Coordination Chemistry of the Amonabactins, Bis(catecholate) Siderophores from *Aeromonas hydrophila*[1]

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The amonabactins are a series of four bis(catecholate) siderophores isolated from the pathogenic organism, *Aeromonas hydrophila*. As tetradentate ligands, they cannot singly satisfy the octahedral coordination sphere of iron. The solution coordination chemistry of the amonabactins has been elucidated using potentiometric and spectrophotometric titrations, circular dichroism, and mass spectroscopy. They form 2:3 metal:ligand complexes at high pH and excess ligand. Their complexation behavior is essentially identical to one another, with log β_{230} = 86.3. At lower pH, they preferentially form a 1:1 bis(catecholato)bis(aqua) iron(III) species, with log β_{110} = 34.3. The 2:3 complexes show a very slight Δ preference in chirality at the metal center, while the 1:1 complexes are achiral. The biological implications of these properties are discussed.

Introduction

The role of siderophores (microbial iron transport agents[2−4]) as growth factors for bacterical pathogens has prompted research into their chemical and structural characterization and transport mechanisms. However, information about a siderophore's potential effectiveness as an iron chelator in competition with other complexing agents also requires solution thermodynamic characterization. The stability of an iron−siderophore complex must be greater than that of iron hydroxide or the siderophore cannot solubilize ferric iron. Siderophores involved in virulence (which are proposed to remove iron from serum transferrin) must have a greater affinity for iron than transferrin proteins in order to be effective. This principle also applies to synthetic iron chelators designed to reduce corporal iron overload. The stability of an iron−siderophore complex influences the $Fe^{III/II}$ reduction potential and is a determinant of the mechanism of iron release from a siderophore.

The amonabactins (Amo) (Figure 1) are a family of four biscatecholate siderophores from *Aeromonas hydrophila*.[5,6] They are composed of either tri- or tetrapeptides in the sequence (gly)-(L)-lys-(L)-lys-(D)-aro, where glycine is the optional amino acid attached to the N^{ϵ} amine of the N terminus lysine and aro is either tryptophan or phenylalanine. The coordinating 2,3-dihydroxybenzamide (DHB) groups are attached to the N^{ϵ} amine of the C terminus lysine and to either glycine, if present, or the N^{ϵ} amine of the N terminus lysine.

The amonabactins have been assumed to participate in the virulence of the organism. To accomplish this function they must be able to sequester and deliver iron in vivo to the microorganism against a strong thermodynamic bias. Therefore they must exhibit three properties: they must be thermodynamically competent to remove iron in vivo from iron sequestering proteins (i.e. transferrin); they must be kinetically competent

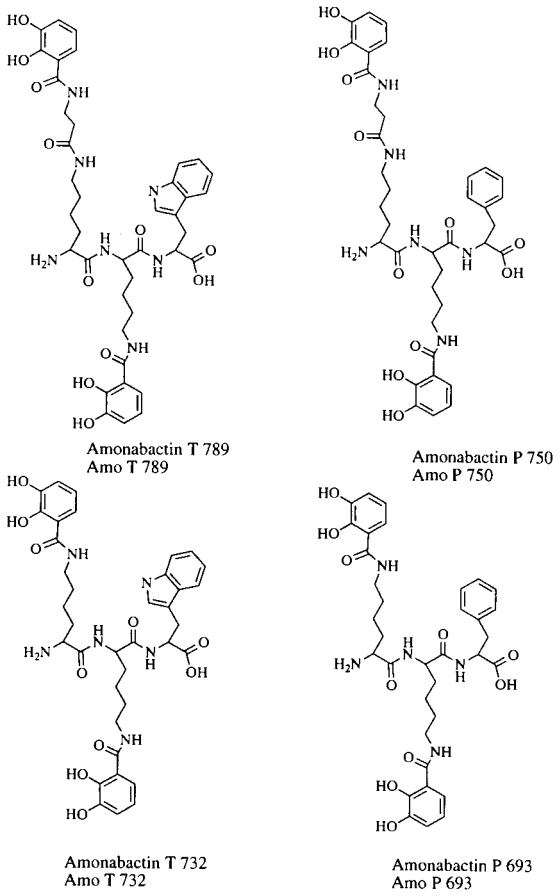

Figure 1. Amonabactins (the names refer to the molecular weight and the aromatic amino acid constituent (tryptophan or phenylalanine).

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to remove the iron; and they must be able to exist in serum for long enough periods of time to perform these functions.

It is known that the amonabactin siderophores can support *A. hydrophila* in both serum and heat inactivated serum (which refers to inactivation of the complement system in serum).[7,8] Previous work has shown catecholate siderophores in general are kinetically competent to remove iron from transferrin.[9,10] Since the amonabactins are only tetradentate ligands, we address the question of whether the amonabactins are thermodynamically able to compete with transferrin for iron.

Since *A. hydrophila* is not an obligate pathogen, it must also be able to live outside a host. The normally free-living *Aeromonas* is a freshwater bacterium; infection occurs only in host organisms under stress or with a decreased ability to fend off infections.[11,12] In both environments, the organism is in competition for available nutrients, including iron. An evaluation of the ferric amonabactin complexes addresses the effectiveness of these siderophores in the environment and in vivo.

Experimental Procedures

General. All solutions were prepared using deionized, distilled water, which was further purified by passing through a Millipore cartridge system to resistivity = 18 MΩ. The water was then degassed by boiling for 30 min while argon was bubbled through it. All solutions were stored under an atmosphere of ascarite-scrubbed argon to prevent absorption of carbonate. The absence of carbonate was confirmed by Gran plots of strong acid/strong base titrations.

Titrant solutions were prepared from Baker Dilut-IT ampule concentrates using freshly distilled, boiled water. The base (0.1 M KOH) was standardized by titrating against potassium hydrogen phthalate, using phenolphthalein as an indicator. The acid was standardized by titrating against the KOH solution, again using phenolphthalein as an indicator. All solutions were maintained at 0.100 M ionic strength by using 0.100 M KCl (Fisher 99.99%). The pH electrode (Orion semimicro Ross combination glass electrode) was calibrated to hydrogen ion concentration.

Syntheses. The isolation and synthesis of the amonabactins have been described elsewhere;[6] N^{α}-{N^{α}-(carbobenzyloxy)-N^{ϵ}-(2,3-dibenzyloxybenzoyl)-(L)-lysyl}-N^{ϵ}-(2,3-dibenzyloxybenzoyl)-(L)-lysinate (**1**) was synthesized as then described.

N^{α}-{N^{ϵ}-(2,3-Dihydroxybenzoyl)-(L)-lysyl}-N^{ϵ}-(2,3-dihydroxybenzoyl)-(L)-lysinate (2). A solution of (**1**) in methanol:ethanol:ethyl acetate (40:40:20) was stirred at room temperature with 10% Pd on carbon catalyst under H_2 (1 atm) for 6 h. Following this, the solution was filtered to remove the catalyst, and the solvent was removed under reduced pressure. The material was then taken up in a few milliliters of methanol, applied to a reverse phase HPLC column and eluted with a mobile phase consisting of 28% acetonitrile and 0.1% TFA in water.

Potentiometric Titrations (Ligand Only). The apparatus and methods for potentiometric titrations have been described in detail elsewhere.[13,14] Titrations were performed in a capped, jacketed titration vessel connected directly to a constant temperature water recirculating bath. The solutions were maintained at constant ionic strength (0.100 M KCl) and temperature (25 ± 0.05 °C). In a typical experiment, 166 data points were collected over the pH range from 5.0 to 11.5. Of these, 122 data points spanning the pH range 6.99−11.5 were used for refinement. An average of 0.01 mM ligand was used in each potentiometric titration. Thermodynamic reversibility of the titrations was confirmed by performing titrations from low to high pH and back to low pH on at least one solution. The data were refined using the nonlinear least-squares fitting program, BETA 90.[13] The results are an average of at least three experiments.

Spectrophotometric Titrations. The apparatus and methods for spectrophotometric titrations have been described in detail elsewhere.[15] When necessary, EDTA (*N*,*N*,*N*-ethylenediaminetetraacetic acid) (Aldrich standardized solution, 0.1004 M) was used as a competitive ligand. All solutions were maintained at constant ionic strength (0.100 M KCl) and constant temperature (25 ± 0.05 °C) by using a cuvette and jacketed titration cell fitted to a constant temperature water recirculating bath. The reversibility of the titrations was confirmed (*vida supra*). The models used to fit the data were refined on the factor analysis and nonlinear least squares program REFSPEC.

Mass Spectroscopy. Negative ion mode electrospray mass spectroscopy was performed at the University of California Department of Analytical Services. Samples (1 mM) of ferric amonabactin P 693, and ferric amonabactin T 732 (ligand:metal, 10:1) were prepared at pH 6.2.

Circular Dichroism. Circular dichroism spectra were recorded using 1 and 10 cm cells on a Jasco J500C spectrometer equipped with an IF-500 II A/D converter. Data collection was controlled by a microcomputer. Ferric complexes of all the amonabactins (18 mM) were prepared at pH 9.1 (phosphate buffer) using a 10:1 ligand:metal stoichiometry, and pH 7.4 (HEPES buffer) at a 1.1:1 stoichiometry. Data acquisition was at 0.2 nm resolution with a scan rate of 1 nm/min and a sensitivity of 25 millidegrees.

Results and Discussion

The solution chemistry of the amonabactin complexes with ferric ion was characterized by spectrophotometric titration; mass spectroscopy was used to elucidate stoichiometry. Simple acid−base spectrophotometric titrations were used to determine protonation constants of the complexes, while overall stability constants were determined by competition titrations. The ferric amonabactin complexes all behave very similarly, thus a discussion of only Amo P 693 is used throughout the text to describe procedures and properties for all four.

Potentiometric Titrations. Attempts to determine directly the protonation constants of the amonabactins were unsuccessful due to low solubility of the ligands. The protonation constants were therefore estimated from the dimer, N^{α}-(N^{ϵ}-di-2,3-dihydroxybenzoyl)-L-lysyl-(N^{ϵ}-di-2,3-dihydroxybenzoyl)-L-lysine (**2**, Figure 2). The additional aromatic residue in the amonabactins significantly reduces their solubility. In contrast, analogue **2** is soluble in aqueous solution; the higher three protonation constants were determined by potentiometric methods. Below pH 6, **2** precipitates, preventing the direct determination of the carboxylate protonation constant. Also, at very high pH (>9), **2** oxidizes in air, and the solution becomes orange. However, this did not affect the results of this study, since the extent of oxidation was negligible.

The amonabactins and **2** are hexaprotic acids with four catecholamide protons, one amine proton, and one carboxylic acid proton. The protonation constants for **2** and the amonabactins are summarized in Table 1. Selected protonation constants for *N*-ethyl-2,3-dihdroxybenzamide (EBA) and enterobactin are included for comparison. The protonation constants log K_{011} and log K_{012} are assigned to the upper two protonation constants of the catecholamides (Table 1). These

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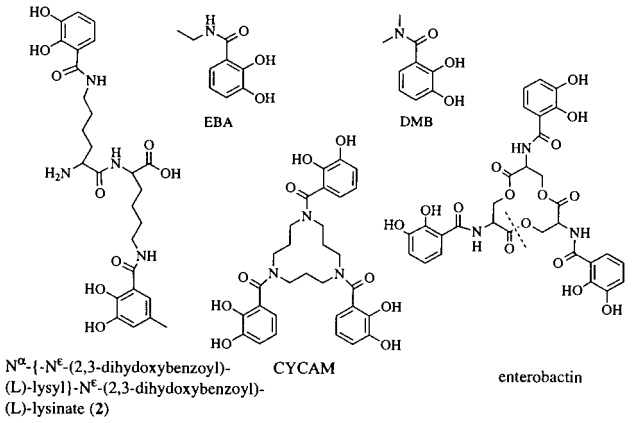

Figure 2. Enterobactin and catecholate siderophore analogues. Abbreviations are given in the text. Hydrolysis at the dashed line on enterobactin gives the DHBS linear trimer, removal of one serine group gives the DHBS linear dimer.

Table 1. Summary of Protonation Constants for the Amonabactins and Analogs (See Figures 2 and 3)[a]

	2	amonabactins		EBA	enterobactin[a]
	Figure 2	Figure 1		Figure 2	Figure 2
K_{011}[b]	12.1	12.1	$K_{012(014)}$	7.34	8.55
K_{012}[b]	12.1	12.1	K_{015}		7.5
K_{013}	8.8(2)[c]	8.8(2)[c]	K_{016}		6.0
K_{014}	7.7(2)	7.7(2)			
K_{015}	7.0(2)	7.0(2)			
β_{015}[d]	47.7(2)	47.7(2)			
β_{014}[e]		38.9(2)	$\beta_{012(016)}$	19.44	58.5
o-hydroxyl[f]		7.35		7.34	7.35

[a] Log values. Numbers in parentheses represent the standard deviation in the last significant digit. Subscripts indicate metal:ligand:proton stoichiometry. [b] Estimated, see the text and ref 17. [c] Assigned as an amine protonation. [d] The carboxylate protonation is not included. [e] Since neither the amine nor the carboxylate participate in binding, their protonation values were not included in the refinement of spectrophotometric data. [f] Average protonation constant of the ortho hydroxyl protons.

values were not directly determined, but rather estimated to be 12.1 based on a closely comparable ligand.[16,17] The highest protonation constant which was observed, log $K_{013} = 8.8$, agrees with the published value (log $K = 8.95$) of the N^{α} amine protonation constant of lysine.[18] The next two protonation constants, log K_{014} and log K_{015}, are again assigned to be catecholamide protons. The average of these protonation constants, 7.35, is in excellent agreement with the lower protonation constant of the ligand EBA (log $K = 7.34$) (Figure 2), and the average of the three lower protonation constants of enterobactin (7.36).

While the ligand was not sufficiently soluble to determine the carboxylic acid log K_a, it can be estimated from analogous amino acids as log $K_{016} \approx 3.6$. With these values, the species distribution of the protonated forms of the amonabactins can be generated (Figure 3). At pH 7−8, the ligand exists predominately as the tetra- and triprotonated forms. The pentaprotonated ligand is a zwitterion with no net charge and the forms above pH 7 are anionic, and thus significantly more soluble.

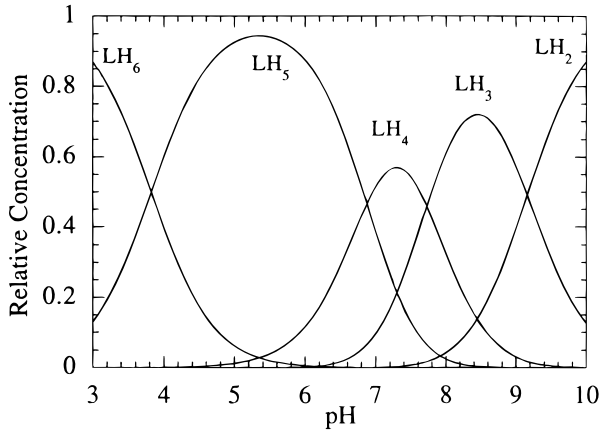

Figure 3. Species distribution curves for the amonabactins as a function of pH.

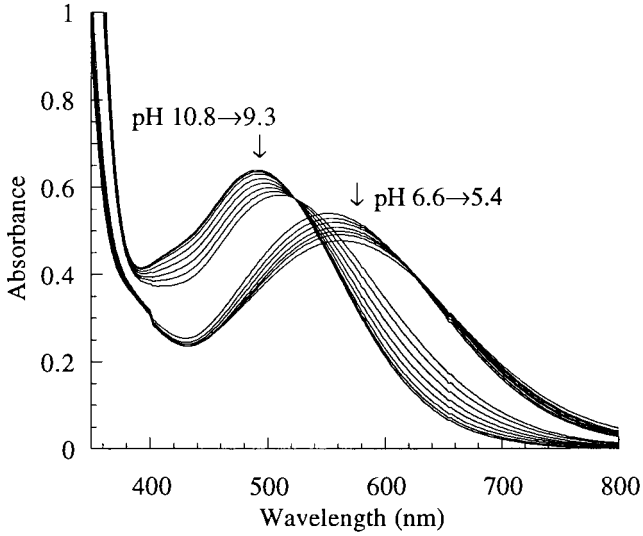

Figure 4. Spectrophotometric titration of ferric amonabactin P 693 (metal:ligand 2:3.2), in absorbance units uncorrected for dilution. The uppermost set of spectra are from the pH range 10.8 (uppermost curve) to 9.3. The lower set of spectra are from the pH range 6.6−5.4. The spectra recorded between these two ranges are not plotted, since they obscure the isosbestic point.

Spectrophotometric Titrations. The spectrophotometric pH titrations of the ferric amonabactins show three buffer regions. The spectra for the titration of ferric amonabactin P 693 are given in Figure 4. The red complex (λ_{max}, 492 nm) that forms at high pH is ascribed to the $Fe_2[Amo]_3^{6-}$ complex. As the pH is lowered from 10 to 9, the absorbance maximum shifts through an isosbestic point to give a purple species. Lowering the pH further, to below 6, results in a second bathochromic shift to give a blue species (λ_{max}, 540 nm) which is assigned as the 1:1 $[Fe(Amo)]^-$ species. On lowering the pH still further a dark blue-black material precipitates. In the titration of ferric enterobactin, a purple precipitate, $[Fe(H_3ent)]^0$, was also observed below pH 4[16,19] and assigned as a tris(salicylato) ferric complex, as recently confirmed.[20] In this case, the species is assigned as $[Fe(HAmo)(H_2O)_2]^0$; the addition of a single proton to the bis(catecholato) complex resulting in a neutral complex which precipitates.

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The proton-independent stability constant for the Fe^{III}/amonabactin ($[Fe_2(Amo)_3]^{6-}$) system can be described as follows:

$$2Fe(H_2O)_6^{3+} + 3Amo^{4-} \rightleftharpoons Fe_2(Amo)_3^{6-}$$

$$\beta_{230} = \frac{[Fe_2(Amo)_3^{6-}]}{[Fe_2(H_2O)_6^{3+}]^2[Amo^{4-}]^3} \quad (1)$$

It was not possible to determine directly the formation constant by simple pH titrations because complete dissociation of the complex never occurs throughout the observable pH range. However, the protonation constant for the metal complex could be determined this way. The 2:3 complex protonates to give $[H_2Fe_2(Amo)_3]^{4-}$, which then dissociates to a 1:1 complex, $[Fe(Amo)]^-$, at lower pH. The protonation of the $Fe_2(Amo)_3]^{6-}$ species can be formally described by the equilibrium equation:

$$Fe_2(Amo)_3^{6-} + 2H^+ \rightleftharpoons H_2Fe_2(Amo)_3^{4-}$$

$$K_{232} = \frac{[H_2Fe_2(Amo)_3^{4-}]}{[Fe_2(Amo)_3^{6-}][H^+]^2} \quad (2)$$

The formation constant of $[Fe(Amo)]^-$ can also be formally described as a proton independent reaction as $[Fe_2(Amo)_3]^{6-}$ is in eq 1.

$$Fe(H_2O)_6^{3+} + Amo^{4-} \rightleftharpoons Fe[(Amo)(H_2O)_2]^-$$

$$\beta_{110} = \frac{[Fe(Amo)(H_2O)_2^-]}{[Fe^{3+}(H_2O)_6][Amo^{4-}]} \quad (3)$$

The formation of $[Fe_2(Amo)_3]^{6-}$ can be described by eq 4 as the proton-dependent formation constant from $[Fe(Amo)]^-$.

$$H_4Amo + 2Fe(Amo)^- \rightleftharpoons Fe_2(Amo)_3^{6-} + 4H^+$$

$$K_{230} = \frac{[Fe_2(Amo)_3^{6-}][H^+]^4}{[H_4Amo][Fe(Amo)^-]^2} \quad (4)$$

As the pH is lowered protonation of the catecholate group results in loss of one ligand, replaced by water. The spectrum of $[Fe(Amo)]^-$ is virtually identical with that of the ferric bis(catecholate) complex, $[Fe(DMB)_2]^-$, where DMB is *N*,*N*′-dimethyl-2,3-dihydroxybenzamide, a monomeric analogue of catecholamide siderophores (Figure 2).[19]

Based on other catecholamide ligands, the proton dependent equilibria were expected to overlap significantly. Since only poorly resolved isosbestic behavior was seen (at lower pH), no attempt was made to analyze the titration data by simple graphical methods. Instead factor analysis and nonlinear least-squares methods were applied to a model based on the species in equilibria 1−3.

Factor analysis of the spectra indicated three components in the spectrophotometric pH titrations. Refinement of the spectrophotometric data fit the above equations quite well. All attempts at data refinement were unsuccessful on a model comprising a four component system $[Fe_2(Amo)_3]^{6-}$, $[HFe_2(Amo)_3]^{5-}$, $[H_2Fe_2(Amo)_3]^{4-}$, and $[Fe(Amo)]^-$, where the protonation steps of eq 2 are separated. The component absorbance of $[HFe_2(Amo)_3]^{5-}$ is a linear combination of the fully deprotonated and diprotonated species; the factor analysis package used in refining the data was unable to extract that constituent. The stepwise protonation and formation constants of the ferric complexes are given in Table 2. Refinements also determined the extinction coefficient spectra of the three species predicted to be in solution during the titration: $[Fe_2(Amo)]_3]^{6-}$, $[H_2Fe_2(Amo)_3]^{4-}$, and $[Fe(Amo)]^-$. These are shown in Figure 5. The pH dependent species distributions predicted by using the protonation constants for the ferric amonabactins are given in Figure 6.

Table 2. Summary of Stability Constants and Spectral Data for the Amonabactins

	Amo T 789	Amo P 750	Amo T 732	Amo P 693	average	transferrin[a]
β_{110}[a]	34.5(5)	34.4(4)	34.2(4)	34.2(5)	34.3(5)	
K_{232}[a]	18.9(3)	18.8(5)	18.8(4)	18.8(5)	18.8(5)	
β_{230}[a]	86.4(3)	86.4(3)	86.3(4)	86.1(5)	86.3(5)	
β_{232}[a]	105.3(4)	105.2(5)	105.1(4)	104.9(4)	105.1(5)	
pM[b]					25.8	23.6
λ_{110}[c]	570 (3.6)	560 (4.0)	562 (4.0)	564 (3.9)		
λ_{230}[c]	490 (8.4)	492 (8.6)	494 (8.8)	494 (8.4)		
λ_{232}[c]	518 (6.6)	516 (6.7)	520 (6.7)	520 (6.6)		

[a] Log values. Numbers in parentheses represent the standard deviation in the last significant digit. Subscripts indicate metal:ligand:proton stoichiometry. [b] For 10^{-6} M total Fe^{3+}, 10^{-5} M total ligand, and pH 7.4. [c] Wavelength at the ligand to metal charge-transfer absorption maximum, nm (molar extinction, 10^{-3} M^{-1} cm^{-1}).

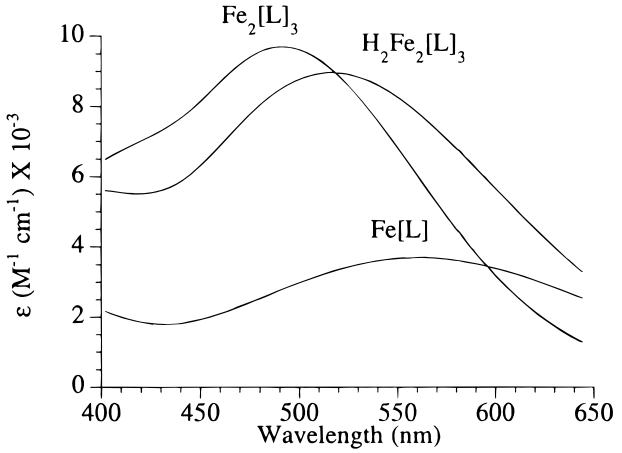

Figure 5. Calculated extinction coefficient spectra from the factor analysis and refinement of the spectrophotometric pH titration of amonabactin P 693.

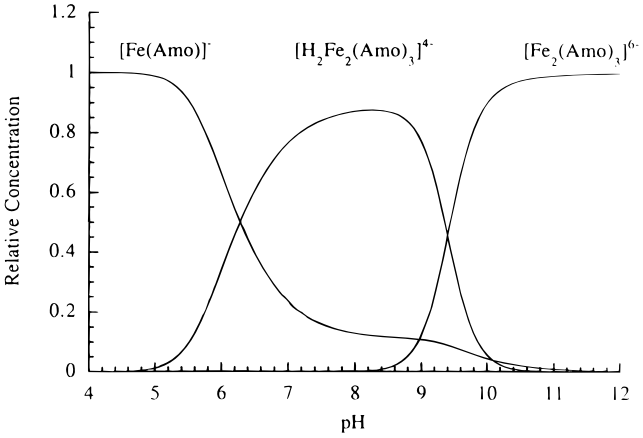

Figure 6. Species distribution as a function of pH for the ferric amonabactin complex (total [Fe] = 1.8×10^{-5}, [L] = 2.7×10^{-5}).

Competition Titrations. The ferric amonabactin complexes are too stable (and insufficiently soluble at low pH) to be able

Table 3. Summary of Circular Dichroism Data for the Ferric Complexes of the Amonabactins and Enterobactin

	amonabactins			
	pH 9.2	pH 7.0	enterobactin[a]	enterobactin linear dimer[a]
assignment	Δ (weak)	achiral	Δ	achiral
$\Delta\epsilon^b$	+0.02 (450)	0 (450)	+6 (410)	+0.7 (410)
$\Delta\epsilon$	−0.1 (550)	0 (550)	−4 (535)	+0.3 (535)

[a] From ref 19. [b] The $\Delta\epsilon$ at CD maximum or minimum (wavelength, nm).

to determine the stability constant β_{110} by direct proton competition. Instead, competition titrations utilizing EDTA were employed to determine the conditional stability constant of the $[Fe_2(Amo)_3]^{6-}$ complex for each of the amonabactins. We have often used this procedure to evaluate very stable complexes.[17,21−23] At high pH the $[Fe_2(Amo)_3]^{6-}$ complex predominates. As the pH is lowered the complex shifts to the protonated $[H_2Fe_2(Amo)_3]^{4-}$ complex and then dissociates to the ferric EDTA complex at pH 5−6. The following model, which includes the measured protonation of the $[Fe_2(Amo)_3]^{6-}$ complex and the known stability of EDTA with ferric ion, was used to refine the stability constant:

$$Fe_2(Amo)_3^{6-} + 2H^+ \rightleftharpoons H_2Fe_2(Amo)_3^{4-} + 2H^+ + EDTA \rightleftharpoons Fe(EDTA) + H_4Amo \quad (5)$$

The extinction coefficient spectra generated by the data refinement package agree with those generated by the spectrophotometric pH titrations for the $Fe_2[Amo]_3$ and the $H_2Fe_2[Amo]_3$ complexes. The average formation constant for the amonabactin complexes was determined to be $\log \beta_{230} = 86.3$, which gives $\log \beta_{232} = 105.1$ and $\log \beta_{110} = 34.3$.

Circular Dichroism. The CD spectra of the ferric amonabactin complexes are summarized in Table 3. The 1:1 ferric:amonabactins are an equal mix of Λ and Δ complexes. The $\Delta\epsilon$ values for the 2:3 complexes are $\Delta\epsilon = -0.1$ (550 nm); about one-tenth the strength as that of ferric enterobactin ($\Delta\epsilon = -4.0$, 535 nm), but of the same sign, and show that there is only a slight preponderence of the Δ complex.[18] The Cotton effect seen at 550 nm in the $[Fe_2(Amo)_3]^{6-}$ and the $[Fe(ent)]^{3-}$ complexes is due to metal centered transitions.[24] The Cotton effect below 360 nm is due to absorbance of the ligand.

Discussion

The metal center chirality of amino acid based siderophores is induced by the catechol group's proximity to the α carbon of the appended amino acid. Every example of DHB appended to the N^α amine of L-lysine gives a metal complex of Δ chirality; DHB appended to the N^α amine of D-lysine invariably gives a metal complex of the opposite (Λ) chirality. The ferric complex of chrysobactin (FeL_3 complex), a monocatecholate siderophore in which DHB is conjugated to the N^α amine of D-lysine, is Λ chirality, the opposite of amonabactin. Ferric complexes of chrysobactin analogues with DHB conjugated to the N^α amine of L-lysine showed the same chirality (Δ) as the 2:3 complexes of amonabactin.[25] In the amonabactins, the catecholamides are four carbons away from the chiral amino acid center and net chirality is probably induced by weak interactions between the three ligand backbones.

Chirality in siderophore complexes is seen only in the tris(catecholate) (or other tris(bidentate)) complexes. Thus, as one amonabactin moiety protonates and dissociates, the slight chirality induced by the backbone is completely eliminated. This behavior is also seen with ferric chrysobactin.[25] The hydrolysis products of enterobactin show varying degrees of induced chirality. The ferric complex of the linear dihydroxybenzoyl serine (DHBS) dimer (a bis(catecholate)) exhibits no chiral preference, even though the linear trimer and enterobactin (both tris(catecholates)) are entirely Δ configuration.[19]

The evaluation of the stability constants and solution equilibria of the amonabactins is somewhat more complicated than that of simple bidentate or hexadentate ligands with iron. The amonabactins are tetradentate and in a 1:1 complex they are unable to coordinatively saturate ferric ion, which prefers hexadentate chelation. Therefore, it is the M_2L_3 complex which fully satisfies the coordination geometry about ferric ion. However, the high $\log K_a$ (about 9.4) of the $[Fe_2(Amo)_3]^{6-}$ complex suggests that it is strained in such a way to destabilize the third catecholate. A typical $\log K_a$ for a tris(catecholate) complex is about 6. The 1:1 $[Fe(Amo)]^-$ complex is very stable, and is the predominate complex at neutral pH. Proof of the monomeric stoichiometry of the complexes (rather than a 2:2 complex) can be seen by titration of the ligand with ferric ion and mass spectroscopy. Plots of the absorbance at 490, 540, and 650 nm show linear behavior with sharp breaks at 0.67 and 1.0 equiv of iron/amonabactin.

The dominant mass seen in electrospray mass spectroscopy of the pH 6.2 ferric amonabactin P 693 complex is at *m*/*z* 372, corresponding to the $[Fe(Amo)]^{2-}$ complex. A peak assigned to the singly charged complex, at *m*/*z* 745, is also seen. Peaks at *m*/*z* 392 and 783, corresponding to the two charged states of the potassium salt of the monomer K[Fe(Amo)] are also seen. Free ligand is observed at *m*/*z* 346. There is no direct evidence for $(Fe(Amo)(H_2O))^-$ or $(Fe(Amo)(H_2O)_2]^-$. The corresponding species are seen in the mass spectrum of the ferric amonabactin T 732 complex.

Studies of other amino acid based siderophores with free carboxylic acid and amine functionalities demonstrate that those functionalities do not participate in metal binding.[19,26] Neither functionality competes effectively with water to coordinate iron. The UV/vis spectra of the amonabactins show no changes in the ligand to metal charge transfer (LMCT) band region observed over the pH regime where the amine protonates. Since the complex precipitates below pH 5.5, no observations were made over the pH regime where the carboxylate would protonate. In the amonabactins, since neither the carboxylate nor the amine bind to the iron and their protonation behavior has no effect on the spectra of the complexes, the equilibrium equations that are given do not include protonation or deprotonation of the amine or carboxylate sites. If the carboxylate or the amine did participate in binding the metal ion, then they (being adjacent to chiral centers) should induce chirality in the ferric complexes, which was not observed by circular dichroism.

The amonabactin ligands, while powerful iron complexing agents, do not form tris(catecholate) complexes as stable as other ligands with the same chelate group. The average formation

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constant of ferric amonabactin, at log $\beta_{230} = 86.3$, is about the same (if normalized to one iron) as that of the simple *N*-ethyl-2,3-dihydroxybenzamide (EBA, $\beta_{130} = 43.7$) (Figure 2) or the linear trimer of enterobactin ($\beta_{110} = 43.0$). The amonabactin complexes exhibit a two-proton reaction at log $K_{232} = 18.8$ before dissociation of one ligand, while the ligand EBA shows only complex dissociation. A high protonation constant is also seen in the enterobactin analogue CYCAM (Figure 2) (log $K_{111} = 9.0$)[21] and in a tris(catecholamide) ligand with a cyclodextrin backbone (log $K_{111} = 8.4$).[27] In the case of CYCAM, the scaffold is a poor ligand motif, and the lack of amide protons significantly decreases the stability of the complex (the amide proton participating in a hydrogen bond to the ortho phenolic oxygen has been shown to play a key role in the stability of catechol complexes).[2,3] The cyclodextrin analogue has secondary amide linkages, like the amonabactins, but the metal binding cavity is too large to easily form a 1:1 complex. Similarly, the amonabactins are poorly designed to form a 2:3 complex; their protonation behavior is a result of their decreased stability.

The behavior of the 1:1 ferric amonabactin complexes should be comparable to that observed in the DHBS linear dimer derived from enterobactin.[18] The formation constant of the $[Fe(Amo)]^-$ complex ($\beta_{110} = 34.3$) is lower than that of the linear dimer of enterobactin ($\beta_{110} = 36$) by about 50-fold, reflecting the linear dimer's superior predisposition to metal binding, but the amonabactins are about an order of magnitude better ligands for iron than the simple bidentate chelate EBA ($\beta_{120} = 33.6$). The pM value (Table 2) of amonabactin is also higher than that of EBA as a result of the lower concentration dependence of a tetradentate ligand. That the stability of amonabactin's iron complex is higher than that of transferrin,[17,26,28] is significant with regard to the role that siderophores and iron play in the virulence of microbes. The amonabactins offer a formidable method of acquiring a limiting nutrient in vivo.

Biological Implications. Amonabactin is one of several siderophores which do not fully satisfy the coordination sphere of ferric ion. Others, such as chrysobactin, Itoic acid, and azotochelin have been reported in the literature, although little has been reported on their solution chemistry.[29−31] The ferric amonabactin complexes in natural environments (water or serum) will certainly be present in extremely low concentrations (micromolar or less), and should exhibit 1:1 ferric ion: siderophore stoichiometry.

Since the complex mixture is not chiral it seems likely that the membrane receptor for the ferric amonabactins is also not chiral. All the amonabactins support growth of *A. hydrophila* under iron restricted conditions. Enterobactin does not, but this could be because there is no receptor capable of internalizing enterobactin, or because the microbe is unable to extricate the iron from the ferric enterobactin complex. The bis(catecholate) siderophore azotochelin and the mono-catecholate ligand EBA also support growth. These data suggest that the receptor, or receptors, on *A. hydrophila* have minimal chiral and structural specificity. This may be an example of a common strategy among bacteria: namely, the expression of multiple siderophore receptors and receptors of low specificity. In this manner, a variety of siderophores can provide the necessary iron for growth and less metabolic energy is spent in its acquisition. Additionally, by incorporation of D-amino acids at the C terminus, and blocking the basic residues of the lysines, the amonabactins have some amount of protection from degradation by proteases. From an evolutionary point of view, *A. hydrophila* has developed a metabolically low cost mechanism of gaining iron which is both resistant to host constitutive defenses and allows for a wide variety of siderophores to be utilized.

Conclusions

The solution chemistry of the ferric amonabactin complexes are all very similar. Each follows a general reaction series with iron and protons of $M_2L_3 \rightarrow H_2M_2L_3 \rightarrow ML$ as a function of pH. The catecholamide coordination sites of amonabactin are many atoms away from stereogenic centers and thus the 1:1 ferric amonabactin complexes are not chiral, while the 2:3 ferric complexes show minimal preference for Δ chirality. The amonabactins are thermodynamically competent to remove iron from the serum protein transferrin, and catecholamides have been shown to be kinetically competent as well.[9,32] These results have significant implications on the role of the siderophore amonabactin in the virulence of *A. hydrophila*.

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Supporting Information Available: Table S1, giving experimental conditions for the spectrophotometric titrations, and Figures S1−S8, showing representative potentiometric titration data for **2**, the spectrophotmetric titration data of amo P 693, the spectrophotometric titration of amo T 789 and the competition titration of amo T 789, as well as circular dichroism spectra of amo T 789 and amo P 750 at high pH, electrospray mass spectra of the amo P 693 and amo T 732 metal complexes; circular dichroism spectra of (a) $Fe_2[Amo]_3$ (pH 9.1) and (b) Fe[Amo] (pH 7.4), circular dichroism spectra of (a) ferric enterobactin and (b) linear dimer, and plot of the absorbance at 490, 540, and 650 nm for the titration of solutions of Amonabactin P 693 with Fe(III) are available (9 pages). Ordering information is given on any current masthead page.

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