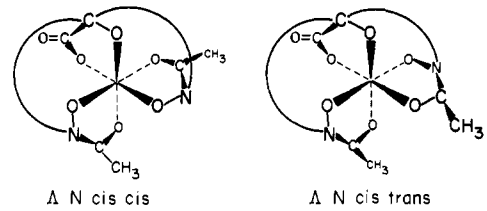

Figure 4. The two possible geometrical isomers of Λ ferric aerobactin.

of the ferric trihydroxamate siderophores, which have a broad visible maximum from 420 to 440 nm and do not exhibit the marked pH-dependent spectral shifts seen with aerobactin. As the pH is lowered, the spectrum shifts to the red so that at pH ~2.7 λ_{max} is 450 nm with an isosbestic point observed at 430 nm. Below pH 2.7, a further shift in the absorption spectra is seen, but no isosbestic point is observed. Finally, as the pH is lowered further the λ_{max} remains constant, but the intensity is rapidly reduced. These spectral changes can be analyzed in terms of four stepwise equilibria (vide infra).

Purified desferriaerobactin absorbs in the ultraviolet region only below 240 nm. Both the ferric and chromic complexes, however, absorb strongly in the ultraviolet at neutral pH, with a peak in the former complex at λ_{max} 300 nm (ϵ 2600 L mol^{-1} cm^{-1}) and a shoulder at ~250 nm in the latter. These absorption peaks have been seen in the structurally related ferric schizokinen complex and are believed to be significant to the predominant metal chelate structure.[15]

CD Spectra. The circular dichroism spectra of ferric aerobactin were also obtained as a function of pH. At near neutral pH three CD peaks are observed in the visible and near-UV region, 430 (+1.0), 345 (−1.0), and 285 (−1.4) nm ($\Delta\epsilon$) (Figure 3). As the pH is lowered, the CD peaks are shifted to the blue and reduced in intensity. Ferrichrome A, which is a trihydroxamatoiron(III) complex, crystallizes in the Λ-cis form, and has a positive CD band at 465 nm (in the region of the absorption max at 440 nm). Likewise, the Λ-cis isomer of tris(benzohydroxamato)iron(III) has been resolved and has a positive CD band at 455 nm (absorption maximum 435 nm).[16] Ferric aerobactin also has a positive CD band at 430 nm (absorption maximum 399 nm) and has qualitatively the same spectrum as does ferrichrome A. Consequently we conclude that the configuration about the iron atom is also Λ. Although the CD spectrum indicates that the Λ optical isomer predominates in solution, there exist two possible geometrical isomers of this configuration, the N-cis,cis and N-cis,trans. These two isomers, shown in Figure 4, are not distinguishable based on the CD or visible-UV spectra. The close similarity of the CD spectra of the ferrichromes, the simple tris(benzohydroxamato)iron(III) complexes, and ferric aerobactin also indicates that the iron is octahedrally coordinated by six oxygen

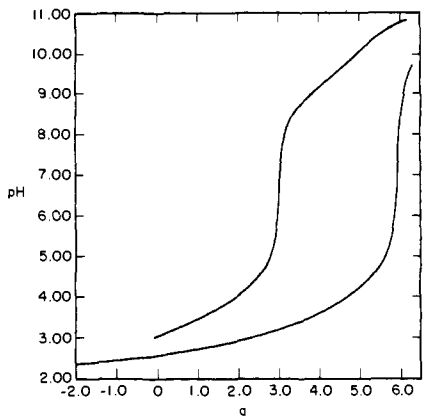

Figure 5. Potentiometric equilibrium curves for aerobactin alone (upper curve) and in the presence of equimolar ferric ion (lower curve).

atoms. In the case of aerobactin, four come from the two hydroxamate groups and two from the citrate moiety. The $\Delta\epsilon$ values observed for the ferric aerobactin are less than those of the corresponding values for ferrichrome A. This may be a consequence of the slightly different coordination sphere in the former or due to lower optical purity. Regardless, ferric aerobactin exists preferentially as the Λ optical isomer in solution, as do all the known hydroxamate siderophores except the dimeric rhodotorulic acid complex.[17] This contention is further supported by the chromic aerobactin CD spectra. Table I shows a comparison of the chromic aerobactin spectra with those of chromic Λ-cis desferriferrichrome and Λ-cis chromic desferriferrichrysin. Based on analogy with the simple chromic hydroxamates, the band at 665 nm is assigned as the $^4A_2 \rightarrow {}^4A_1$ transition, the band at 567 nm as the $^4A_2 \rightarrow {}^4E_a$, and that at 416 nm as the $^4A_2 \rightarrow {}^4E_b$ transition.[18] Since the low-energy, E_a, CD band at 567 nm is positive, it is clear that the chromic aerobactin complex exists at least primarily as the Λ optical isomer, but that its optical purity (based on chromium) is a great deal less than that of the ferrichromes.

Electrochemistry. The redox potential of ferric aerobactin was measured by cyclic voltammetry over the range of pH from 6 to 11. Over most of this range quasireversible one-electron reduction waves were observed with a peak-to-peak separation averaging ~90 mV. For a completely reversible one-electron reduction, a separation of 59 mV is expected. At pH 7 a redox potential of −336 mV vs. the normal hydrogen electrode was obtained. This is compared to several other hydroxamate siderophores in Table II. The pH dependence of the redox potential of ferric aerobactin showed a −59 mV/pH unit change, which is consistent with protonation of the reduced species.

Ligand Protonation Equilibria. The potentiometric equilibrium curve for aerobactin alone is shown as the upper curve in Figure 5. The data below pH 5 represent the loss of three acidic carboxylate protons, followed by deprotonation of the two more basic hydroxamic acid groups between pH 7 and 10. Ligand protonation constants were determined by nonlinear least-squares refinement of the titration data to optimize the fit of $p[H]_{calcd}$ vs. $p[H]_{obsd}$.[20] These constants are defined by eq 1 and have values of 9.44 (1), 8.93 (1), 4.31 (6), 3.48 (10), and 3.11 (13):

$$K_H{}^n = \frac{[H_nL]}{[H][H_{n-1}L]} \quad (1)$$

These protonation constants have been previously reported by Gibson and Magrath[7] as 10.33, 9.31, 4.51, 3.85, and 3.01. There is a significant difference between the two sets of hydroxamate log Ks. The ligand protonation constants have been reported for several trihydroxamic acids, and the values range from 8.1 to 10.0, with an average log K_H of around 9.2.[21] As one might expect, this average is close to the log $K_H{}^1$ of 9.35 of acethydroxamic acid.[22] In addition, ligand protonation constants for one other dihydroxamate, rhodotorulic acid, have been reported as 9.44 and 8.49.[19] Thus the constants 9.44 and 8.93 reported here are much more consistent with the rest of the literature than the unusually high values of Gibson et al.

The very shallow slope of the titration curve below $a = 3$, where a = moles of base per mole of ligand, makes it more difficult to calculate precise protonation constants for the carboxylate groups. This is reflected in the larger standard deviations in log $K_H{}^3$ − log $K_H{}^5$. This is not due to a poorer fit in $p[H]_{obsd} - p[H]_{calcd}$, as this difference is ±0.001 over this entire region. The higher standard deviations are a direct result of the lower sensitivity of the fit toward small changes in the log K_H values.

The carboxylate protonation constants of aerobactin (4.31, 3.48, and 3.11) can be compared to the values for citric acid of 5.69, 4.35, and 2.87.[23] The decrease can be attributed both to the inductive effects of the amide groups and to the wider separation of negative charge in aerobactin. Presumably, the aerobactin log K_H values of 3.11 and 3.48 are primarily related to ionization of the two terminal carboxylates, and the very small difference between these two constants indicates that these two deprotonations are essentially independent of one another. In addition, work on simple dipeptides has shown that the protonation constant for a carboxylate group α to an amide linkage is almost always in the range of 3.05-3.15,[24] which agrees well with the $K_H{}^1$ of 3.11 reported for aerobactin.

Metal-Ligand Equilibria. The potentiometric equilibrium curve for an equimolar solution of aerobactin and ferric ion is shown as the lower curve in Figure 5. One important feature of this curve is that the break does not occur until $a = 6$, where a = moles of base added per mole of metal. This indicates that in addition to the five readily titratable ligand groups (three carboxylates and two hydroxamates), there is one other group which is dissociating to produce a proton only in the presence of ferric ion, to give a species which will be referred to herein as $Fe(H_{-1}L)$.

The visible spectra of ferric aerobactin over a wide range of pH are shown in Figure 2. Around pH 2, the solution has a

Table I. Spectral Parameters for the Chromic Complexes of Desferriferrichrome, Desferriferrichrysin, and Aerobactin

complex	absorption max, nm (ϵ)	CD max, nm ($\Delta\epsilon$)
Λ-cis chromic desferriferrichrome	427 (70.1)	432 (−2.49)
	583 (81.0)	571 (7.47)
		661 (−1.74)
Λ-cis chromic desferriferrichrysin	427 (66.9)	431 (−2.48)
	583 (79.7)	570 (7.31)
		659 (−1.86)
chromic aerobactin	420 (68.8)	415 (−0.12)
	589 (55.8)	574 (0.25)
		668 (−0.10)

Table II. Formal Potentials for a Series of Hydroxamate Siderophores

complex	ref	$E_f{}^a$ vs. NHE, mV
ferrioxamine B	11	−468
ferrichrome A	11	−448
ferric rhodotorulic acid	19	−359
ferric aerobactin	this work	−336

[a] pH 7, 10 mM phosphate, 10 mM borate buffer, 1 M KCl, 1 mM $[Fe^{3+}]$.

λ_{max} of 470 nm and a molar absorptivity of ~2100. The only other well-characterized dihydroxamic acid siderophore is rhodotorulic acid, which has a λ_{max} 480 and ϵ 1750.[17] The λ_{max} for a second dihydroxamate, fusaranine A, has been reported as 475 at pH 2.[25] On the other hand, ferric monohydroxamate complexes have λ_{max} values of 500 to 530 nm, with molar absorptivities of only 900–1100.[26] Thus, it is clear that both the hydroxamic acid groups of aerobactin coordinate in the initial low pH complexation reaction. Even at pH 1.0, there is no shift in λ_{max} past 475 nm toward longer wavelength, which would have indicated the presence of a monohydroxamate complex.

The ferric aerobactin spectra in Figure 2 show an isosbestic point at 430 nm from pH 7 down to 2.7. From the titration curve, it is seen that this pH range corresponds to more than the addition of 1 equiv of H^+ to the $FeH_{-1}L$ complex (pH at $a = 5$ is ~3.9). Thus, the isosbestic point is retained, even though more than two species are involved in the equilibria, indicating that various protonated chelates have very similar spectra. This is easily understood if one considers that two of the aerobactin carboxylate groups are sterically prohibited from coordinating to the iron. Thus, two 1 equiv deprotonations, which are reflected in the titration curve, do not involve a change in the coordination sphere of the metal and therefore do not result in any noticeable changes in the spectrum.

The spectral data were interpreted based on the following model. The spectrum at pH 7 is that of $[Fe(H_{-1}L)]^{3-}$, with λ_{max} 398 nm (ϵ 2170). The initial spectral changes observed as the pH is lowered are due solely to the reaction $Fe(H_{-1}L)^{3-} + H^+ \rightarrow FeL^{2-}$. The FeL^{2-} complex undergoes two further protonations to give $FeHL^-$ and FeH_2L, but these reactions probably involve remote noncoordinating carboxylate groups. Therefore, it was assumed that these chelate protonation constants would be equal to the corresponding ligand protonation constants, i.e.,

$$K^H_{MHL} = \frac{[MHL]}{[ML][H]} = \frac{[H_4L]}{[H_3L][H]} = 10^{3.48} \quad (2)$$

$$K^H_{MH_2L} = \frac{[MH_2L]}{[MHL][H]} = \frac{[H_5L]}{[H_4L][H]} = 10^{3.10} \quad (3)$$

Such an approximation is justified by two particular features of the aerobactin complex. First, the charges are the same for deprotonation of the free ligand and for the chelate, i.e., $K^H_{MH_2L}$ and $K_H{}^1$ both involve equilibria between neutral and monoanionic species. Therefore, one need not consider simple electrostatic effects on the protonation constants. In addition, there are at least eight atoms between the two noncoordinating carboxylates and the nearest atom bound directly to the iron, so one could not expect any significant inductive effects. Since the only difference between FeL, FeHL, and FeH_2L is the protonation of uncoordinated carboxylate groups, it was also assumed that $\epsilon_{ML} = \epsilon_{MHL} = \epsilon_{MH_2L}$.

The spectral data from pH 4 to 5 can be treated as a two-component system, described by the equations:

$$M = [MH_{-1}L] + [ML] \quad (4)$$

$$\text{Abs} = \epsilon_{MH_{-1}L}[MH_{-1}L] + \epsilon_{ML}[ML] \quad (5)$$

where M represents the total iron concentration and Abs is the measured absorbance.

These can be rearranged and combined with the equilibrium expression $K^H_{MH_{-1}L} = [ML]/[MH_{-1}L][H]$ to give the equation:

$$\epsilon_{obsd} = \epsilon_{ML} + \frac{(\epsilon_{MH_{-1}L} - \epsilon_{obsd})}{[H^+]} K^H_{MH_{-1}L} \quad (6)$$

where ϵ_{obsd} is the measured absorbance divided by the analytical iron concentration, i.e., $\epsilon_{obsd} = \text{Abs}/M$. Plots of ϵ_{obsd} vs. $(\epsilon_{MH_{-1}L} - \epsilon_{obsd})/[H]$ were linear both at 470 and 400 nm (the λ_{max} for $MH_{-1}L$ and ML) and gave an averaged value of log $K_{MH_{-1}L}$ of 4.27 and $\epsilon_{ML} = 1878$.

Table III. Overall Formation and Stepwise Protonation Constants for the Ferric Aerobactin Complexes Determined from Spectral and Potentiometric Data

	spectra	titration	Δ
$\beta_{113}{}^a$	31.74 (0.12)	31.74 (0.12)	
β_{112}	29.05	29.70 (0.12)	0.65
β_{111}	25.94	26.68 (0.12)	0.72
β_{110}	22.46 (0.21)	23.06 (0.12)	0.60
$\beta_{11\bar{1}}$	18.19	18.48 (0.12)	0.28
$K^H_{MH_3L}$	2.69	2.04 (0.08)	0.65
$K^H_{MH_2L}$	3.11	3.02 (0.06)	−0.09
K^H_{MHL}	3.48	3.62 (0.07)	0.14
K_{ML}	22.46	23.06 (0.12)	0.60
$K^H_{MH_{-1}L}$	4.27	4.58 (0.08)	0.31

[a] See eq 10 in text.

The ferric aerobactin system can be described at any pH by the following set of equations:

$$M = \alpha_{ML}[ML] + \alpha_m[m] \quad (7)$$

$$L = \alpha_{ML}[ML] + \alpha_L[l] \quad (8)$$

$$\text{Abs} = [ML](\epsilon_{ML} + K^H_{MHL}[H]\epsilon_{MHL} + K^H_{MH_2L}[H]^2\epsilon_{MH_2L} + \ldots) \quad (9)$$

where the αs are the normal functions of protonation and hydrolysis constants and hydrogen ion concentration (and the βs are defined in eq 10), e.g., $\alpha_L = 1 + \beta_{011}[H] + \beta_{012}[H]^2 + \beta_{013}[H]^3 + \ldots$). Using these three equations, the absorbance can be calculated as a function of the observables M, L, and [H] and ten parameters. Of these ten parameters, $K_{MH_{-1}L}$ and ϵ_{ML} were determined graphically as described above. The value of $\epsilon_{MH_{-1}L}$ is measured directly, and the assumptions discussed above lead to values for ϵ_{MHL}, ϵ_{MH_2L}, K^H_{MHL}, and $K^H_{MH_2L}$. One is left with the unknown parameters $K^H_{MH_3L}$, K_{ML}, and ϵ_{MH_3L}. These were determined by a nonlinear least-squares fit of Abs^{calcd} to Abs. The average percent deviation [defined as $100(\text{Abs} - \text{Abs}^{calcd})/\text{Abs}$] was 2.0, with no single point exceeding 5% deviation. The molar absorptivities determined for the various iron species at 470 nm are: $\epsilon_{MH_{-1}L} = 1100$, $\epsilon_{ML} = \epsilon_{MHL} = \epsilon_{MH_2L} = 1878$, and $\epsilon_{MH_3L} = 2152$.

The set of equilibrium constants obtained from the spectral data is listed in Table III. The overall formation constants are expressed as β_{ijk} for the reaction:

$$iFe + jL + kH \rightleftharpoons Fe_iL_jH_k$$

$$\beta_{ijk} = \frac{[Fe_iL_jH_k]}{[Fe]^i[L]^j[H]^k} \quad (10)$$

The low pH obtained in the titration of ferric aerobactin simply by mixing the reagents indicates that there is a considerable degree of chelate formation prior to the addition of any base. Since there is not a significant fraction of free metal present, it is not possible to obtain an overall formation constant from the titration data alone. However, given the value of β_{113} determined spectrophotometrically, the titration data are sufficient to calculate the sequential chelate deprotonation constants. Only β_{113} and the ligand protonation constants were held fixed. All other metal-ligand constants were varied during refinement. The resulting set of equilibrium constants is listed in Table III. In general, the agreement between the two methods is fairly good. Since the titration values are less directly dependent on the assumptions made in the treatment of

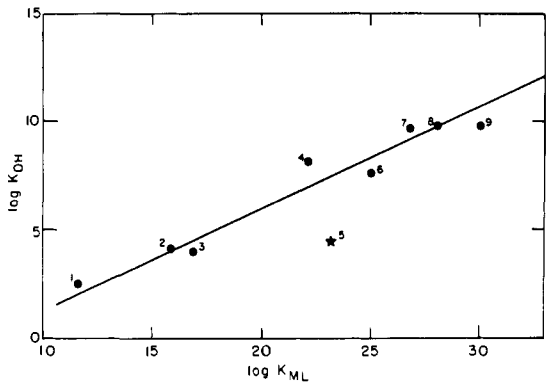

Figure 6. Plot of log K_{OH} vs. log K_{ML} for a series of ferric complexes. K_{OH} = [ML]/[MLOH][H]. Point 1, HIDA; 2, NTA; 3, EDDA; 4, ethylenedinitrilo-*N,N'*-diacethydroxamic *N,N'*-diacetic acid; 5, aerobactin; 6, EDTA; 7, TTHA; 8, DTPA; 9, CDTA.

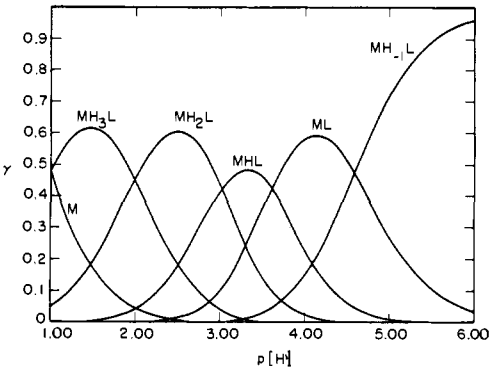

Figure 7. Species distribution curves for the ferric aerobactin system from pH 1.00 to 6.00, where γ represents the fraction of total iron represented by any individual species.

Table IV. Formation Constants of Ferric Dihydroxamic Acid Complexes

ligand	log *K*	ligand	log *K*
aerobactin	22.93	ferrioxamine D_1	21.6[b]
rhodotorulic acid	21.99[c]	ferrioxamine E	22.50[b]
acethydroxamic	21.10[a]	ferrichrome	20.7[b]
ferrioxamine B	21.7[b]		

[a] $K \equiv \beta_2$ = [ML]/[M][L]^2, ref 4. [b] $K = K^{M}_{MHL}$ = [MHL]/[M]·[HL], ref 3. [c] Reference 5.

Table V. Values of p[M] for Solutions of a Series of Ferric Complexes of Biological Ligands

ligand	$p[M]_1$[a]	$p[M]_2$[b]	$p[M]_3$[c]
enterobactin	37.6	35.52	29.30
ferrioxamine E	29.73	27.68	25.55
ferrioxamine B	28.64	26.59	24.48
N-acetylferrioxamine B	28.50	26.45	24.33
ferrichrysin	27.89	25.84	23.76
ferrichrome	27.25	25.20	23.13
transferrin	25.62	23.60	21.42
aerobactin	25.37	23.32	21.17
rhodotorulic acid	25.00	21.90	20.85

[a] $T_M = 10^{-6}$ M, $T_L = 10^{-3}$ M, pH 7.40. [b] $T_M = 10^{-6}$ M, $T_L = 10^{-5}$ M, pH 7.40. [c] $T_M = 10^{-6}$ M, $T_L = 10^{-3}$ M, pH 6.00.

the spectral data, they should be considered the more accurate of the two sets of constants.

By far the largest discrepancy between the spectral and potentiometric results is in the values of $K^{H}_{MH_3L}$. Since the titration values are based on a fixed value of β_{113}, the difference in $K^{H}_{MH_3L}$ is directly reflected by a large difference in the K_{ML} values as well. The values of K_{ML} for aerobactin are in the range expected for a dihydroxamic complex, as shown by the data in Table IV. The values listed for the ferrioxamines and other trihydroxamic acids correspond to the reaction of the monoprotonated ligand with Fe^{3+} ion:

$$Fe^{3+} + HL^{2-} \rightarrow FeHL^{+} \qquad (11)$$

The aerobactin complex has the largest log K_{ML} of any known dihydroxamate complex, which reflects the increased stability due to coordination of one of the carboxylate groups. None of the other ligands listed contains any auxiliary ligating groups.

Based on the available data, it is difficult to conclusively identify the $FeH_{-1}L$ species, but there are only two obvious possibilities: (a) Coordination and deprotonation of the citrate hydroxyl group to form $[Fe^{3+}(Aero^{6-})]^{3-}$ or (b) deprotonation of a coordinated water molecule to form a monohydroxo complex $[Fe^{3+}(Aero^{5-})(OH^{-})]^{3-}$. There is frequently a direct relationship between the magnitude of the formation constant K_{ML} and the corresponding chelate hydrolysis constant K_{OH}, where K_{OH} = [ML]/[MOHL][H]. This is demonstrated for ferric ion by the plot shown in Figure 6. Given the large formation constant for aerobactin, one would expect a hydrolysis constant of about seven. Thus, the log $K_{MH_{-1}L}$ of 4.58 appears to be too low to be assigned to a simple hydrolysis. In addition, there are other data which indicate that the hydroxyl group of citric acid itself is coordinated in complexes of Cu^{2+}, Fe^{3+}, and Ga^{3+}.[27-30] Therefore, it appears that the central hydroxyl group is coordinated in the $FeH_{-1}L$ complex of aerobactin.

It is not possible to prove which carboxylate is coordinating to the iron. Molecular models show that any one of the three is sterically capable of coordinating when both hydroxamates are bound. However, there is considerably more strain involved in bonding of either of the two terminal carboxylates. In addition, such a model contains implausibly large chelate rings, whereas the central carboxylate and citrate -OH group are able to easily form a stable five-membered ring. Thus, the most reasonable bonding model involves the two hydroxamates, the central carboxylate, and the citrate hydroxyl group, to give an octahedral six-oxygen coordination sphere.

Because of the number of overlapping protonation constants, ferric aerobactin represents a complicated equilibrium system. A species distribution curve for ferric aerobactin is shown in Figure 7. It is clear from inspection of the curve for free iron why K_{ML} could not be calculated from the titration data. At pH 2.3, which is the lowest pH measured in the titration of ferric aerobactin, only about 3.5% of the total iron is in the form of free, uncoordinated ferric iron. The aerobactin ligand is so effective at coordinating ferric ion in acidic solution that even at pH 1.0, almost half the total iron is chelated. Up to pH 4.5, the protonation equilibria are so strongly overlapping that no single complex ever contains more than 60% of the iron. However, by pH 7 all the iron is present as the single $(FeH_{-1}L)^{3-}$ aerobactin complex, and this is the biologically active species.

Discussion

It is difficult to judge the relative effectiveness of the various biological iron chelators. Because of variations in ligand protonation constants and concentration dependencies, it is not meaningful simply to compare the magnitudes of the overall formation constants. For this reason the concentrations of free ferric ion in equilibrium with a series of siderophores have been calculated for three sets of conditions, and the results are shown in Table V. These p[M] values provide a direct comparison of

the relative abilities of these ligands to complex iron under the specified conditions, i.e., the higher the p[M], the more effective the ligand.

Enterobactin, a cyclic triester of *N*-2,3-dihydroxybenzoylserine, is obviously the most effective chelator at or above neutral pH. However, because of the very high ligand protonation constants and the sixth-order dependence on [H] in the reaction of enterobactin with ferric ion, the ability of this ligand to coordinate iron drops rapidly with decreasing pH. However, even at pH 6, its $p[M]_3$ value is still greater than those of the ferrioxamines.

The desferriferrioxamines are the most effective hydroxamate ligands, with p[M] values about 1 log unit higher than ferrichrome and ferrichrysin. Because of the obvious similarities between these ligands, changing the pH or ligand:metal ratio produces no change in the relative effectiveness of these ligands.

The dihydroxamates aerobactin and rhodotorulic acid, with $p[M]_1$ values of about 25, are able to compete for iron more effectively than is indicated by their β_{110} formation constants. In the case of aerobactin, the log β_{110} of 23.06 is 6-9 orders of magnitude smaller than the trihydroxamate values, but additional coordination of the citrate hydroxyl group above pH 6 decreases the difference in p[M] to only 2-5 orders of magnitude. The β_{110} constant for rhodotorulic acid is only 21.99,[19] but above pH 4, the iron exists as a Fe_2RA_3 dimer, which is sufficiently stable to raise the $p[M]_1$ value to 25.0.

The transferrin values are based on the constants recently reported by Aisen, Leibman, and Zweier,[31] using the blood serum bicarbonate concentration of 0.024 M. The p[M] values at pH 7.4 should accurately represent the ability of a given ligand to compete with serum transferrin for iron. It is clear that transferrin, with a $p[M]_1$ value of 25.6, cannot compete thermodynamically with enterobactin or any of the trihydroxamate siderophores, and is only slightly more effective than aerobactin. Because of the dimeric nature of the rhodotorulate complex, the competitive equilibrium with transferrin is strongly dependent on the concentration and ligand:metal ratios. With a tenfold excess of ligand, the transferrin $p[M]_2$ is 1.7 units higher than rhodotorulic acid. However, increasing the ligand concentration to 1 mM cuts the transferrin advantage to only 0.62 log unit. Higher ligand:metal ratios or higher iron concentrations would be expected to shift the p[M] value of rhodotorulic acid above that of transferrin.

Aerobacter aerogenes 62-1 produces the catecholate siderophore enterobactin in addition to aerobactin, described here.[3] A great deal more aerobactin than enterobactin is produced. The stability constant for enterobactin has been determined and log β found to be ~52,[32] which is some 29 orders of magnitude larger than the value we report here for aerobactin. Speaking teleologically, the question arises as to why the organism should produce the inferior iron chelator aerobactin when it can produce the "super" iron chelator enterobactin.

It has been found that enterobactin is destroyed enzymatically after fulfilling its iron transport function, whereas aerobactin can be separated from the iron under mild conditions and be reused by the organism. We and others have previously suggested that the iron can be reduced and removed from the hydroxamate siderophores via physiological reductants, while it cannot be from enterobactin. This concept was supported by our observed redox potentials of enterobactin (~−0.700 V vs. NHE) and ferrichrome (~−0.450 V).[11] In the case of aerobactin and enterobactin, both derived from the same organism, the case is made even more clear as the redox potential of aerobactin (−0.336 V) is almost 0.5 V more positive (easier to reduce) than enterobactin. A qualitative picture thus emerges for the siderophore-mediated iron transport in *A. aerogenes*. Under conditions of mild iron stress the organism excretes aerobactin, a moderately good iron chelator which can compete effectively for iron and deliver it to the cell. The iron thus obtained can be released from aerobactin via reduction and the aerobactin reused. However, if the availability of iron becomes extremely short (growth limiting), *A. aerogenes* can excrete enterobactin, which then can remove iron from almost all known physiological/chemical sources. By excreting this extremely powerful chelator, *Aerobacter* can compete favorably for the available iron with any competing organisms. But for this ability it must pay a price in metabolic energy, since to remove iron from enterobactin, destruction of the molecule is necessary. Thus, *Aerobacter* possesses both high affinity-low capacity (enterobactin) and low affinity-high capacity (aerobactin) iron transport systems, which can provide the organism with iron with the least expenditure of metabolic energy under a variety of conditions.

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