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Iron(III) complexes of chrysobactin, the siderophore of *Erwinia chrysanthemi*

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The phytopathogenic bacterium *Erwinia chrysanthemi* produces the monocatecholate siderophore chrysobactin under conditions of iron deprivation. Only the catecholate hydroxyl groups participate in metal coordination, and chrysobactin is therefore unable to provide full 1:1 coordination of Fe(III). The stoichiometry in aqueous solution is a variable dependent on pH and metal/ligand ratio, in addition to being concentration dependent. At neutral pH and concentrations of about 0.1 mM, ferric chrysobactin exists as a mixture of bis and tris complexes. Chrysobactin and its isomers form optically active tris complexes. The dominant configuration depends on the chirality of the amino acid to which the catecholate moiety is attached.

Keywords: *Erwinia chrysanthemi*, chrysobactin, catechol, iron complex, coordination

Introduction

Under aerobic conditions at neutral pH, the availability of free iron is extremely limited due to the insolubility of ferric oxyhydroxides ($K_{sp} = 10^{-38}$, $[Fe^{3+}] \approx 10^{-17}$ M). To overcome this deprivation, microorganisms excrete siderophores, which are chelators virtually specific for ferric iron (Hider 1984, Neilands 1989). Ferric siderophores then re-enter the cells via cognate membrane receptors (Bagg & Neilands 1987, Matzanke *et al.* 1989). Although many siderophores possess six ligands, several microbial chelators of the hydroxamate and thiazoline types with fewer ligands have been described. The coordination chemistry of some of these has been thoroughly studied (Barclay *et al.* 1984, Carrano & Raymond 1978, Hahn *et al.* 1991, Jalal *et al.* 1986).

To the group of potential siderophores which can not form 1:1 octahedral complexes with hexacoordinate Fe(III) belong an increasing number of amino acids or peptides monosubstituted with dihydroxybenzoic acid—DHBA (Barghouthi *et al.* 1989, Chakraborty *et al.* 1990, Kobaru *et al.* 1983, Patel *et al.* 1988, Persmark *et al.* 1989 and references therein, Skorupska *et al.* 1988). The potential of bidentate ligands as true siderophores may have been overlooked, as they are much less efficient chelators than their hexadentate counterparts (Hider 1984, Harris & Raymond 1979). Furthermore, as many microorganisms produce both bidentate and hexadentate chelators, the former may even occasionally have been regarded as degradation products.

Some microorganisms, however, do not appear to synthesize any hexadentate siderophores. One example is the plant pathogen *Erwinia chrysanthemi*, from which we recently isolated and structurally characterized the monocatecholate siderophore chrysobactin (Cb; [N^{α}-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine]; Figure 1; Persmark *et al.* 1989). The interaction of catecholates with ferric iron has been well-studied analytically (McBryde 1964, Tsin-Jao *et al.* 1961). Bidentate catecholates have also served as model compounds for the hexadentate catecholate siderophore enterobactin (Anderson *et al.* 1976, Avdeef *et al.* 1978, Harris *et al.* 1979, McArdle *et al.* 1978). Few studies have focused directly on the stoichiometry or mode of metal coordination of natural monocatecholate siderophores (Buckingham *et al.* 1982). Our present study therefore aimed to answer these questions for ferric Cb, in parallel to an investigation of its biological activity in *E. chrysanthemi* (our unpublished results).

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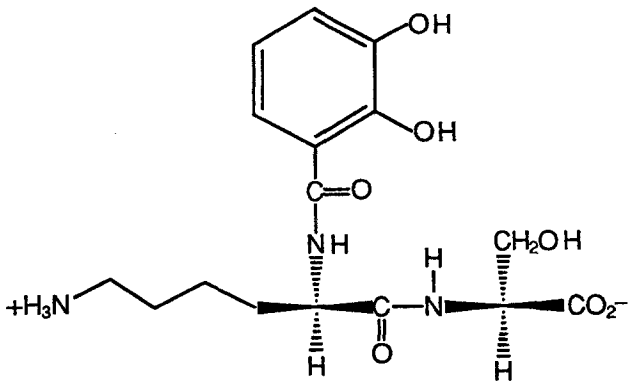

Figure 1. Structure of chrysobactin, N^{α}-(2,3-dihydroxybenzoyl)-D-lysyl-L-serine.

We report here that Cb chelates Fe(III) only through the catechol hydroxyl groups. In addition to being concentration dependent, the stoichiometry varies with pH and metal/ligand (M:L) ratio. At low millimolar concentrations, physiological pH and M:L ratios between 1:2 and 1:4, ferric Cb exists mainly as the bis (1:2) complex, and at M:L from 1:4 to 1:10 as a mixture of bis and tris (1:3) complexes. The Cb and DHBA-Lys isomers used in this study formed optically active ferric tris complexes, whereas bis complexes are optically inactive. The preferred chirality in solution depended on the chirality of the α-carbon of the lysine residue to which the DHBA moiety was attached.

Materials and methods

Materials

When applicable, glassware were washed in 6 M HCl and rinsed excessively with double-distilled water prior to use. D-Serine methyl ester hydrochloride was obtained from Chemical Dynamics Corp. $Ga_2(SO_4)_3$ was purchased from Fluka.

Spectroscopy

Bausch and Lomb Spectronic 2000 or Shimadzu UV160 spectrophotometers were used for ultraviolet and visible spectrophotometry. Circular dichroic (CD) spectra were recorded on an AVIV 60 DS spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AM spectrometer operating at 400.13 MHz proton frequency, at the NMR facility of the University of California at Berkeley. Mass spectra were obtained at the mass spectrometry facility of the University of California at Berkeley.

Chemical synthesis

The synthesis of chrysobactin (D-L-Cb), its L-lysine-D-serine (L-D-Cb) and L-lysine-L-serine (L-L-Cb) isomers and the two isomers of N^{α}-(2,3-dihydroxybenzoyl)-lysine (DHBA-D-Lys and DHBA-L-Lys) were performed as described (Persmark *et al.* 1989), with the following exceptions. For the L-D-Cb: to N^{2}-(2,3-dibenzoylbenzoic acid)-benzyloxycarbonyl-L-lysine in acetonitrile was added 1.05 mol/mol of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; this was stirred briefly before the addition of an acetonitrile solution containing 1 mol/mol each of D-serine methyl ester HCl and triethylamine. The solution was refluxed by gentle warming overnight. Solvent was removed by rotary evaporation and the oil taken up in methanol. The methyl group was removed by hydrolysis with 1.1 mol/mol of NaOH with stirring for 2 h at 4 °C. After acidification, the benzoyl protective groups were removed by catalytic hydrogenation with palladium on activated charcoal. For DHBA-D-Lys and DHBA-L-Lys, reductive deprotection was done after coupling ε-benzyloxycarbonyl-lysine to the activated ester of DHBA, as described (Persmark *et al.* 1989). Purity of the compounds was checked by fast-atom-bombardment mass spectrometry, NMR and CD.

Spectrophotometric measurements

Ferric complexes were studied spectrophotometrically over a range of pH values and M:L ratios using the Vareille method (Avdeef *et al.* 1978, McBryde 1964). Complexes were formed by mixing degassed and nitrogen-purged aqueous solutions of $FeSO_4$ and Cb and, after a brief incubation to allow complex formation, the pH was raised to the desired value by addition of buffer. The final Fe(III) concentration was 0.165 mM and the M:L ratio was 1:4. To study the variation of stoichiometry with ligand concentration, samples with M:L ratios of 1:2, 1:3, 1:4, 1:6 and 1:10 at 0.022 mM Fe(III) were prepared. The following buffers were used; acetate pH 4.0–5.6 (six values); Mops pH 6.0–7.5 (five values); Tris/HCl 8.0–9.0 (five values). Spectra were recorded over 375–775 nm. Cb concentrations were estimated spectrophotometrically from $\varepsilon_{318} = 3.0\ mM^{-1}\ cm^{-1}$ at pH 6.5. CD spectra over 400–600 nm of ferric complexes were obtained in 0.1 M Tris/HCl pH 8.7 at 0.1 mM Fe and 0.5 mM ligand concentration.

Gallium chrysobactin

$Ga(Cb)_2$ was prepared by mixing Ga(III) and Cb in a 1:2 ratio in degassed and nitrogen-purged distilled water. The solution was lyophilized and taken up in 0.1 M phosphate pH 6.0. ^{1}H- and ^{13}C-NMR spectra were obtained on the complex in both D_2O and D_2O/H_2O (9:1). NMR spectra of the free ligand were taken under the same conditions as a reference.

Size of ferric complexes

Sizing of the ferric Cb complexes was done by gel filtration on a column (1 × 90 cm) of Bio-Gel P2. The bis complex formed at a 1:2 M:L ratio was chromatographed at pH 5.6.

The tris complex formed at a 1:4 M:L ratio was chromatographed at pH 8.0. The column was calibrated with a bichromate/blue dextran mixture; ferrichrome A and ferric enterobactin were used a molecular mass markers.

Results and discussion

Characterization of ferric chrysobactin

Visible spectra of ferric Cb (M:L = 1:4) are shown in Figure 2. At pH values ≥8 the solution had a red color, with an absorbance maximum at 493 nm ($\varepsilon_{Fe,tris} = 5.2\ mM^{-1}\ cm^{-1}$). The solution acquired a blue color as the pH was lowered to 5.4, concomitant with a spectral shift through an isosbestic point at 551 nm to a new maximum at 567 nm ($\varepsilon_{Fe,bis} = 3.8\ mM^{-1}\ cm^{-1}$). The blue color remained as the pH was lowered to 4.0, although the intensity decreased and no new isosbestic point was observed. To verify the sizes of the bis and tris complexes, both were prepared independently under conditions where either one was almost exclusively present. The calculated M_r of bis(Cb) (790) and tris(Cb) (1157), correlated well with the experimentally found values of 810 and 1137, respectively. The result makes it unlikely that bis(Cb) would be an oxo-bridged binuclear complex. This form of complex has been observed for ferric siderophores, in crystals and organic solvents but not in aqueous media (Anderson *et al.* 1976, Barclay *et al.* 1982).

In the Vareille method, absorption coefficients at certain wavelengths are plotted against pH. Individual complex species are then identified by horizontal lines. The Vareille plot of ferric Cb at M:L = 1:4 (Figure 3) showed that at pH ≥ 8, the ferric tris(Cb) complex was almost exclusively present. Between pH ≈ 5.6 and 8, a mixture of bis and tris complexes was found, whereas the ferric bis(Cb) complex dominated in a narrow pH region between 5 and 5.6. The presence of multiple species was also indicated by Jobs plots at different pH values (data not shown). In the case of a similar compound, ferric *N*,*N*-dimethyl-2,3-dihydroxybenzamide, an isosbestic point for the mono and bis complexes appeared around 688 nm (Harris *et al.* 1979). In a previous study involving several monocatecholates, some compounds showed complex distribution curves whereas, for others, pH ranges for unique species could be identified (Adveef *et al.* 1978). Complex distribution curves were also observed for the monohydroxamate siderophores *cis*- and *trans*-fusarinine, where a mixture of mono, bis and tris complexes existed below pH 5 (Jalal *et al.* 1986). The stability of the ferric trishydroxamates down to pH 5 was in contrast to the very pronounced [H^+] sensitivity of the catechol complexes, where the tris complexes began to disproportionate slightly below pH 8 (Figure 2).

From the Vareille plot, values of $\varepsilon_{Fe,bis}$ and $\varepsilon_{Fe,tris}$ were measured. However, $\varepsilon_{Fe,mono}$ could not be estimated because the bis complex disintegrated into several species below pH 5, as shown by the lack of a new isosbestic point. Below pH 5, presumably a

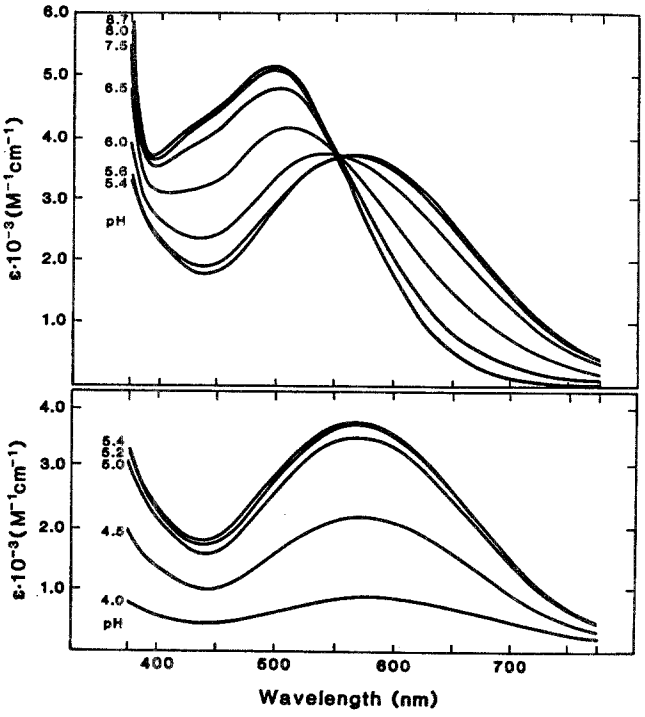

Figure 2. Visible absorption spectrum of ferric chrysobactin (M:L = 1:4) in aqueous solution as a function of pH. For clarity, spectra at selected pH values are shown.

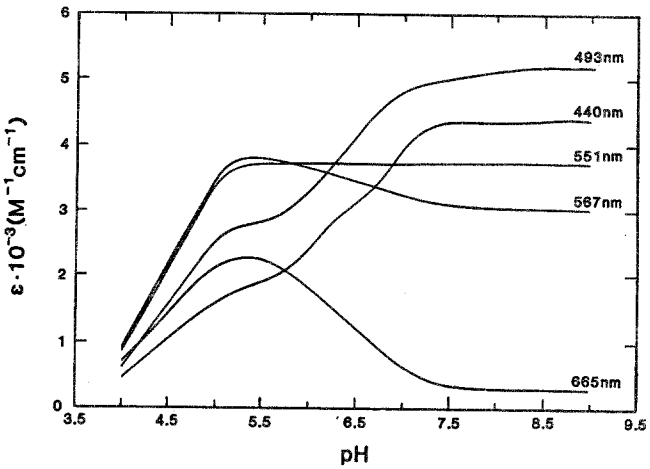

Figure 3. Vareille plots of the molar absorptivity of ferric chrysobactin (M:L = 1:4) at certain wavelengths as a function of pH.

mixture of bis and mono complexes of various degrees of protonation was present (Figure 2). Equilibrium constants could therefore not be estimated without an assumption of $\varepsilon_{Fe,mono}$. However, by potentiometric titration a log value for the cumulative equilibrium constant β_3 of 35.4 was calculated for the overall reaction

$$[M(HL)_3^{3-}]/[M^{3+}][HL^{2-}]^3$$

(Martell AE, personal communication). This was lower than values reported for other bidentate catecholates (Avdeef *et al.* 1978, Harris & Raymond 1979).

The stoichiometry of ferric Cb also depended on the M:L ratio. Figure 4 shows a section of a Vareille plot at 665 nm for ferric Cb solutions with M:L ratios from 1:2 to 1:10. At this wavelength the absorption of the tris complex was very low, and the figure hence shows, in essence, the appearance and disappearance of the bis complex with increasing pH. Clearly, increasing ligand concentration shifted the pH range over which the bis and tris complexes were stable at lower pH values. Thus, at physiological pH and M:L ratios of 1:4 to 1:10, iron existed as a mixture of bis(Cb) and tris(Cb).

Iron-center configuration

The absolute configuration around the metal center has in several studies been found to be a major factor in ferric siderophore–receptor recognition (Bergeron & Weimar 1990, Matzanke *et al.* 1989, Neilands *et al.* 1981, Winkelmann & Huschka 1987). By a combination of crystallography and the formation of kinetically inert siderophore-metal complexes, CD curves can be correlated with an absolute metal center configuration (Matzanke *et al.* 1989, Raymond *et al.* 1980, van der Helm *et al.* 1987). The CD spectra of the ferric DHBA-D-Lys-X and DHBA-L-Lys-X derivatives were essentially mirror images (Figure 5). D-L-Cb and D-Lys show positive CD bands at about 550 nm ($\Delta\varepsilon \approx 0.8\ M^{-1}\ cm^{-1}$) whereas the three DHBA-L-lysine derivatives (L-D-Cb, L-L-Cab and L-Lys) show negative CD bands at this wavelength ($\Delta\varepsilon \approx -0.8\ M^{-1}\ cm^{-1}$), which corresponds to low energy transitions in the visible spectrum (Figure 2). The CD curves of the D-Lys catecholates resembled those of L-parabactin (Bergeron & Weimar 1990) and *enantio*-enterobactin (Rastetter *et al.* 1981), which have been shown to exist in solution mainly in the Λ-form. The L-Lys compounds, on the other hand, gave CD curves which resembled those of D-parabactin (Bergeron & Weimar 1990) and enterobactin (Rastetter *et al.* 1981), which show a right-handed propeller, or Δ optical configuration in solution. The CD spectra furthermore resemble those of the isolated optical isomers of the tris(catechol) Cr and Rh complexes (Raymond *et al.* 1980). The $\Delta\varepsilon$ values for the peaks at 550 nm in the CD spectra were about 40% and 70% lower for parabactin A and parabactin, respectively, although the absorption coefficient of ferric tris(Cb) is about 1.4-times higher (Bergeron & Weimar 1990). Although the latter strictly are not true tricatecholates, the lower $\Delta\varepsilon$ values for the Cb analogues likely represent an equilibrium between Λ and Δ forms with one form in moderate excess, depending on the chirality of lysine. In contrast to the chiral Cb analogues, the ferric tris complexes of the monohydroxamate siderophores *cis*- and *trans*-fusarinine, which are achiral, showed very weak CD

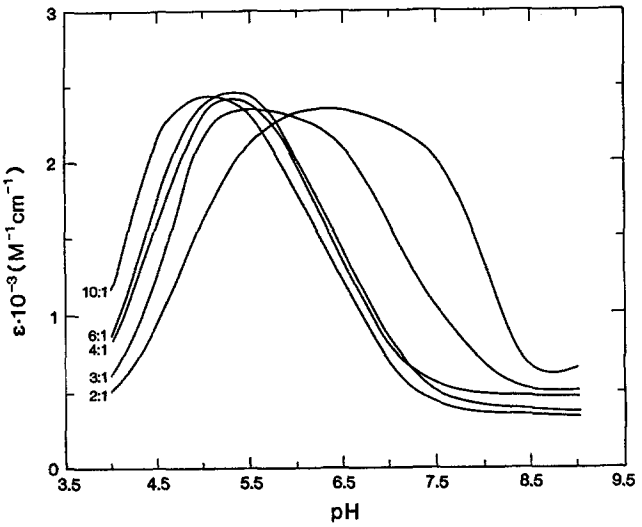

Figure 4. Vareille plots of ferric chrysobactin at 0.02 mM Fe(III) and ligand/metal ratios from 2:1 to 10:1 at a wavelength of 665 nm.

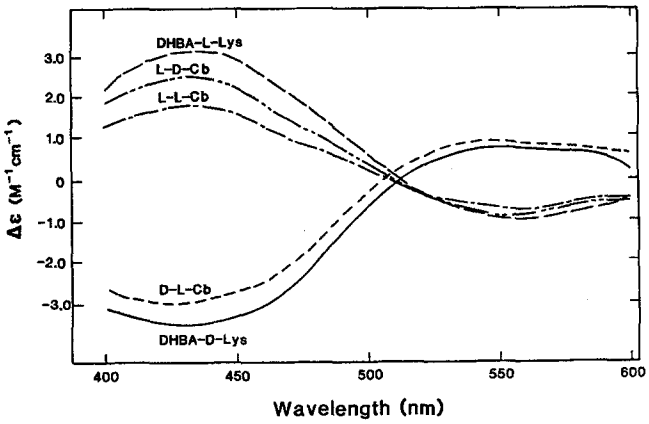

Figure 5. Circular dichroic spectra of the ferric tris complexes of five DHBA derivatives (M:L = 1:5) at 0.1 mM Fe(III) in 0.1 M Tris/HCl pH 8.7.

spectra. This indicated an equilibrium close to unity for the Λ and Δ optical isomers (Jalal *et al.* 1986).

The bis complexes were, however, not optically active. The $[H^+]$ dependence of the CD spectrum is shown in Figure 6. A comparison with the visible spectrum and its Vareille plot (Figures 2 & 3) showed that the increase in optical activity corresponded to formation of ferric tris(Cb). As a parallel study (our unpublished work) showed no difference in biological activity between the different DHBA derivatives, it is tempting to speculate that the bis complex was biologically active. However, due to the kinetic lability of ferric iron, rapid isomerization of a biologically active tris complex cannot be excluded.

Gallium chrysobactin

Since the Vareille plot suggested that ferric bis(Cb) may be the predominant species at physiological conditions and thus be biologically active, it was of interest to know how ferric iron was coordinated. We had previously speculated (Persmark *et al.* 1989) that the seryl hydroxyl may be involved in chelation, as this would give a tridentate ligand and thus afford a more stable and well defined complex, rather than a hydrated tetradentate complex. In the latter, Fe(III) would only be chelated by the four benzoyl hydroxyls. To investigate this, the gallium complex was formed under conditions known from the studies with iron to give almost exclusively a bis(Cb) complex. Gallium(III) was chosen as it has an identical charge and a radius very similar to that of Fe(III), in addition to being diamagnetic and thus permitting NMR analysis.

The ^1H and ^{13}C shifts of Cb and the 1:2 Ga(III)–Cb complex are shown in Table 1 and 2, respectively. The chemical shifts of the tricatechol siderophore enterobactin and Ga-enterobactin have been included for comparison (Llinás *et al.* 1973). Qualitatively the chemical shift changes upon binding of Ga(III) in Cb and enterobactin are very similar, although quantitatively slightly less pronounced in the former. This may be explained in part by the constraints imposed on the configuration of the enterobactin catechol groups around the metal ion by the cyclic triester backbone.

In the ^1H-NMR spectra, the major difference upon chelation is the downfield shift of 1.72 ppm of the amide proton on the nitrogen to which DHBA is bonded, indicating a deshielding of the amide bond.

Far the most striking feature of the ^{13}C-NMR spectra is the very large downfield shift of 8.0 ppm and 9.1 ppm of the *ortho* and *para* hydroxyl carbons, respectively. Notable also is the relatively large upfield shift of the three aromatic carbons which may indicate an increased electron delocalization in the benzene ring of the chelate as compared to the free ligand, as mentioned previously (Llinás *et al.* 1973).

The chemical shifts of both the carbonyl and β-carbon of the serine moiety were essentially identical in both the free ligand and the Ga(III) complex. This indicated that neither the serine hydroxyl or carboxylate groups were involved in chelation. The remaining two sites in the bis complex presumably were occupied by water or solvent anions. Thus, according to NMR and spectrophotometric data, the mode of metal coordination in the Cb complex was via the catechol hydroxyls only. Similar observations were made for monocatecholate model compounds. These studies furthermore showed that protonation of ferric tris-(monocatecholate) complexes lead to a dissociation of one of the bidentate ligands. In hexadentate catecholates, protonation was suggested to lead to a salicylate model of bonding (Cass *et al.* 1989, Harris & Raymond 1979, Harris *et al.* 1979). In contrast to Cb, infrared studies of itoic acid (DHBA-glycine) indicated that the carboxylate oxygen participated in Fe(III) coordination in solution (Buckingham *et al.* 1982).

A putative receptor for ferric Cb has been identified (Enard *et al.* 1988). We have also shown (unpublished results) that ferric Cb very effectively delivered iron to *E. chrysanthemi* cells. This study has answered some questions concerning the mode of Fe(III) coordination by Cb. However, due to the kinetic lability of ferric iron complexes, and the presence of both bis(Cb) and tris(Cb) under physiological conditions, the nature of the biologically active species warrants further study.

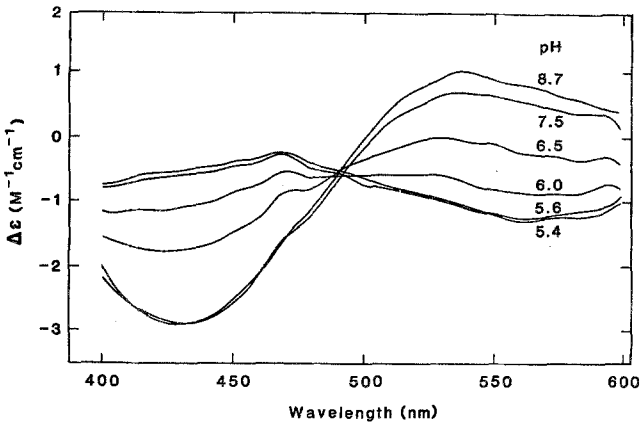

Figure 6. Variation of the CD spectrum of Fe(chrysobactin) with pH at 0.1 mM Fe(III) (M:L = 1:5).

Table 1. ^{1}H-NMR chemical shifts of chrysobactin and Ga(chrysobactin)$_2$ in 0.1 M phosphate pH 6.0 compared with those for enterobactin and its Ga complex

Proton	δ (ppm)		Δδ (ppm)	δ (ppm)		Δδ(ppm)
	Chrysobactin	Ga-chrysobactin		Enterobactin	Ga-enterobactin	
Lysyl						
CαH	4.65	4.49	−0.16			
CβH	1.95	1.82	−0.13			
CγH	1.53	1.25	−0.28			
CδH	1.73	1.45	−0.28			
CεH	3.00	2.61	−0.39			
CαNH	9.10	10.82	1.72			
Seryl						
CαH	4.37	4.31	−0.06	4.94	5.14	0.20
CβH	3.87	3.82	−0.05	4.66	5.22	0.56
				4.41	3.80	−0.61
CαNH	8.10	8.06	−0.04	9.06	11.72	2.66
Benzoyl						
o-CH	7.29	7.13	−0.17	7.34	6.84	−0.50
m-CH	6.84	6.62	−0.27	6.73	6.13	−0.60
p-CH	7.07	6.85	−0.25	6.98	6.44	−0.54

Chemical shifts are referred to an internal standard of acetone, equal to 2.225 ppm.
Values for enterobactin are taken from Llinás *et al.* (1973).

Table 2. ^{13}C-NMR chemical shifts of chrysobactin and Ga(chrysobactin)$_2$ in 0.1 M phosphate pH 6.0 compared with those for enterobactin and its Ga complex

Carbon	δ (ppm)		Δδ (ppm)	δ (ppm)		Δδ(ppm)
	Chrysobactin	Ga-chrysobactin		Enterobactin	Ga-enterobactin	
Lysyl						
Cα	54.6	54.6	0			
Cβ	31.6	31.6	0			
Cγ	22.9	22.9	0			
Cδ	27.1	27.0	−0.1			
Cε	40.0	39.9	−0.1			
C=O	174.3	175.1	0.8			
Seryl						
Cα	57.5	57.9	0.4	51.6	52.1	0.5
Cβ	62.6	63.3	0.7	63.8	65.3	1.5
C=O	176.2	176.9	0.7	169.4	170.2	0.8
Benzoyl						
o-COH	147.4	156.5	9.1	148.8	158.6	9.8
m-COH	145.4	153.4	8.0	146.3	155.0	8.7
C1H	117.8	114.9	−2.9	115.7	112.5	−3.2
C4H	120.6	117.2	−3.4	118.5	113.7	−4.8
C5H	120.5	116.6	−3.9	119.3	114.8	−4.5
C6H	120.3	116.3	−4.0	118.5	113.3	−5.2
C=O	170.8	171.4	0.6	169.0	168.8	−0.2

Chemical shifts are referred to an internal standard of dioxane, equal to 67.4 ppm.
Values for enterobactin are from Llinás *et al.* (1973).

Acknowledgments

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