The Salicylate Mode of Bonding in Protonated Ferric Enterobactin Analogues[1]

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Abstract: The coordination environment of protonated forms of Fe(III) catechoylamides has been probed using paramagnetic NMR. The deuteriomethyl $^2H_9$-MECAM-Me ligand (1,3,5-tris[[[4-(deuteriomethyl)-2,3-dihydroxybenzoyl]amino]methyl]benzene ligand) was synthesized and coordinated to Fe(III), and the paramagnetic shift of the CD_3 resonance in the ^2H NMR was monitored as a function of pH. At high pH one paramagnetically shifted resonance is observed at +21 ppm (downfield from TMS), which is assigned to the three equivalent CD_3 groups of the $^2H_9$-MECAM-Me ligand bound to Fe(III) through the six catechol oxygens. At a pH below the first protonation constant (log K = 7.99, determined from the spectrophotometric titration of $[Fe(MECAM\text{-}Me)]^{3-}$) monoprotonation of the complex yields the $[Fe(H^2H_9\text{-}MECAM\text{-}Me)]^{2-}$ species; a second paramagnetically shifted resonance is observed at −8 ppm, and the intensity of the +21 ppm resonance is observed to decrease. As the pH is lowered further, the resonance at −8 ppm grows in intensity, and the resonance at +21 ppm is further decreased. The large change in the paramagnetic shift of the ligand is indicative of a change in coordination geometry. Previously we proposed that protonation of ferric complexes of tricatechoylamide ligands resulted in a sequential shift in coordination of the catechoylamide binding subunits from the two catechol oxygens to the ortho-hydroxyl oxygen and carbonyl oxygen of the amide in what has been referred to as a salicylate mode of bonding. In order to establish that the resonance at −8 ppm resulted from catecholyamide subunit(s) bound to Fe(III) in a salicylate fashion, the model ligand H-4-(methyl-d_3)salicylic acid ligand [which contains CD_3 groups in equivalent positions to $^2H_9$-MECAM-Me and which can coordinate only in a salicylate mode] was synthesized. When coordinated to Fe(III) the CD_3 groups on the ligand are paramagnetically shifted to −8 ppm, identical with the shifts observed for the protonated forms of $[Fe(H^2H_9\text{-}MECAM\text{-}Me)]^{2-}$ and $[Fe(H_2{}^2H_9\text{-}MECAM\text{-}Me)]^{-}$. The results of these experiments establish that protonation of ferric enterobactin and related model compounds result in a shift of the catechoylamides from a catecholate to a salicylate mode of bonding.

Enterobactin (Figure 1a) is a naturally occurring low molecular weight iron chelating agent (siderophore) produced by *E. coli* and other enteric bacteria to bind and assimilate extracellular iron.[2] Its ferric complex has the highest formation constant known (K_f = 10^{52}) for any Fe(III) species. Previous work has established that ferric enterobactin and its synthetic analogues are recognized by a specific receptor protein.[3] The recognition mechanism is both stereoselective and highly sensitive to the ligand structure near the metal binding subunits.[4,5] Once recognized, the complex is taken into the cell by active transport.[3,5,6] Iron from ferric enterobactin is eventually found as Fe(II), presumably in the cytoplasm. The mechanism by which iron is reduced and released from enterobactin is not well understood because the highly negative reduction potential (−1.03 V vs SCE, −0.79 V vs NHE at pH 7.4)[7] precludes reduction of the intact, unprotonated Fe(III)-enterobactin complex by naturally occurring reductants at physiological pH. Three mechanisms which may facilitate the release of iron from ferric enterobactin have been proposed: (a) hydrolysis of the three ester linkages in the ligand backbone and subsequent reduction on the ferric tris complex of 2,3-dihydroxybenzoylserine;[8,9] (b) protonation in a medium of low dielectric constant followed by an internal electron-transfer reaction to yield Fe(II),[10] or (c) protonation concurrent with reduction of the ferric enterobactin complex in a low pH environment in vivo.[7]

We have studied the solution protonation reactions of ferric enterobactin and its analogues in order to determine the feasibility of mechanism (c). The protonation behavior of these tris-catechoylamides has been the subject of some controversy in the literature. We first showed that protonation of enterobactin[11,12] and some synthetic analogues[13-15] such as MECAM (1,3,5-tris[[(2,3-dihydroxybenzoyl)amino]methyl]benzene) (Figure 3) proceeds in three sequential one-proton steps. We proposed that sequential protonation of ferric enterobactin and its analogues resulted in protonation of the meta-hydroxyl oxygen of the catechol ring, concomitant with a shift in coordination of Fe(III) from the two catechol oxygens to the ortho-hydroxyl oxygen and the oxygen of the amide carbonyl, in what we have referred to as a salicylate mode of bonding (Figure 2). A series of subsequent studies in our laboratories has been consistent with this model. At this same time (1979) Hider et al. proposed a formulation of $[Fe^{II}(H_4ent)]$ for "Iron(II) enterobactin, the major form at pH 4.0".[10] The structure suggested for this neutral product of the protonation of ferric enterobactin included an unprecedented trifurcated hydrogen bond which capped the iron complex. Hider et al. subsequently (1981) supported the formulation of the protonated aqueous species as $[FeH_3ent]^0$ but maintained that it is formed in a discrete three-proton step and that internal electron transfer

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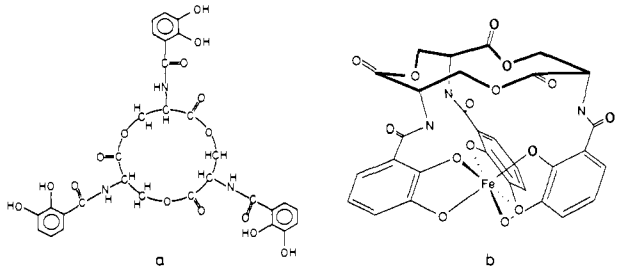

Figure 1. The structures of (a) neutral enterobactin and (b) ferric enterobactin.

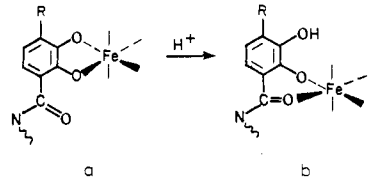

Figure 2. (a) Catecholate and (b) salicylate modes of bonding for the pH dependent coordination environments of Fe(III) bound to catechoylamide ligands.

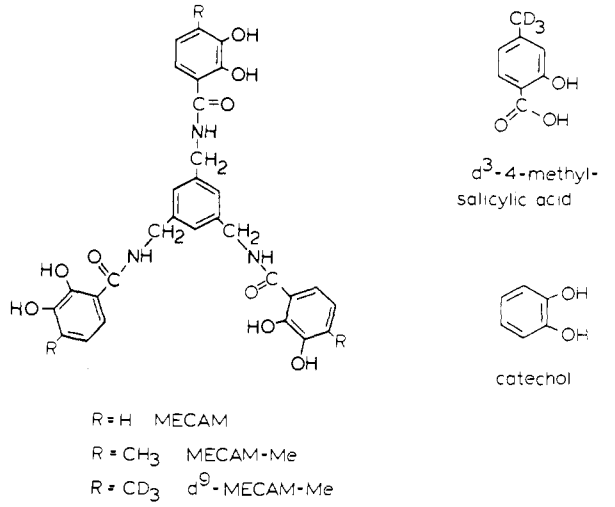

Figure 3. The structures of the ligands used and discussed in this study.

produces a ferrous–semiquinone/catechol complex.[16] Mössbauer studies of this product have shown that it contains exclusively ferric ion[17] and reevaluation of the Hider data[18] has shown that the protonation proceeds via sequential, one-proton steps (1983). In the third paper of their series, Hider et al. acknowledged that the protonated ferric enterobactin which precipitates from aqueous solution is the ferric complex $[Fe^{III}(H_3ent)]$ but disputed the structure proposed for it and the protonated ferric MECAM analogue.[19] Each of the three papers by Hider et al.[10,16,19] has presented a different characterization of the protonated ferric enterobactin complex, with no explicit reference to the previous, superseded characterization.

While there now seems to be agreement on the formulation of the protonated complex, the structure has remained in question. An infrared study on the low pH forms of the tricatechoylamides showed no C=O stretching frequency indicative of an uncoordinated ligand arm in the di- and triprotonated forms of enterobactin analogues consistent with our salicylate model.[18] Subsequently, R. C. Hider and J. Silver asserted that a pH dependent shift in coordination from a catecholate to a salicylate mode of bonding is improbable for ferric-MECAM due to the considerable strain that would result in the protonated forms of the complex; they proposed instead that protons bind to the meta-hydroxyl oxygens without causing a shift in the mode of coordination of the ligand binding subunits to the metal center (Figure 4).[19]

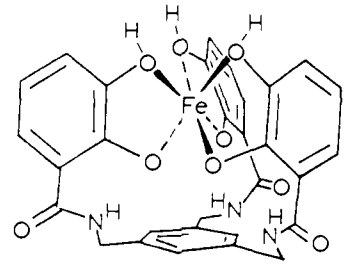

Figure 4. The structure of the triprotonated $Fe(H_3MECAM)$ species proposed by R. C. Hider and co-workers.

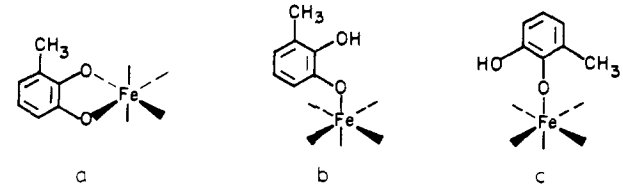

Figure 5. The structure of ferric complexes of 3-methylcatechol: (a) chelated to Fe(III) through two catechol oxygens; (b) monocoordinated through O(1); and (c) monocoordinated through O(2).

A method to distinguish between these two alternative mechanisms was inspired by the work of Que and co-workers, who showed that the mode of coordination of methyl-substituted catechols can be determined from the paramagnetic shift of the methyl resonance in the ^1H NMR. The CH_3 resonance of 3-methyl catechol was observed at 32 ppm (downfield from TMS) for the ligand chelated to Fe(III) (Figure 5a), at −28 ppm when monocoordinated through O(1) (Figure 5b) and at 89 ppm when monocoordinated through O(2) (Figure 5c).[20,21] This suggests that an NMR study of enterobactin and its synthetic catechoylamide analogues would yield comparable results. One analogue of enterobactin particularly suited for such a paramagnetic study is $^2H_9$-MECAM-Me (Figure 3). The $^2H_9$-MECAM-Me ligand has deuteriomethyl groups adjacent to the catechol oxygens, which should be paramagnetically shifted (with better resolution than CH_3 groups vide infra) when the ligand is bound to the high-spin ferric ion. Furthermore, the protonated form of the complex should show a different paramagnetic shift than the unprotonated form, regardless of whether the protonation results in a salicylate or protonated catecholate species. Through a comparison of appropriate model complexes, it should be possible to distinguish between a salicylate mode of bonding and the protonated catecholate species proposed by Hider et al.

Experimental Section

Chemicals. Boron tribromide (Aldrich) was vacuum distilled and stored under argon at −5 °C prior to use. Dichloromethane was distilled over CaH_2, under dried nitrogen. Perdeuteriated dimethyl sulfoxide (Aldrich, 99.9 atom %) was dried over 4 Å molecular sieves, under argon for at least 24 h prior to use. A $FeCl_3$ solution ($[Fe^{3+}]$ = 0.09964 (8) M, approximate $[H^+]$ = 0.096 M) was prepared and standardized in $[Fe^{3+}]$ by titration with a standardized EDTA[22] solution by using a

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(21) Note: the paramagnetic shifts reported here are reported here with the convention that a positive chemical shift is downfield from TMS. This is the same convention used by Que and co-workers et al. in ref 32 but is opposite in sign to Que's earlier work in ref 20.
(22) Abbreviations used in the text include: EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; β_{rst}, cumulative formation constant $[M_rL_sH_t]/[M]^r[L]^s[H]^t$; $R = \{\Sigma(Y^o - Y_c)^2/\Sigma Y_o^2\}^{1/2}$ Y is the dependent variable; ent, enterobactin.

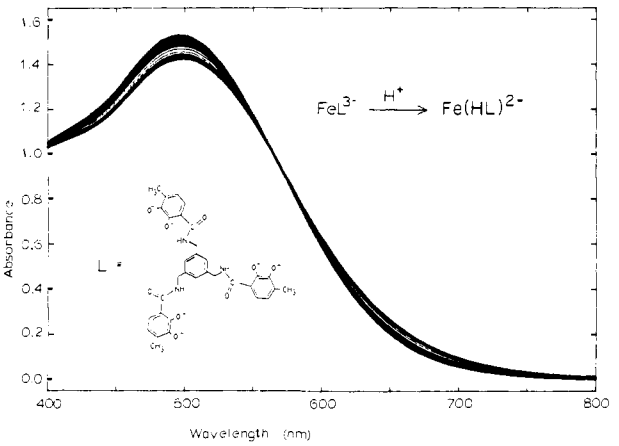


Figure 6. Visible spectra in the isosbestic region (pH 8.88–7.62) in the titration of $[Fe(MECAM\text{-}Me)]^{3-}$ to $[Fe(HMECAM\text{-}Me)]^{2-}$.

variamine blue indicator. A second $FeCl_3$ solution, standardized in $[Fe^{3+}]$ by the same procedure and of known $[H^+]$ concentration ($[Fe^{3+}]$ = 0.05029 (8) M, $[H^+]$ = 0.1000 (2) M), was used in the preparation of Fe(MECAM-Me) solutions for spectrophotometric titration experiments. All water used in the preparation of samples or solutions used in paramagnetic NMR or spectrophotometric titration experiments was first distilled and deionized (resistivity 18 × 10^6 ohms-cm) and then boiled while degassed with argon to remove oxygen, CO_2, and other dissolved gases. The degassed water was stored under a flow of argon and used within 6 h after deoxygenation. Acetone was dried over 4 Å molecular sieves prior to use. Other starting materials were of reagent grade and were not further purified. The ligands MECAM-Me and 4-methyl-2,3-dimethoxybenzoic acid were synthesized in this laboratory as previously reported.[5]

Physical Measurements. Microanalyses and electron impact and high resolution electron impact mass spectra were performed by the Analytical Services Laboratory at the Chemistry Department, University of California, Berkeley. All NMR spectra were recorded at the University of California, Berkeley NMR Facility. Routine ^1H NMR spectra were recorded on the UCB-200 MHz FT NMR spectrometer. ^2H NMR spectra were recorded on the UCB-180 MHz FT NMR spectrometer operated at a frequency of 27.64 MHz with a deuterium probe. Paramagnetic ^2H NMR spectra were recorded with the following parameters: sweep width 50000 Hz and aquistion time 41 ms or sweep width 62 500 Hz and aquisition time 33 ms. Approximately 1000 scans were collected for each sample, exponentially multiplied with a line broadening parameter of 10.0 Hz, and Fourier transformed.

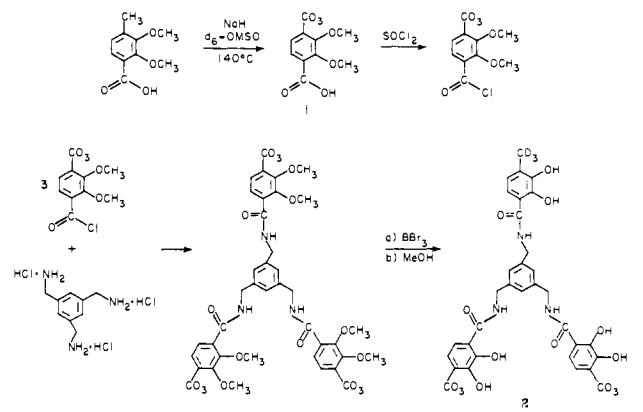
Spectrophotometric Titration of Fe(MECAM-Me). The Fe-MECAM-Me complex was prepared in an oxygen-free environment by using Schlenk techniques.[23] The MECAM-Me ligand (5.610 mg, 9.11 × 10^{-6} mol) was placed in a 25-mL pear-shaped sidearm flask under an argon atmosphere; 1.077 mL of 0.1016 M KOH and, subsequently, 0.181 mL of 0.05029 M $FeCl_3$ (9.11 × 10^{-6} mol) were slowly added, while the solution was vigorously stirred. An aliquot of this $Fe^{(III)}$-MECAM-Me solution was transferred with a 2.000-mL Gilmont buret to 50.00 mL of 0.1 N KCl (prepared from low iron KCl and degassed water) in a spectrophotometric titration cell. The solution temperature was maintained at 25.0 ± 0.5 °C with a circulating water bath. The pH of the solution was monitored with a Fisher Scientific Accumet pH meter (Model 825 MP) equipped with a Corning semimicro calomel combination electrode. The electrode was calibrated in concentration units with degassed solutions of $10^{-2.30}$ M HCl and $10^{-2.99}$ M KOH at 0.1 M ionic strength (KCl). The appropriate value of $10^{-13.78}$ for K_w was used.[24] The titration was run under a flow of water-saturated argon to prevent oxidation and evaporation. The solution was titrated with 0.100 N HCl (prepared from a Baker Dilut-It ampule and stored under argon) with an automatic spectrophotometric titrator. The cell and automatic titrator have been described elsewhere. Spectra were recorded on a Hewlett-Packard 8540A UV–vis multichannel spectrophotometer approximately every 0.04 pH units. Data (84 spectra) were collected between 400 and 800 nm from pH 9.63 to 5.28 over the course of 10 h. The isosbestic region of the titration from pH 8.88 to 7.62 is shown in Figure 6. A Schwarzenbach plot ($\{A_0 - A_{obsd}\}/[H^+]^n$ vs A_{obsd}) for the data in the isosbestic region is shown in Figure 7. A factor analysis and a nonlinear least-squares refinement program, REFSPEC,[25,26] was used to determine the first protonation constant of the ferric MECAM-Me complex from 25 spectra taken from the isosbestic region. The first protonation constant for the single proton protonation of Fe-MECAM-Me to Fe-HMECAM-Me refined to log K_{MLH} = 7.97 (2) (R = 0.1%) in 10 cycles of least-squares refinement.

A second data set for the same protonation constant was recorded with 0.234 mL of a 7.25 × 10^{-3} M solution of $Fe^{(III)}$-MECAM-Me^{3-} (prepared from 4.856 mg of MECAM-Me (7.883 × 10^{-6} mol), 0.930 mL of 0.1016 M KOH, and 0.157 mL of 0.05029 M $FeCl_3$ (7.883 × 10^{-6} mol) in 50.00 mL of 0.1 N KCl. Data (95 spectra) were recorded over the course of 12.5 h from pH 9.81 to 4.64. The first protonation constant for the monoprotonation of Fe-MECAM-Me to Fe-HMECAM-Me refined to log K_{MLH} = 8.01 (2) (R = 0.35%) in six cycles of least-squares refinement on 19 spectra in the isosobestic region from pH 8.89 to 7.61.

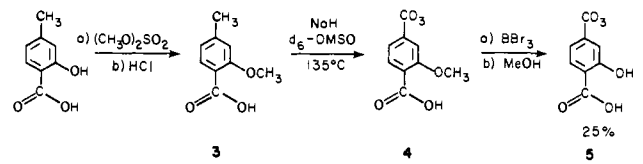
Synthesis of $^2H_9$-MECAM-Me (2). The reactions used in the synthesis of **2** are shown in Scheme I.

Synthesis of 4-(Deuteriomethyl)-2,3-dimethoxybenzoic Acid (1). The 4-methyl group of 4-methyl-2,3-dimethoxybenzoic acid was deuteriated by a procedure analogous to that used in the deuteriation of toluene, mesitylene, and *o*-, *m*-, and *p*-xylene;[27] NaH (0.163 g, approximately 6.6 × 10^{-3} mol in a 50% paraffin suspension) was added to a stirred solution of 4-methyl-2,3-dimethoxybenzoic acid (1.00 g, 5.1 × 10^{-3} mol) in DMSO-d_6 under a flow of argon (Note: increasing the amount of NaH drastically decreases the yield of the reaction). The reaction mixture was maintained at 140 °C with constant stirring for 28 h under a positive argon pressure, DMSO was removed in vacuo, and the product was dissolved in H_2O and precipitated with HCl to pH 1. This process converted a substantial fraction of the reaction mixture to a monomethoxy-4-methylbenzoic acid species; therefore, the reaction mixture was remethylated in refluxing dimethyl sulfate (1.6 × 10^{-2} mol), dry acetone, and K_2CO_3 (1.05 × 10^{-2} mol) by a previously described procedure.[7] Dimethyl sulfate and acetone were removed in vacuo, and the product was refluxed in dilute NaOH for 12 h and then precipitated with concentrated HCl (to pH 1). The precipitate was dissolved in ethyl acetate, and this solution was filtered through diatomaceous earth, dried over $MgSO_4$, and filtered, and the solvent was evaporated. The product (**1**) was recrystallized from an ethanol/water mixture: yield 80%; ^1H NMR (CD_3OD) δ 7.46 ppm (d, Ar-H, 1), 6.99 ppm (d, Ar-H, 1), 4.93 ppm (H_2O), 3.88 ppm (s, O-CH_3, 3), 3.82 ppm (s, O-CH_3, 3), 2.28 ppm (s, residual -CH_3, 0.3); mass spectral data: parent ion peak m/e = 199; M^+ = CD_3-$C_6H_2(OCH_3)_2(COOH)$.

Scheme I



Scheme II



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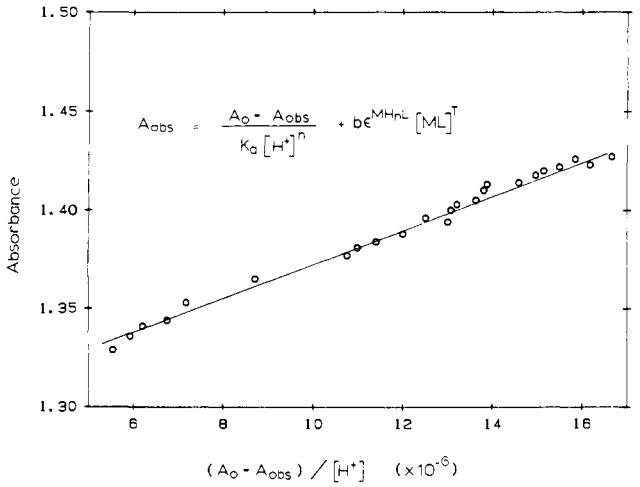

Figure 7. Schwarzenbach plot for the one-step protonation of [Fe(MECAM-Me)]$^{3-}$ to [Fe(HMECAM-Me)]$^{2-}$. Values of A_{obsd} are an average absorbance between 464 and 474 nm for a titration data set between pH 8.88 and 7.62; A_0 is the initial absorbance of the [Fe(MECAM-Me)]$^{3-}$ complex, n the proton stoichiometry, b the cell path length (10 cm), ϵ_{MH_nL}, the molar extinction coefficient of the protonated complex, $[ML]^T$ the total concentration of metal–ligand species, and K_a the protonation constant for the reaction.

Synthesis of 1,3,5-Tris[[[2,3-dihydroxy-4-(deuteriomethyl)benzoyl]-amino]methyl]benzene (2). The synthesis of **2** was carried out in the series of reactions shown in Scheme I by the same procedure exactly as in the synthesis of 1,3,5-tris[[(2,3-dihydroxy-4-methylbenzoyl)amino]methyl]-benzene from 2,3-dihydroxy-4-methylbenzoic acid:[7] overall yield 48%; ^{1}H NMR (in CD_3OD) δ 7.24 ppm (s, mesitylene ring Ar-H, 1), 7.10 ppm (d, catechol ring Ar-H, 1), 6.56 ppm (d, catechol ring Ar-H, 1), 4.96 ppm (H_2O), 4.51 ppm (s, -CH_2-, 2); ^{2}H NMR (105 mg of **2** in 3.0 mL of spectral grade methanol, referenced to CD_3OD at 3.30 ppm) δ 2.16 ppm (s, CD_3).

Synthesis of 4-(Methyl-d_3)salicylic Acid (5). The reactions used in the synthesis of **5** are shown in Scheme II. The synthesis was not optimized.

Synthesis of 4-Methyl-2-methoxybenzoic Acid (3). The starting material (4-methylsalicylic acid, 2.79×10^{-2} mol) was heated at a reflux in dimethyl sulfate (5.81×10^{-2} mol) with dried K_2CO_3 (5.62×10^{-2} mol) and 50 mL of acetone (dried over 4 Å molecular sieves) under a positive pressure of argon for 24 h. Acetone and dimethyl sulfate were removed by trap-to-trap distillation in vacuo. The remaining solid was dissolved in 50 mL of 1 M NaOH and heated at reflux under argon of 24 h. Concentrated HCl was added (to pH 1) to precipitate **3**. The precipitate was dried in vacuo and then recrystallized from an ethanol-/water mixture: yield 61%; ^{1}H NMR (DMSO-d_6) δ 7.58 ppm (dd, Ar-H, 1), 6.95 ppm (s, Ar-H, 1), 6.81 ppm (dd, Ar-H, 1), 3.80 ppm (s, O-CH_3, 3), 2.34 ppm (s, -CH_3, 3).

Synthesis of 4-(Methyl-d_3)-2-methoxybenzoic Acid (4). To **3** (1.6×10^{-2} mol) was added NaH (in a 50% paraffin suspension, 0.859 g, approximately 1.8×10^{-2} mol) and 30 mL of DMSO-d_6. The solution was heated at 135 °C with constant stirring for 24 h. DMSO was removed by vacuum trap-to-trap distillation. The crude product was dissolved in H_2O and reprecipitated by addition of concentrated HCl. TLC indicated both 4-(methyl-d_3)-2-methoxybenzoic acid and 4-(methyl-d_3)salicylic acid were present in the product mixture, and so the product was again methylated with dimethyl sulfate: yield of **4** from **3**, 28%; ^{1}H NMR (DMSO-d_6) δ 7.57 ppm (d, Ar-H, 1), 6.94 ppm (s, Ar-H, 1), 6.82 ppm (d, Ar-H, 1), 3.80 ppm (s, O-CH_3, 3), approximately 2.4 ppm (s, residual CH_3, 0.12).

Synthesis of 4-(Methyl-d_3)salicylic Acid (5). Deprotection of **4** was accomplished by the dropwise addition of BBr_3 (7.8×10^{-2} mol) in 50 mL of freshly distilled CH_2Cl_2 into a solution of **4** (7.8×10^{-3} mol) in 30 mL of distilled CH_2Cl_2 under argon at 0 °C. The reaction mixture was stirred for 6 h at 0 °C. Distilled water (30 mL) was added, and the reaction mixture was stirred overnight to hydrolyze excess BBr_3. Water and CH_2Cl_2 were removed by rotoevaporation. The product was dissolved repeatedly (14 times) in 50 mL of methanol and heated to boiling, and the solvent was evaporated in order to volatilize and remove all boron species: yield of **5** from **4**, 83%; overall yield; 25%; ^{1}H NMR (CD_3OD) δ 7.72 ppm (d, Ar-H, 1), 6.73 ppm (s, Ar-H, 1), 6.68 ppm (d, Ar-H, 1); ^{2}H NMR (80 mg of **5** in approximately 4 mL of spectral grade methanol in a 10 mm NMR tube, CD_3OD reference) δ 2.26 ppm (s, CD_3 resonance of **5**); ^{2}H NMR in H_2O, pH 10.0 (referenced to acetone-d_6) δ 2.25 ppm (s, CD_3 resonance of **5**); mass spectral data: parent ion peak m/e = 155.02 (100% rel intensity), $M^+ = CD_3C_6H_3(OH)(COOH)$.

Preparation of an Fe(^{2}H$_9$-MECAM-Me) Sample for the Paramagnetic ^{2}H NMR Experiment. In an argon atmosphere, 176 mg (2.82×10^{-4} mol) of $^2H_9$-MECAM-Me (**2**) was dissolved in 2.00 mL of degassed H_2O and 1.000 mL of 2.062 M NaOH. Under an argon flow 2.826 mL of a 0.09964 M Fe^{3+} solution was introduced. The solution was stirred and then filtered into an argon-filled 10-mm NMR tube. The pH of the solution was measured (12.0), and the ^{2}H NMR spectrum was recorded (Figure 8a). The pH of the solution was then lowered to pH 7.7 by addition of 1.002 M HCl, and a second ^{2}H NMR spectrum was recorded (Figure 8b). To check for reversibility, 82 μL of 2.062 M NaOH was added to bring the pH of the solution to pH 8.3; the ^{2}H NMR spectrum (not shown) confirmed the reversiblity. The pH of the solution was lowered to pH 5.3 by addition of 300 μL of 1.002 M HCl; the sample was mixed, sonicated, and filtered into a second argon-filled 10-mm NMR tube. The ^{2}H NMR spectrum of this solution is shown in Figure 8c.

Preparation of a Ferric (4-(Methyl-d_3)salicylic acid)$_3^{3-}$ Solution for the Paramagnetic ^{2}H NMR Experiment. A species distribution calculation was performed from the published equilibrium constants (log β_{011} = 14.26, log β_{012} = 17.23, log β_{110} = 17.3, log β_{120} = 32.9, log β_{111} = 19.0)[28] and an estimated value of β_{130} = 44.3 (based on the drop between K_{120} and K_{130} for 3-methylsalicylic acid) in order to determine the concentration condiions necessary to prepare a solution of the ferric tris complex of 4-methylsalicylic acid. The species distribution plot for the Fe^{3+} (0.016 M)/4-(methyl-d_3)salicylic acid (0.164 M) solution present in the NMR sample is shown in Figure 10. Under an argon atmosphere, **5** (447 mg, 2.884×10^{-3} mol) was dissolved in 1.950 mL of 2.04 M KOH. An aqueous 0.09964 M Fe^{3+} solution (2.894 mL, 2.884×10^{-4} mol) was slowly added with stirring under a flow of argon. The pH was adjusted to 10.4, and the volume of the solution was brought to 5.680 mL. An aliquot (2 mL) of this solution was diluted to 6 mL with degassed water under inert atmosphere (pH = 10.1). The solution was filtered into an argon-filled 10-mm NMR tube, and the ^{2}H NMR spectrum was recorded (Figure 9). After recording the spectrum, 4 drops of pyridine-d_5 were added to the sample and the spectrum was rerecorded and referenced to the peak at 8.7 ppm. Reported chemical shifts are referenced to this value.

Results and Discussion

The $^2H_9$-MECAM-Me ligand was synthesized by the series of reactions shown in Scheme I. Specific deuteriation of the methyl groups was accomplished through a procedure developed for the deuteriation of the methyl groups of toluene, mesitylene, and *o*-, *m*-, and *p*-xylene.[27] The ^{2}H NMR spectrum of $^2H_9$-MECAM-Me in methanol shows one singlet resonance at 2.16 ppm for the three equivalent CD_3 groups. The singlet at 2.16 ppm is absent in the ^{1}H NMR spectrum. The ^{1}H NMR spectra of $^2H_9$-MECAM-Me and nondeuteriated MECAM-Me are otherwise identical.

The 4-(methyl-d_3)salicylic acid ligand (Figure 3) was prepared by the reactions shown in Scheme II. One singlet (2.26 ppm) is observed in the ^{2}H NMR spectrum for the 4-(methyl-d_3)salicylic acid ligand in methanol, confirming that only the methyl group was deuteriated. This resonance is absent in the ^{1}H NMR spectrum. The ^{1}H NMR spectra of 4-(methyl-d_3)salicylic acid and the undeuteriated 4-methylsalicylic acid are otherwise identical. At pH 10.1 in aqueous solution, 4-(methyl-d_3)salicylic acid shows one resonance at 2.25 ppm for the monoprotonated form (pK_{a1} = 2.9, pK_{a2} = 14.3 for 4-methylsalicylic acid)[27] in the deuterium NMR spectrum.

Above pH 10 the Fe(MECAM-Me)$^{3-}$ complex is unprotonated. The [Fe(MECAM-Me)]$^{3-}$ (λ = 496 nm ϵ = 4700 L cm^{-1} mol^{-1}) complex has a similar spectrum to the trianionic [Fe(ent)]$^{3-}$, [Fe(MECAM)]$^{3-}$ and [Fe(catechol)$_3$]$^{3-}$ complexes,[29,14,30] all of which have six catechol oxygens bound to the high-spin ferric ion. Isosbestic behavior is observed between pH 8.9 and 7.6, with an isosbestic point at 563 nm accompanying a small spectral change from λ = 496 nm (FeL^{3-}) to λ = 498 nm ($FeHL^{2-}$). A

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Schwarzenbach plot ($[A_0 - A_{obsd}]/[H^+]^n$ vs A_{obsd}) is linear for two independent data sets when $n = 1$, indicating that the observed spectral change arises from a one-proton reaction. A Schwarzenbach plot for one data set and the visible spectra in the isosbestic region are shown in Figures 7 and 6, respectively. The log K of the protonation was determined to have a value of approximately 8.0 from the slope of the Schwarzenbach equation (Figure 7); refinement of the equilibrium constant from the spectrophotometric titration data between pH 8.9 and 7.6 [using a factor analysis and nonlinear least-squares refinement program, REFSPEC, and two independent data sets] gave an average value of 7.98 (2). Determination of the second and third protonation constants was not possible due to the subsequent lack of significant spectral changes between 400 and 800 nm and the eventual precipitation of the complex.

Structural information on the protonated forms of ferric tricatechoylamides was obtained by monitoring the paramagnetic shift of the CD_3 resonance in Fe($^2H_9$-MECAM-Me). Large shifts have been seen in these types of complexes, resulting from the π delocalization of unpaired spin density in a dominant contact shift interaction.[20,31] Relatively well-resolved spectra are often observed, due to the large isotropic shifts and the relatively short electron spin relaxation time of Fe(III) in these complexes. As discussed above, large differences in the paramagnetic shift have been observed as a function of the mode of coordination of the ligand.[20,32] Therefore a discernable change is expected in the chemical shift upon protonation of the ferric complex. Since the chemical shift expected in going from a catecholate to protonated catecholate ligand was expected to be smaller than that seen in going to a salicylate mode of bonding, it was particularly important to obtain well resolved spectra.

Rather than monitor the shift of the CH_3 resonance of Fe(MECAM-Me), we chose to monitor the shift of the CD_3 resonance in the deuteriomethyl analogue of Fe(MECAM-Me), Fe($^2H_9$-MECAM-Me). Site specific deuteriation of the ligand proved to have several other experimental advantages. Only the CD_3 resonances were observed in the ^2H NMR (other than D_2O and the added acetone-d_6 reference). The ^2H NMR spectrum is uncomplicated by other paramagnetically shifted or unshifted ligand resonances. Monitoring the ^2H NMR also allowed us to work in water, with aqueous acids and bases, and simplified the pH measurement. Finally, crucial to our ability to observe well-resolved spectra, the paramagnetic line widths of the deuterium resonances are significantly narrower than the corresponding proton resonances. The paramagnetic peak width has a squared dependence on the gyromagnetic ratio of the NMR nucleus. Theoretically, the paramagnetic line width of deuterium resonances should be 42.5 times (a factor of γ_H^2/γ_D^2) narrower than corresponding proton resonances.[33] We observed a resonance line width 24 times narrower for the CD_3 resonance of Fe($^2H_9$-MECAM-Me)$^{3-}$ relative to the CH_3 resonance in Fe(MECAM-Me)$^{3-}$. Other studies have reported a decrease in paramagnetic peak widths for deuterium versus proton resonances by factors ranging from 12 to 42.[33]

Above pH 10, the [Fe($^2H_9$-MECAM-Me)]$^{3-}$ complex is unprotonated, as determined by the spectrophotometric titration of ferric MECAM-Me. At high pH a single paramagnetically shifted CD_3 resonance is observed at +21 ppm in the ^2H NMR of a 1:1 solution of Fe^{3+} and $^2H_9$-MECAM-Me (Figure 8a) which is assigned to the three CD_3 groups of the three equivalent catecholyamide subunits all chelated to Fe(III) through two catechol oxygens (Figure 11a). The two peaks in the diamagnetic region are assigned to D_2O (4.66 ppm) and acetone-d_6 (2.04 ppm, added as reference). As the pH is lowered, the complex is protonated in a one-proton step with a pK_a of 7.99 (2). The first pK_a for ferric

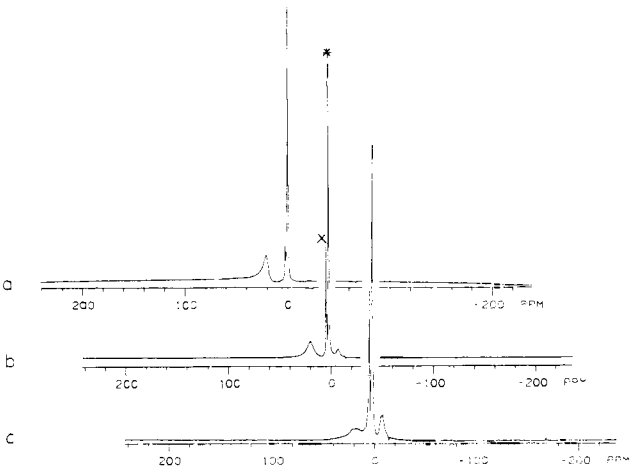

Figure 8. ^2H NMR spectra of a 1:1 solution of Fe^{3+} (0.048 M) and $^2H_9$-MECAM-Me (0.048 M) at (a) pH 12.0, (b) pH 7.7, and (c) pH 5.3. The spectrum is referenced to acetone-d_6 (*) at 2.04 ppm. D_2O (×) is also observed in the diamagnetic region of the spectrum at 4.66 ppm. The spectrum was recorded at 27.64 Mz, at a sweep width of 50 000 Hz and an acquisition time of 41 ms.

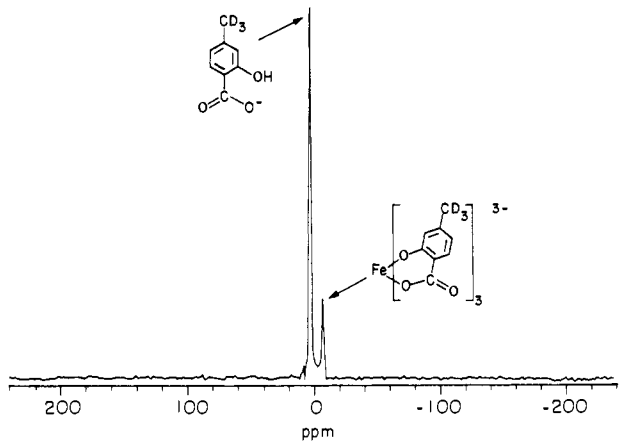

Figure 9. ^2H NMR spectrum of a 10/1 solution of 4-(methyl-d_3)salicylic acid (0.164 M) and Fe^{3+} (0.0164 M) at pH 10.1. A species distribution calculation indicates that under these conditions [Fe(4-(methyl-d_3)-salicylic acid)$_3$]$^{3-}$ represents more than 99% of the iron containing species in solution.

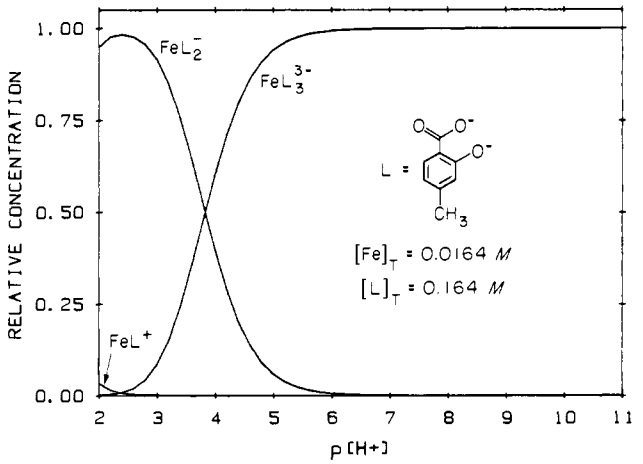

Figure 10. A species distribution plot for a 10/1 solution of 4-(methyl-d_3)salicylic acid (0.164 M) and Fe^{3+} (0.0164 M) at pH 10.1.

MECAM-Me is more basic than that measured for ferric MECAM (7.1)[14] as anticipated from the electron donor properties of the methyl substituents of MECAM-Me. Below the first pK_a of the complex a new paramagnetically shifted resonance is ob-

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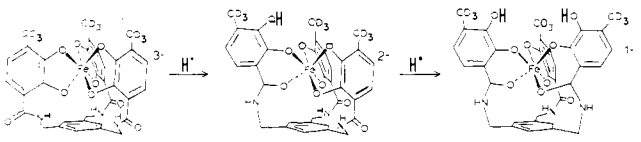

Figure 11. The protonation scheme for ferric $^2H_9$-MECAM-Me. Structures of (a) the tris(catecholate) $[Fe(MECAM\text{-}Me)]^{3-}$ complex, (b) the mono(salicylate), bis(catechoylate) $[Fe(HMECAM\text{-}Me)]^{2-}$ complex, and (c) the bis(salicylate), mono(catecholate) $[Fe(H_2MECAM\text{-}Me)]^-$ complex.

served at −8 ppm (Figure 8b). This new resonance suggests that a shift in coordination of the ligand has occurred upon protonation. A species distribution calculation using the first protonation constant indicates that 66% of the complex should be in the monoprotonated form ($FeHL^{2-}$) and 34% of the complex should be unprotonated at the pH of the NMR sample (pH = 7.7). Therefore 78% of the catechoylamide binding subunits should be chelated to Fe(III) through the catechol oxygens and 22% protonated and bound to Fe(III) in an alternative fashion. This agrees with the relative intensities of the paramagnetically shifted resonances at +21 and −8 ppm in the ^2H NMR spectrum. As the pH is lowered further to pH = 5.3, the resonance at −8 ppm grows in intensity while the peak at +21 ppm decreases in intensity. Addition of a second proton, therefore, results in an identical change in coordination for the second binding subunit as the first.[34] This is consistent with our previous solution thermodynamic and infrared studies.[11-14,16,17] Further protonation of the complex results in the formation of the neutral $[Fe(H_3\text{-}^2H_9\text{-}MECAM\text{-}Me)]$ complex which precipitates from solution.

A model ligand, 4-(methyl-d_3)salicylic acid, was synthesized which, like $^2H_9$-MECAM-Me, has a deuteriomethyl substituent para to the carbonyl. However, unlike $^2H_9$-MECAM-Me, 4-(methyl-d_3)salicylic acid can bond to Fe(III) only in a salicylate fashion. The ^2H NMR spectrum of the ferric tris complex correspondingly shows only one paramagnetically shifted resonance at −8 ppm, which is assigned to the three CD_3 groups on three equivalent ligands chelated to Fe(III) through the phenolic oxygen and one carboxylate oxygen. This shift is identical with that seen in the protonated forms of ferric $^2H_9$-MECAM-Me, establishing that in the protonated forms of ferric $^2H_9$-MECAM-Me catechoylamide subunits are bound to the ferric ion in a salicylate mode.

(34) A slight change (less than 2 ppm) in the chemical shift from the peak of +21 ppm (due to FeL^{3-}) may occur for $Fe(HL)^{2-}$ or $Fe(H_2L)^-$ but would not be observable at this resolution.

A priori, it might be expected that the two complexes with additional hydroxy groups on $^2H_9$-MECAM-Me (which are absent in the model ligand) would have different chemical shifts. However, our results are consistent with those of Que and his co-workers, who observed only small differences between the paramagnetically shifted CH_3 resonances of monocoordinated methyl catechols and equivalent methyl substituted phenols.[20] Therefore, on the basis of the identical shift for the protonated forms of ferric $^2H_9$-MECAM-Me and ferric tris(4-(methyl-d_3)-salicylic acid) we conclude that $[Fe(H^2H_9\text{-}MECAM\text{-}Me)^{2-}$ is a mono(salicylate), bis(catecholate) complex as shown in Figure 11b and $[Fe(H_2\text{-}MECAM\text{-}Me)]^-$ is a bis(salicylate), mono(catecholate) complex (Figure 11c). CPK models of the $Fe(HL)^{2-}$ and $Fe(H_2L)^-$ complexes with catechoylamide subunits bound in salicylate fashion show no evidence of strain in the ligand backbone.

Previous work has shown that the spectral changes upon addition of a third proton parallel those seen with addition of the first and second. Thus any shift in the ligand coordination upon addition of the third proton must parallel the shift upon mono- and diprotonation. Since the mono- and diprotonated species are mono- and bis-salicylate complexes, the triprotonated compound is assigned as a tris(salicylate) complex. While the data reported here establish the salicylate mode of coordination and deny a structure such as that proposed by Hider et al.,[35] whether that tris(salicylate) structure is monomeric or polymeric cannot be distinguished.

Conclusions

In conclusion, we have observed a large shift in the CD_3 resonance of Fe($^2H_9$-MECAM-Me) as the pH is lowered, corresponding to a large change in the coordination geometry. Addition of a second proton causes an identical shift in the CD_3 resonance, indicating an identical shift in coordination geometry (for the second ligand arm). By comparison with model ligands and complexes of known coordination mode, the ligand coordination upon protonation is assigned to a salicylate mode of bonding. The triprotonated form of ferric enterobactin and its analogues must be a tris-salicylate species.

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(35) Figure 5 in ref 19 above.