Coordination Chemistry of the Carboxylate Type Siderophore Rhizoferrin: The Iron(III) Complex and Its Metal Analogs

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Rhizoferrin is a member of a new class of siderophores (microbial iron transport compounds) based on carboxylate and hydroxy donor groups rather than the commonly encountered hydroxamates and catecholates. We have studied the coordination chemistry of rhizoferrin (Rf), as a representative of this group, with Fe^{3+}, Rh^{3+}, Cr^{3+}, Al^{3+}, Ga^{3+}, VO^{2+}, and Cu^{2+}. The metal complexes have been studied by UV−vis, CD, NMR, and EPR spectroscopies and mass spectrometry. The formation constants for the iron complex have also been measured and yield a log K_{LFe} of 25.3. The Rh and Cr rhizoferrin complexes are unusual in that they appear to adopt a chirality about the metal center that is the opposite of the native iron analog. Several of the alternative metal ion complexes are found to have biological activity toward *Morganella morganii* in a plate type assay.

Introduction

Siderophores are low molecular weight iron binding compounds excreted by nearly all microorganisms in response to the low levels of this essential element available in the environment.[1] These low levels of available iron stem from its profound insolubility at neutral pH in an aerobic atmosphere ($K_{sp}(Fe(OH)_3)$ = ca. 10^{-38}). The siderophores serve not only to solubilize Fe^{3+} but also to transport, and in some cases store,[2] it in microbial cells. Most of the known siderophores employ either the hydroxamate or catecholate groups in forming very specific and extremely stable octahedral complexes with the ferric ion.[3−7] An increasing number of mixed-ligand complexes containing either the hydroxamate or catecholate groups along with another functional group or atom (i.e. citrate hydroxy group, thiazoline ring nitrogen, etc.) are also known.[8,9] The coordination chemistry, binding constants, and transport properties of these molecules have already been extensively studied.

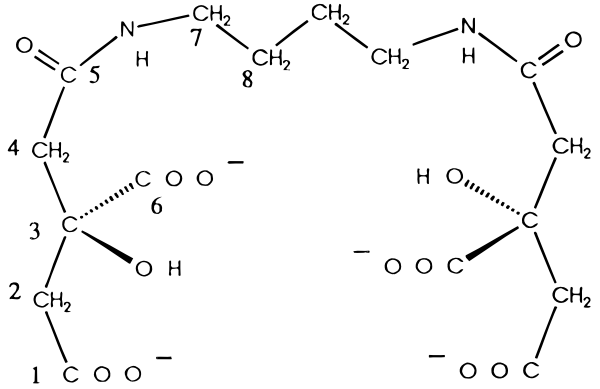

Figure 1. Structural formula of rhizoferrin.

Recently, however, a totally new class of siderophores, which employs neither hydroxamate nor catecholate groups, was isolated from various microorganisms.[8−12] These siderophores, dubbed "complexones",[8] of which rhizoferrin[11,13,14] (Rf) may be viewed as a representative example (Figure 1), contain only aliphatic amines and/or carboxylate and hydroxy donor groups, yet they bind iron strongly and clearly function to transport this metal into the cell.[12] Despite this, the structures of the iron complex of these carboxylate siderophores remain a matter of conjecture, and even less is known about their coordination chemistry with other metals. The detailed mechanisms of rhizoferrin-mediated transport by microorganisms also remain unclear. Indeed, since this class of siderophores more closely resembles the generic chelator EDTA or the so-called phytosiderophores,[15] both of which can bind more strongly to some

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other metal ions than to Fe^{3+}, the question of how the organisms can achieve any specificity of uptake needs to be addressed.

One approach to obtaining information about the basic uptake mechanisms which may be operative in siderophore-mediated iron transport is to employ alternative metal ion complexes. In work originally pioneered by Raymond and co-workers,[16−18] kinetically inert metal ions such as Cr^{3+} or Rh^{3+} have proven to be particularly informative in this regard since they are not readily exchanged or reduced. In this report we describe the coordination chemistry of rhizoferrin with Fe^{3+}, Ga^{3+}, Al^{3+}, VO^{2+}, Cu^{2+}, Cr^{3+}, and Rh^{3+} as studied by potentiometric and spectrophotometric titrations, UV−vis, NMR, EPR, and circular dichroism spectroscopies, and mass spectrometry, which provide the structural data necessary as a prelude for detailed biological studies. Such biological studies are already in progress and will be reported separately.[19]

Experimental Section

Chromatography and Analytical Methods. Free rhizoferrin was isolated and purified as previously described.[11] $CrCl_3 \cdot 3THF$ was synthesized according to the literature,[20] while $RhCl_3 \cdot 3H_2O$ was purchased from Aldrich. Reagent grade chemicals were used as received. Column chromatography was performed using both aminoethyl Bio-Gel P-2 ion exchange resin and Bio-Gel P-2 size exclusion gel, both from Bio-Rad. It was found that the separation of the desired complexes on unmodified Bio-Gel P-2 was unsatisfactory due to some nonspecific interactions with this material. However, gel treated with 0.1 M NaOH overnight followed by washing with 0.1 M HCl and then H_2O yielded a material with better resolution. Partial hydrolysis of the acrylamide polymer leads (presumably) to the introduction of a small number of free carboxy groups which appeared to be sufficient to eliminate the nonspecific interactions.

High-voltage electrophoresis was performed on Cellogel strips (Serva) using 50 mM Tris buffer, pH 7.4 at 300 V. ^1H and ^{13}C NMR spectra were recorded from D_2O solutions using Bruker 250, 400, and 600 MHz spectrometers. Molecular modeling and evaluation of NOE data were done on a Silicon Graphics Iris Indigo computer running BIOSYM software modules Discover, Insight II, and Felix, respectively. Electrospray mass spectrometry employed a Sciex API III triple-quadrupole instrument in the negative ion mode with a flow rate of 2−5 μL/min. CD and UV−vis spectra were recorded on a JASCO J600 spectropolarimeter and a Pharmacia Ultraspec III, respectively, with each instrument interfaced to a PC. EPR and Mössbauer spectroscopic measurements were made as previously described.[21] Metal analyses were obtained by atomic absorption spectrophotometry using a PE 400 instrument equipped with graphite furnace and autosampler. Biotests were performed according to literature protocols.[22]

Potentiometric and Spectrophotometric Experiments. The solutions were prepared with deionized water under argon, and the ionic strength was fixed at 2.0 M using sodium perchlorate (Merck, p.a.). Purified and characterized solid samples of rhizoferrin were dissolved in degassed water (to give a 1.0 mM solution) and placed in a jacketed cell (10 mL) maintained at 25.0 ± 0.1 °C by the flow of a HAAKE FJ thermostat. The solution was deoxygenated and continuously flushed with argon during the titration. The hydrogen ion concentration was measured with an Ag/AgCl combined glass electrode (Tacussel, High Alkalinity) and a Tacussel Isis 20,000 millivoltmeter. The millivoltmeter was standardized and the linearity of the electrode verified with concentrated solutions of sodium hydroxide (Merck, p.a.) and perchloric acid (Merck, p.a.). The titration of the free siderophore ($1.8 \leq pH \leq 12.0$) was carried out by addition of known volumes of sodium hydroxide (0.1 M) with a Manostat microburet. The potentiometric data obtained during the titration were processed with the Miniquad program,[23] which uses an iterative least-squares Marquardt refinement.

The iron(III) complex of rhizoferrin was prepared with a stock solution of $FeCl_3 \cdot 6H_2O$ (Merck, p.a.) standardized by classical methods.[24] The concentration of the ferric complex used was 1.7×10^{-4} M, and titrations were performed between pH 1.8 and 9.0 by addition of known volumes of perchloric acid (0.1 M) or sodium hydroxide (0.1 M). Spectrophotometric measurements were recorded (220−550 nm) for each solution using a Kontron Uvikon 860 spectrophotometer and Hellma quartz optical cells (1 cm). The simultaneous potentiometric and spectrophotometric data recorded at various values of pH were fitted with the Letagrop-Spefo[25] program, which uses a Raphson−Newton algorithm and a pit-mapping method to calculate the thermodynamic constants of the absorbing species and their corresponding electronic spectra.

Synthesis of Metal Complexes. The kinetically labile Fe^{3+}, Al^{3+}, Cu^{2+}, VO^{2+}, and Ga^{3+} complexes were prepared in aqueous solution at low pH using a slight excess of ligand. The pH was subsequently adjusted to the desired value with 0.1 M NaOH.

The chromium(III) complexes were synthesized by dissolving free rhizoferin (50 mg) in dry DMSO (1 mL) with heating (90 °C) and adding 1 equiv of $CrCl_3 \cdot 3THF$. The initially violet solution turned bright green, whereupon solid sodium bicarbonate (20 mg) was added. The heating was continued for an additional 6 h and the solution then allowed to stir overnight. The crude complex was precipitated with 5 volumes of acetonitrile and collected by centrifugation. It was then redissolved in water and applied to a AE P-2 column. A pale gray-green band was eluted with water while a more polar material was eluted as a violet band with 5% ammonia. Each fraction was then freeze-dried and redissolved in a minimum amount of water, and the solution was applied to a P-2 column for desalting. The major colored fractions (a single one in each case) were collected and analyzed in solution by a variety of techniques.

The rhodium(III) complex could be prepared by a similar route using $RhCl_3 \cdot 3H_2O$ as the metal source, but an improved procedure used water as the solvent. To rhizoferrin (50 mg) dissolved in H_2O (2 mL) was added sufficient solid sodium bicarbonate to bring the pH to nearly neutral. $RhCl_3 \cdot 3H_2O$ (33 mg) was added to the neutralized solution, and the pH again was adjusted to 7 with solid sodium bicarbonate. The dark reddish brown solution was stirred for 16 h, during which the color gradually lightened to a bright orange. No heat was applied, however, since if the aqueous solution was heated above 40 °C it turned black and deposited what appeared to be Rh metal. This apparent redox reaction was not observed in DMSO solution. Isolation and purification followed procedures previously described for the Cr complexes except that one major and several minor fractions were isolated from the P-2 column. Unfortunately, only the major fraction contained sufficient material for detailed characterization.

Results

Thermodynamics and Spectrophotometry. Six protonation constants for the free siderophore were determined at 25.0 °C in 2.0 M $NaClO_4$ and are presented in Table 1. The corresponding distribution curves obtained with the Haltafall program[26] for known concentrations of rhizoferrin at various pH values are presented in Figure 2a. where the fully deprotonated form of rhizoferrin is denoted L^{6-}. The constants K_1 and K_2

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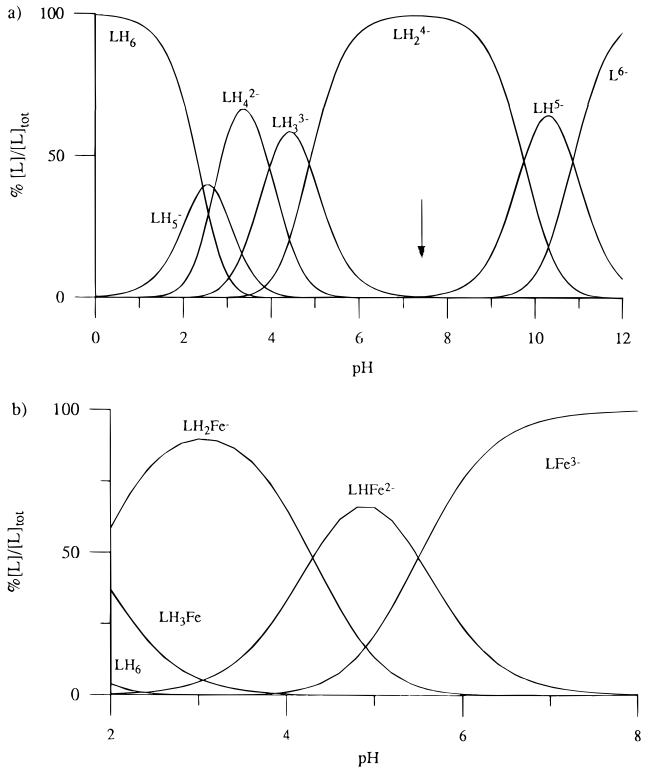

Figure 2. Species distribution curves at 25.0 °C and $I = 2.0$ for (a) free rhizoferrin (1 mM) and (b) ferric rhizoferrin (0.17 mM).

Table 1. Ligand Protonation Constants for Rhizoferrin ($I = 2.0$ M, $T = 25$ °C)[a]

log K_1	log K_2	log K_3	log K_4	log K_5	log K_6
11.3(1)	10.05(1)	5.25(3)	4.21(4)	3.05(6)	2.86(7)

[a] Values in parentheses are $\pm 2\sigma$ errors.

are readily assigned to the hydroxyl groups, as their values are of the same order of magnitude as the protonation constant of the hydroxyl site of citric acid[27] (pK = 11.6). The small decrease of the rhizoferrin protonation constants compared to those of citric acid is due to the higher ionic strength ($I = 2.0$) used in this study as well as to the statistical effects of having two hydroxyl groups. The third and fourth protonation constants agree very well with the values observed for classical carboxylic acids and with the corresponding values determined for the carboxylic sites of citric acid[27] (pK = 5.49, pK = 4.39). We assigned the K_5 and K_6 values to the carboxylic sites of the α-hydroxy carboxylic acids of rhizoferrin. The decrease of about 2 orders of magnitude of these constants compared to the protonation constants of the simple carboxylic acids is similar to the decrease observed between α-hydroxyacetic acid (pK = 3.8) and acetic acid protonation constants[28] (pK = 4.7) and is due to the inductive effect of the hydroxyl group in a position α to the carboxylic group.

The spectrophotometric titration of the ferric rhizoferrin species in water over the pH range 1.8−9.0 shows that at pH 2.0 only a shoulder at 280 nm is observable. Upon an increase in the pH however, this shoulder decreases and a ligand-to-metal charge transfer band (*vide infra*) appears at 335 nm. This charge transfer band reaches its maximum at pH 6.0. The spectrophotometric and potentiometric data obtained were processed with the Letagrop-Spefo program. This program takes into account the protonation constants of the free ligand

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Table 2. Overall Metal−Ligand Formation Constants for Iron Rhizoferrin at $I = 2.0$ M and $T = 25$ °C[a]

log K_{LFe}	log β_{HLFe}	log β_{H_2LFe}	log β_{H_3LFe}
25.3(3)	30.8(2)	35.1(1)	36.9(2)

[a] Values in parentheses are $\pm 2\sigma$ errors. L = Rf. $K = [FeL]/[Fe][L]$. $\beta_{H_nLFe} = [H_nLFe]/[H_{n-1}LFe][H]^n$.

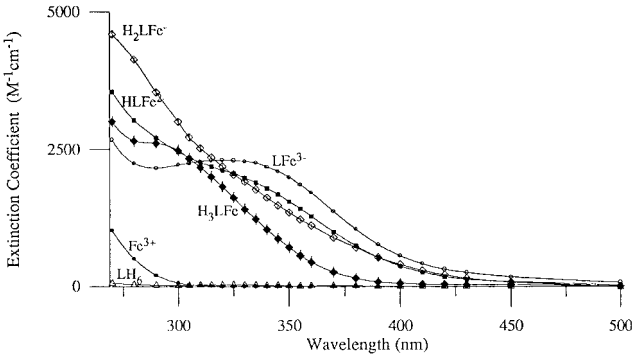

Figure 3. Calculated electronic spectra for the various ferric rhizoferrin species in water at 25 °C and $I = 2.0$ (see text).

(Table 1) and the hydrolysis constants related to $Fe(OH)^+$, $Fe(OH)_2^+$, and $Fe_2(OH)_2^{4+}$ species.[29−31] The thermodynamic constants $K_{LFe^{III}}$, β_{LHFe}, $\beta_{LH_2Fe^{III}}$, and $\beta_{LH_3Fe^{III}}$ (which are for the fully deprotonated, monoprotonated, diprotonated, and triprotonated ferric complexes, respectively) were determined, and the values are listed in Table 2. The distribution curves for the rhizoferrin ferric complex at various pH values are presented in Figure 2b, while the corresponding calculated electronic spectra for each pure species are shown in Figure 3.

The first protonation constant for Fe-aerobactin of 4.8 has been attributed to the protonation of the single citrate hydroxy oxygen.[32] The first two protonation constants for FeRf of 5.5 and 4.3 average to 4.9, nearly the same as for Fe-aerobactin. Therefore we suggest the sites of the first two protonations to be the two citrate hydroxys in FeRf as well. The disappearance of the characteristic optical band at 335 nm, assigned as a citrate hydroxy oxygen to Fe LMCT, as the first two protons are added to the FeRf complex is also consistent with this interpretation. The third protonation constant $K^H_{H_3LFe}$ can then be assigned to the protonation of one of the carboxylic groups of rhizoferrin, since this value is very similar to the second protonation constant of ferric citrate.

Synthesis and Characterization. Metal complexes of rhizoferrin can be prepared in either aqueous or DMSO solution and lead to similar product distributions. We were unable to obtain satisfactory elemental analyses for the complexes due both to the small amounts of material available and to their extreme hydrophilicity which rendered it problematic to completely desalt them. Nevertheless they have been extensively characterized in solution by a variety of techniques. The kinetically inert complexes tested (Cr and Rh) ran in relatively broad bands as anions with similar mobilities upon high-voltage electrophoresis. Unfortunately, no suitably reliable standards were found to allow an accurate assignment of the absolute value of their charge. The kinetically labile complexes, on the other hand, showed only smears on electrophoresis, indicative of the simultaneous presence of several interconverting species at pH 7.4. The

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extreme hydrophilicity of the complexes (insoluble in all solvents but water) also thwarted attempts to separate isomers via HPLC, as no adequate solvent system or solid phase could be found. In the end, we had to rely on the classical ion exchange and size exclusion techniques.

Iron(III) Rhizoferrin. Negative-ion electrospray mass spectrometry reveals a strong peak at 488 amu corresponding to a singly charged, mononuclear iron rhizoferrin complex. No evidence for higher charged or polynuclear species was seen even under a variety of ionization conditions.

UV−vis and CD spectra for FeRf have previously been reported and are consistent with a high-spin ferric ion in an octahedral O_6 environment.[13] Since there are no spin-allowed d−d transitions for high-spin Fe^{3+}, on the basis of the magnitude of its extinction coefficient, the band at 335 nm is tentatively assigned as an oxygen to iron CT transition. This presumably arises from the bound and deprotonated alkoxo groups, as such transitions are not characteristic of carboxylate donors but are observed in staphyloferrin A[33] and ferric dicitrate,[34] both of which also presumably involve alkoxo coordination to iron. However, definitive proof of these assignments will have to await detailed resonance Raman spectroscopic analysis. Nevertheless involvement of the deprotonated citrate hydroxy groups in the coordination of iron by rhizoferrin is also indicated by the spectrophotometric titration data.

Electronically, both the EPR and Mössbauer spectra of FeRf are interpretable on the basis of a simple mononuclear high-spin ferric ion in a rhombically distorted environment. The EPR spectra of FeRf taken at a variety of temperatures displays the strong classical $g = 4.3$ signal along with the weaker transitions at $g \approx 9$ and 2 expected for rhombic iron. Analysis of the temperature dependence of the spectra gives an E/D value of 0.33, similar to that of most other siderophores.[35] The Mössbauer spectrum at 4.2 K consists of a very sharp, magnetically split, six-line pattern characteristic of high-spin ferric ion in the slow-relaxation limit. The internal field of 51.7 T is smaller than fields found in the hydroxamate and catecholate siderophores. Fits of the spectra as a function of temperature again yield E/D values near 0.33. The other Mössbauer parameters for FeRf are distinct from, but similar to, those of other ferric siderophores.[35]

Copper(II), Vanadyl, and Cobalt(III) Rhizoferrins. Attempts to prepare Co(III) complexes of rhizoferrin were unsuccessful due to a redox reaction which yielded only Co(II) and (presumably) oxidized ligand. That Co(III) in an all-oxygen coordination environment should be a good oxidant is hardly surprising, and indeed complexes of Co(III) with the hydroxamate siderophores are also unstable with respect to an internal redox reaction.[16] A small amount of what appeared to be the desired complex was isolated; however, the small amounts produced precluded detailed characterization and these complexes were not pursued further. Since they were not required for biological studies, the kinetically labile Cu^{2+} and VO^{2+} complexes were not characterized as extensively as those of the inert metal complexes. However, they appear to form readily in solution, have characteristic optical spectra (Supporting Information), and gave the expected molecular ions via electrospray mass spectrometry. The mass spectrum of the copper complex in particular showed the presence of several distinct protonation states in solution, all of which, however, are readily assigned. Oxidation of the blue-violet vanadyl Rf with hydrogen peroxide yielded an orange-yellow V(V)−Rf complex whose optical spectrum shows an LMCT band near 400 nm characteristic of a $V^{V}O$ moiety coordinated to an alkoxide ion.[36] From this we infer deprotonation and coordination of the citrate hydroxy group in the V(V) complex.

Gallium(III) Rhizoferrin. Gallium(III) and aluminum(III) complexes of siderophores are widely used as NMR-active analogs for native ferric complexes which are unobservable by this technique due to their paramagnetism and resultant unfavorable electron/nuclear spin-relaxation properties. Gallium complexes of rhizoferrin are readily prepared in solution and appear to be fully formed at pH values greater than 6. Negative-ion electrospray mass spectrometry reveals a strong parent ion peak cluster at 501, 502, and 503 amu due to the presence of the gallium-69, -70, and -71 isotopes.

Proton NMR spectra were obtained on the gallium complex (the aluminum complex gave similar results) in D_2O. The assignment of peaks in free rhizoferrin has already been reported,[13] and we utilize the same nomenclature here. The primary effects seen on complexation are as follows: (1) There is a splitting of the inner methylene protons from broad singlets in the free ligand, where the protons are equivalent, into two multiplets centered at 1.6 and 1.85 ppm, indicating their inequivalence upon complexation. A similar situation ensues with the outer methylene protons which upon complexation appear as multiplets at 3.2 and 3.5 ppm. (2) The citrate methylene signals, which behave as an AA′XX′ system in the free ligand specrum, collapse into an AB quartet and a strong singlet in the spectrum of the gallium complex. These assignments have been confirmed by the expected couplings as determined by 2D COSY spectra. The only change visible in the proton NMR spectra between pH 5.5 and 9.0 is the beginning of a collapse, at the lowest pH, of the inequivalent inner and outer methylene signals into the broad singlets characteristic of the free ligand.

The ^{13}C spectra of rhizoferrin and its gallium complex were also recorded and were most informative (Table 4). First, as with the free ligand, only eight carbon signals are observed for the gallium complex, confirming that the 2-fold symmetry in the free ligand is not lost upon complexation. The major change, as compared to the spectrum of the free ligand, is the 3−6 ppm downfield shift of the carboxylate carbon resonances for the complex. The magnitudes of these coordination-induced shifts (CIS) are entirely in keeping with those expected for carboxylate coordination to high-valent metals and indicate that all the carboxy groups are deprotonated and coordinated in the final complex.[37,38] Of particular note is the resonance for the citrate quaternary carbon, which at 77 ppm appears to be unshifted from its position in the spectrum of the free ligand. This appears to indicate that, although the citrate hydroxy groups are probably coordinated in order to achieve an octahedral geometry around the metal, they remain in their protonated form from pH 5.5 to 9.0.

We have attempted to construct a solution structure for GaRf via NOESY-derived, through-space proton−proton distances and geometrical constraints based on reasonable gallium(III) coordination geometries and distances. We considered only the three geometrical isomers which simple model building indicated were possible given the (*R,R*)-rhizoferrin ligand (*vide infra*) and

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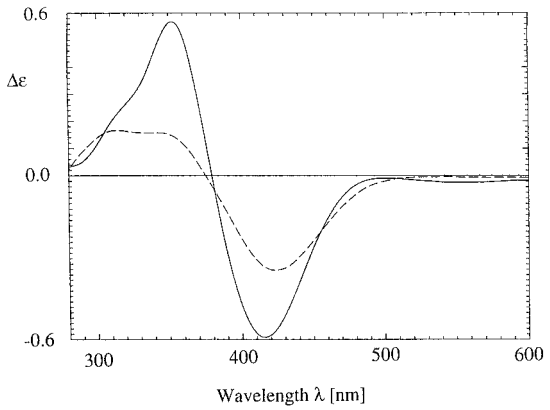

Figure 4. Circular dichroism spectra of the two isomers of RhRf: solid line, major isomer, $c = 2.5$ mM in H_2O, $T = 298$ K; broken line, minor isomer, $c = 5.7$ mM in H_2O.

constrained the Ga−O bonds to 2.0 Å with a regular octahedral geometry. The results indicated that both the isomer with the "outer" or 1-position carboxylate oxygens trans to each other and the one with the "inner" carboxylate oxygens trans had nearly the same energies with that of the trans hydroxy much higher. Therefore the latter could safely be eliminated from further consideration. Between the two remaining isomers, the simple minimized structure of the "trans outer" was the closest to that required by the NOE constraints. Nevertheless, it is not really possible to make a clear choice between the isomers at this level of sophistication.

Rhodium(III) Rhizoferrin. While gallium is a useful probe for iron(III), since it is both nonreducible and allows the use of NMR spectroscopy in characterization, it remains kinetically labile, which limits its utility as a biological tool. Rh(III), on the other hand, shares the nonreducibility and NMR activity of Ga, but its complexes are kinetically inert. Hence we have prepared Rh(III) complexes of rhizoferrin for both physical and biological studies. One major and one minor bright yellow complex was isolated by a combination of ion exchange and size exclusion chromatography. Negative-ion electrospray mass spectrometry on both complexes revealed the formation of the expected RhRf monomers with molecular ions at 536 amu, suggesting that the two fractions were geometrical isomers. The UV−vis spectra for both showed a single band at 400 nm with an extinction coefficent of ca. 100 M^{-1} cm^{-1} which, in analogy to spectra of other complexes of Rh(III) in an octahedral O_6 environment, is assigned as the $^1A_{1g}$ to $^1T_{1g}$ d−d transition. CD spectra were also recorded for each isomer and are similar but nevertheless distinct, particularly in the higher energy region. We find that, for both geometrical isomers, one optical isomer predominates in solution (Figure 4). However, to our surprise, these isomers appear to have a chirality *opposite* to that of the native iron rhizoferrin.[13] Again, on the basis of the anionic O_6 complexes $Rh(cat)_3^{3-}$, $Rh(ox)_3^{3-}$, and $Rh(ent)^{3-}$, the negative CD band near 420 nm assigns the chirality of RhRf as predominantly Δ or more generally, using IUPAC nomenclature, clockwise or "*C*".[39] On the basis of a comparison of the magnitudes of the extinction coefficients of RhRf and $Rh(cat)_3^{3-}$, the preference for the Δ chirality is quite strong.[40] Examination of the CD spectra of all the other minor isomers found in the RhRf reaction gave no evidence for the Λ or "*A*" optical isomer. To our knowledge, this is the first example of an alternative metal complex of a natural siderophore that displays a strong chiral preference for the *chirality opposite* to that of the native iron complex.[11] It should be noted that the rules used to assign the absolute stereochemistry at the metal do not strictly apply for rhizoferrin complexes, since the ligand is not a tris bidentate type leading to formal D_3 symmetry about the metal. However, applying the rules uniformly to both Fe and Rh still leads to the conclusion that the configurations about the two metals must differ in chirality.

Table 3. ^1H NMR Data for Ga and Rh Rhizoferrins in D_2O at pD 7[a]

chem shift (ppm)	J_{12} (Hz)	J_{13} (Hz)	assignment
	Ga Complex		
1.60 (d, 2H)	15	0	inner methylene
1.85 (dd, 2H)	15	12	inner methylene
2.53 (d, 2H)	15		citrate
2.77 (s, 4H)			citrate
2.95 (d, 2H)	15		citrate
3.20 (dd, 2H)	12	12	outer methylene
3.50 (dd, 2H)	12	12	outer methylene
	Rh Complex		
1.48 (b, 3H)	nd		inner methylene
1.68 (b, 1H)	nd		inner methylene
2.40 (d, 4H)	18	0	citrate
2.58 (d, 4H)	16	0	citrate
3.17 (b, 4H)	nd		outer methylene

[a] "Apparent" multiplicity: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; q, quartet; m, multiplet; b, broad. nd = not determined.

Table 4. ^{13}C NMR Data for Ga and Rh Rhizoferrins

chem shift (ppm)			
free Rf	Ga	Rh	assignt[a]
181.6	188.0	192.3	C6
178.3	181.9	182.7	C1
174.6	176.5	176.4	C5
77.1	77.1	83.3	C3
47.4	49.3	49.9	C2
46.5	46.5	47.5	C4
41.6	42.0	42.0	C7
28.4	27.5	27.1	C8

[a] See Figure 1 for numbering scheme.

Proton NMR spectra were also recorded for the major RhRf species in both H_2O and D_2O solutions. As in the case of gallium, both the inner and outer methylene proton signals become inequivalent and split on complexation, indicating that the bridge is fixed in a more rigid configuration. However, the facts that this inequivalence is much smaller for Rh and that the signals for the bridge protons are very broad both indicate that the bridge conformation is more flexible than for the Ga. The citrate region consists of a pair of AB quartets which can be satisfactorily simulated using the chemical shift and coupling constants given in Table 3. Assignments have again been confirmed by the 2D COSY and HSQC spectra. Unfortunately, due to the lack of sufficient usable NOESY cross peaks for the Rh complex, derivation of an NOE-derived solution structure was impossible.

The ^{13}C spectrum again proved to be the most informative in assigning which atoms of Rf were involved in complexation (Table 4). The most revealing piece of data is the shift in the citrate quaternary carbon signal from 77 to 83 ppm upon complexation. The +6 ppm CIS is close to that expected for deprotonation and complexation of an alcohol functionality, demonstrating conclusively that, unlike those of the Ga complex but similar to those of the Fe complex, the hydroxy groups of citrate are both deprotonated and coordinated to Rh(III).[41,42] The remainder of the spectrum is similar to that seen for the Ga(III) complex, with the carboxylate carbon signals all shifted 4−10

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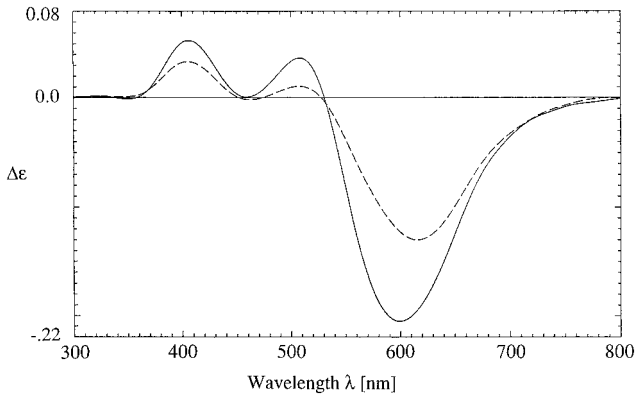

Figure 5. Circular dichroism spectra of the two isomers of CrRf: solid line, violet isomer, $c = 17.6$ mM in H_2O; broken line, green isomer, $c = 14.2$ mM in H_2O.

ppm downfield from their respective values for the free ligand, as expected for the deprotonation and complexation of these groups to Rh. The peak at 182.7 ppm is associated with the terminal carboxylate group, as its CIS (4.4 ppm) is similar in magnitude to that seen for the Ga complex (3.6 ppm), while the peak at 192.3 ppm is assigned to the α-hydroxy carboxylate carbon, whose CIS is much larger (10.7 ppm vs 6.4 ppm) than that for the Ga complex. We attribute this difference to the fact that deprotonation accompanies the complexation of the adjacent α-hydroxy group in the Rh complex while it remains protonated for the Ga complex.

Chromium(III) Rhizoferrin. The chromium complexes of rhizoferrin are more difficult to prepare than those of the other metal ions, requiring nonaqueous solvents, elevated temperature, and more reactive starting materials. In addition, two major complexes could be isolated rather than one. The ratio of the two complexes appeared to depend on the strength of the base used in the synthesis. Triethylamine or sodium carbonate gave primarily a violet complex. Use of the weaker base, sodium bicarbonate, gave a larger percentage of a green, less polar complex (ca. 50%). The two complexes, once formed, could not, however, be interconverted by changes in pH. Indeed, the optical and CD spectra of both species were pH independent. Both complexes also gave virtually the same negative-ion electrospray mass spectra with the peak at 484 amu expected for a mononuclear CrRf, suggesting that the two were geometrical isomers. The optical spectra of both were similar, with two bands assignable as the spin-allowed $^4A_{2g}$ to $^4T_{1g}$ and $^4A_{2g}$ to $^4T_{2g}$ transitions in "octahedral" symmetry. The ligand field parameters derived from these spectra suggest that the less polar, green complex has essentially the same ligand field strength as the violet one (17.42×10^3 vs 17.48×10^3 cm^{-1}) but a somewhat larger interelectronic repulsion parameter, *B* (829 vs 713 cm^{-1}).[43] The CD spectra of the two species were also very similar (Figure 5), differing only in the low-wavelength transitions which are split, suggesting that both are "trans" geometrical isomers.[44] Once again the optical chirality preference of both isomers was found to be Δ or C, the opposite of the native iron complex but the same as that of the Rh. On the basis of extinction coefficients, however, the degree of preference for Δ over Λ is much weaker, only about 5%.[44] The EPR spectra of the complexes were measured as a function of temperature. Both isomers showed essentially axial spectra with relatively broad features at 4 K around $g = 2$, 4, and 6. The sharp $g = 2$ signal is due to the presence of a small amount of some higher symmetry impurity (likely hexaaquochromium(III)). As the temperature is raised to 60 K, the nature of the spectra changes dramatically with the axial features at $g = 6$ and 4 disappearing and the broad feature near $g = 2$ growing concomitantly. A detailed analysis of these spectra is complex and will be reported later.

Biotests. Although detailed biological studies will be presented elsewhere,[19] we report here evidence that the metal ion complexes prepared do indeed have biological activity. The plate assay described by Winkelmann et al.[22] using *Morganella morganii* 13 was slightly modified for semiquantitative assay of the ability of alternative metal ion complexes to antagonize rhizoferrin-mediated iron uptake. In this assay, the organism is seeded into agar growth medium made iron deficient with 300 μM bipyridyl. A fixed concentration of FeRf placed directly on the surface of the agar will relieve the iron deficiency and cause a ring of growth of predictable size. Mixing alternative metal ion complexes with the FeRf in various ratios of molar excess will lead to a reduced ring size or no growth at all if the specific complex is capable of antagonizing the effects of FeRf. As expected, at least some of the Rh and Cr complexes were able to antagonize the ability of FeRf to alleviate iron limited growth in the agar plate assay. Both Rh isomers appeared to be effective, while only the green isomer of Cr was so. Gallium and aluminum rhizoferrins not only inhibited the ability of FeRf to deliver iron but functioned to give a concentration-dependent killing zone.

Discussion

Rhizoferrin is a chiral, potentially hexadentate ligand that can be viewed as a pair of facially coordinating citric acid moieties tied together with a diaminobutane linker. In principle, assuming only facial coordination is possible, there are five geometric isomers, designated as (the numbers in the parentheses are the recommended IUPAC configuration indices[39]), trans citrate hydroxy (*OC*-6-2′3), trans inner carboxy (*OC*-6-1′2), and trans outer carboxy (*OC*-6-3′1′), along with an all-trans and an all-cis isomer (a schematic representation of all the isomers is available as supplementary material). However, the last two can be eliminated by the steric constraints of the *R*,*R* ligand. Since the ligand itself is chiral and its coordination leads to non-superimposible mirror images, all the remaining isomers are chiral and can exist as diastereomeric pairs with either *A* or *C* (again adopting the recommended IUPAC nomenclature[39]) chirality at the metal center.

Although the coordination chemistry of rhizoferrin with iron might be expected to be similar to that of citrate itself, there is one significant difference between the two ligands. While citrate can form octahedral 2:1 complexes with various metal ions via facial coordination using the two carboxylates and the hydroxy oxygen donor atoms, an additional carboxylate donor remains which can lead to the formation of polynuclear species. Thus, despite extensive study, the detailed nature of the coordination chemistry of iron and other trivalent metals with citrate remains somewhat unclear. On the basis of various pieces of evidence, mononuclear, dinuclear, and polynuclear complexes have all been suggested to be the dominant species in solution.[34,45−47] The role played by the citrate hydroxy groups in complex

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formation is also obscure.[48−49] Even the recent reports by Lippard[50] and co-workers of the X-ray structures of two different iron citrate complexes, showing them to be dinuclear with bridging alkoxides, and that of Barron et al.,[51] showing the aluminum complex to be trinuclear, are unlikely to end the discussion as to the nature of the species *in solution*. However, in rhizoferrin, the third carboxylate group is tied up in the amide bond of the backbone, which should strongly suppress the formation of any polynuclear species. Thus, among the major questions we sought to answer for the iron rhizoferrin complex were the following: (1) Is the complex in solution mononuclear or is a dimer with bridging alkoxides formed? (2) Are the citrate hydroxy oxygens deprotonated and coordinated in the final complex?

In answer to the first question, it is clear from the EPR, Mössbauer, and mass spectrometry data that only mononuclear species are formed for any of the metals examined. The answer to the second question, however, seems to be that the contribution of the hydroxy oxygens to complexation is metal ion dependent. Although the citrate hydroxy groups in the free ligand have pK_a's in excess of 10, in a complex their deprotonation will be assisted by the Lewis acidity of the metal. For example, the pK_a of water when coordinated to Fe^{3+} falls from 14 to 2.19. The general trend for acidity of water coordinated to the trivalent cations varies as Fe > Rh > Ga > Cr > Al.[52,53] Hence it is not unreasonable to suppose that the citrate hydroxy groups would be coordinated and deprotonated for the Fe and Rh complexes, as was indeed demonstrated by the titration data (Fe) and NMR (Rh). The fact that in the Ga complex these groups remain protonated, as determined by NMR, is surprising and would indicate that this should also be the case for the even less Lewis acidic Cr complex (where a direct determination is impossible). However, high-voltage electrophoresis indicates that the Cr complex has the same charge as the Rh complex, which suggests the same protonation state for both. It is of course possible that a variety of conflicting effects fortuitously cancel in the Ga complex, giving rise to no observable CIS despite the fact that the citrate hydroxyl group is both deprotonated and coordinated.

It is difficult to compare the overall formation constant of iron rhizoferrin to that of either citrate itself or other siderophores due to differences in ligand pK_a's and/or stoichiometry. Therefore, the pM value has been proposed as a means for more direct comparison. The pM values are expressed under particular sets of experimental conditions, typically at a total ligand concentration of 10^{-5} M, a total iron concentration of 10^{-6} M, and a pH of 7.4 (Table 5). Although rhizoferrin is not a particularly powerful iron chelator as compared to the tris(hydroxamate) or tris(catecholate) siderophore at this pH, it becomes much more competitive at pH values near 4−5, the growth and transport optimum for the producing fungi, due to the use of the carboxylate group as opposed to the more basic hydroxamate or catecholate as donor. Thus the ligand is well designed for the environment in which it was meant to function.

Although a fully deprotonated complex is formed between Fe and rhizoferrin at pH values in excess of 7.5, such a species may not be the one actually transported in the producing organisms, since fungi are known to strongly acidify their growth environment by the excretion of various organic acids.[54,55] The pH optimum for growth is usually near 5. Indeed, *Absidia spinosa* FeRf uptake[19] shows a very strong pH dependence with a transport optimum near pH 4.5 and virtually no uptake at pH 7, which nicely tracks the species distribution diagram. At the pH optimum for transport, the major species (>80%) is the monoprotonated complex with the remainder in the diprotonated form. Of course, all the protonated forms are in rapid equilibrium and the local pH at the membrane surface may not be the same as for the bulk solution. It will therefore be difficult to prove if the protonation state of the complex is important for recognition and transport.

Table 5. pFe Values for Various Fe(III) Siderophore Complexes[a]

complex	pM_1	pM_2	pM_3
rhizoferrin[b]	19.7	17.0	13.2
enterobactin[c]	35.5	29.3	
ferrichrome[d]	25.2		
ferrioxamine B[d]	26.5	22.3	16.3
rhodotorulic acid[e]	21.8		
aerobactin[f]	23.3	21.2	
citrate[g]	17.7		

[a] $pM_1 = -\log [Fe^{3+}]$ where $[L]_0 = 10^{-5}$ M, $[Fe]_0 = 10^{-6}$ M, and pH = 7.4; pM_2, pH = 6.0; pM_3, pH = 4.0. pM values are based on published stability constants (see footnotes *b*−*g*). [b] This work. [c] Loomis, L. D.; Raymond, K. M. *Inorg. Chem.* **1991**, *30*, 906. [d] Anderegg, G.; L'Eplattenier, F.; Schwarzenbach, G. *Helv. Chim. Acta* **1963**, *46*, 1409. [e] Carrano, C. J.; Cooper, S. R.; Raymond, K. N. *J. Am. Chem. Soc.* **1979**, *101*, 599. [f] Reference 32. [g] Reference 34.

The chirality of the rhizoferrin complexes at the metal center is surprising and deserves some comment. On the basis of extinction coefficients, and in analogy to other O_6 pseudooctahedrally coordinated siderophores, the chirality of the fully deprotonated iron complex is nearly completely Λ. This configuration must be essentially independent of the ligand backbone structure, since various analogs obtained by directed fermentation all show the same chiral preference. However, the CD spectra show that the chirality is pH dependent,[13] disappearing at lower pH despite the fact that titration studies indicate that the metal remains complexed to the ligand. Thus there appears to be a reduced chiral preference for the various protonated species vis-à-vis the fully deprotonated one. We point out that the few divalent metals we examined, namely Cu^{2+} and Co^{2+}, which are inherently less Lewis acidic than the trivalent cations, show essentially no CD spectra even though complexes are formed. Nevertheless, the differing chirality between Fe on the one hand and Rh and Cr on the other remains unexplained and unprecedented for siderophore complexes. This observation suggested the attractive hypothesis that the producing organisms might use the metal center chirality as a means to discriminate between the desired iron complex and fortuitious formation of alternative metal complexes. However, such an idea has to be discarded in light of the recognition and transport of the opposite-chirality Rh and Cr species.[19] In addition, since Rh and Cr, which are respectively larger and smaller than Fe, both adopt the Δ configuration, atomic size is apparently not the source for the differing metal center chirality. It must be pointed out that the change in sign of the CD spectra for Cr and Rh as compared to Fe could be due to fundamental differences in the nature of the electronic transitions involved rather than a change in metal center chirality since the optical transition involved is an LMCT for Fe while it is d−d in the

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Rh and Cr cases. However, such has not proven to be the case for other siderophores.

The biological effects on iron-limited cultures of *M. morganii* 13 by the alternative metal ion complexes of rhizoferrin are also very interesting. While the Rh and Cr complexes gave results more or less in line with expectations, i.e. competitive inhibition with iron rhizoferrin's ability to promote growth on iron-limited media and evidence for some stereospecificity in this process, Ga and Al complexes behaved anomalously. As indicated under results, GaRf not only inhibited FeRf's ability to stimulate growth but also it produced a concentration-dependent halo or killing zone around the site of application. Thus it behaves as an antibiotic toward *M. morganii*. The concept of using alternative metal ion complexes of siderophores as antibiotics is not new.[56] Two potential modes of action were originally envisioned. First a simple blockade of the uptake of the essential nutrient, iron, could be expected to exert a bacteriostatic effect itself. In addition, if the appropriate metal ion was chosen, the iron uptake system could be tricked into delivering a toxic metal. Unfortunately, neither of these hopes has yet been realized in experimental systems. The fundamental problems appear to be that (1) almost all microorganisms have multiple pathways for iron uptake, blocking only one of them hence being ineffective, and (2) the toxic, alternative metal ion complexes either are not recognized by the transport system or are unstable, exchange with iron leading to the ironic situation that many of these potential "antibiotics" actually stimulate microbial growth. Thus it was unexpected that GaRf should appear to function in just the manner long hoped for. It remains to be seen if GaRf will function in a similar manner with the Zygomycetes class of fungi which produce this siderophore. If such proved to be the case, then a potentially effective new class of antifungal agents for this group of organisms, which includes a number of known human pathogens, may have been found.

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Conclusions

We have delineated for the first time the coordination chemistry of rhizoferrin, a member of the new carboxylate class of siderophores, and have measured its iron-binding constants. The Cr and Rh rhizoferrin complexes are unusual in that they appear to be the first alternative metal siderophore complexes reported that adopt a chirality about the metal center *opposite* of that found in the native iron analog. These kinetically inert complexes should be useful tools for detailed investigations into the mechanisms of rhizoferrin-mediated iron uptake. Finally, some of the alternative metal ion complexes of rhizoferrin have been found to have unexpected biological properties which will bear further examination.

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Supporting Information Available: The pH-dependent optical spectra whose data were used in the determination of the FeRf formation constants, uv−vis spectra of the Cu^{2+} , VO^{2+} , and V^{5+} rhizoferrin complexes, uv−vis spectra of the isomers of Cr^{3+}Rf, and a schematic representation of the possible geometric and optical isomers of octahedral metal complexes of rhizoferrin (5 pages). Ordering information is given on any current masthead page.

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