Potentiometric, Spectrophotometric, and ^1H NMR Study of Four Desferrioxamine B Derivatives and Their Ferric Complexes

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Received September 13, 1993®

Abstract: A new octadentate terephthalamide desferrioxamine B (DFO) derivative, *N*-(2,3-dihydroxy-4-(methylamido)-benzoyl)desferrioxamine B (DFOMTA), has been prepared. This ligand and three other DFO derivatives [*N*-(2,3-dihydroxybenzoyl)desferrioxamine B (DFOCAM), *N*-(2,3-dihydroxy-4-carboxybenzoyl)desferrioxamine B (DFO-CAMC), and *N*-((1,2-dihydro-1-hydroxy-2-oxopyridin-6-yl)carbonyl)desferrioxamine B (DFOHOPO)] as well as their ferric complexes have been studied by potentiomeric, spectrophotomeric, and proton NMR titrations. All four DFO derivatives form six-coordinate 1:1 ferric complexes in aqueous solution. The catecholate derivatives are trishydroxamate complexes at low pH. At higher pH they form bishydroxamate–monocatecholate complexes by replacement of the terminal hydroxamate group with the catecholate group. In contrast, DFOHOPO forms a trishydroxamate complex at high pH and a bishydroxamate–monohydroxypyridinoate complex at low pH. These transformations parallel the relative acidity of the ligand functional groups: hydroxypyridinonate > hydroxamate > catecholate. The formation constants of the ferric complexes have been determined by spectrophotometric competition titrations (log K values): DFOMTA, 34.8 (4); DFOCAM, 35.4 (4); DFOCAMC, 34.9 (2); DFOHOPO, 30.7 (3). All four derivatives are more powerful than their parent compound DFO as iron chelators at pH 7.4. The catecholate derivatives and their formation constants are the first determined for Fe(III) bound by two hydroxamate and one catecholate group and show UV/vis spectra characteristic of this coordination; these are good models for the pyoverdin siderophores.

Introduction

Iron is an essential element for almost all living organisms. However it is also very toxic when in excess. The most common iron overload is due to regular blood transfusions, particularly in the treatment of β-thalassemia (Cooley's anemia), which constitutes a major health problem in North America.[1,2] The toxicity of excess iron in the body can be ameliorated by administration of an iron chelating agent which is able to remove iron *in vivo* from transferrin, ferritin, and other iron stores.[3] The current drug of choice for chelation therapy is the methane sulfonate salt of deferrioxamine B (Desferal). While Desferal has been shown to increase iron excretion and to reduce liver iron in β-thalassemic patients, its drawbacks include a lack of oral activity and a short body retention time, which necessitates its administration by one of the cumbersome and expensive methods of slow subcutaneous or intravenous infusion. For the last two decades, considerable effort has been invested in developing a more effective iron removal agent;[4-7] however, the treatment has remained essentially unchanged. Desferrioxamine B (DFO) (Figure 1), one of a class of microbially produced iron chelating agents known as siderophores,[8] has excellent affinity and selectivity for Fe^{3+}. However it is kinetically ineffective at iron removal from transferrin both *in vitro* and *in vivo*,[9] which may account for why DFO is not more clinically effective.

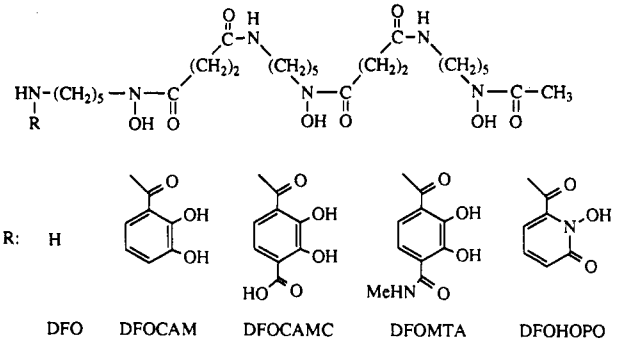

Figure 1. Desferrioxamine B (DFO) and its four derivatives: DFOMTA, *N*-(2,3-dihydroxy-4-(methylamido)benzoyl)desferrioxamine B; DFO-CAM, *N*-(2,3-dihydroxybenzoyl)desferrioxamine B; DFOCAMC, *N*-(2,3-dihydroxy-4-carboxybenzoyl)desferrioxamine B; DFOHOPO, *N*-(1,2-dihydro-1-hydroxy-2-oxopyridine-6-yl)carbonyl)desferrioxamine B.

Recently studies on transferrin have provided more information for understanding the mechanism by which iron is removed from the protein by chelators. It has been established that the cleft of the iron-binding site is closed in the stable iron transferrin complex (which includes a synergistic carbonate anion), so that the release of iron requires a conformational change to open the cleft and make the iron site accessible to chelators.[10,11] Kinetic studies have shown that the protein conformational change can be promoted by so called "mediator anions".[12] Apparently catechol-based ligands are themselves synergistic anions and thus display rapid kinetics in removing transferrin bound iron. Hydroxamic acid-based ligands, such as DFO, are not synergistic anions, they are thermodynamically capable of removing iron

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® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

(1) Kirking, M. H. *Clin. Pharm.* **1991**, *10*, 775.
(2) Pippard, M. J. *Acta Haemat.* **1987**, *78*, 206.
(3) Raymond, K. N. *Coord. Chem. Rev.* **1990**, *105*, 135.
(4) Proceedings, the Third NIH-Sponsored Symposium on the Development of Iron Chelators for Clinical Use; Gainsville, FL, May 20–22, 1992, in press.
(5) Bergeron, R. J., Liu, Z-R.; McManis, J. S.; Wiegand, J. *J. Med. Chem.* **1992**, *35*–4739.
(6) Motekaitis, R. J.; Sun, Y.; Martell, A. E. *Inorg. Chim. Acta* **1992**, *200*, 421.
(7) Sheppard, L. N.; Kontoghiorghes, G. J. *Inorg. Chem. Acta* **1992**, *188*, 177.
(8) Matzanke, B. F.; Müller-Matzanke, G.; Raymond, K. N. In *Iron Carriers and Iron Proteins*, Physical Bioinorganic Chemistry Series; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; Vol. 5, pp 1–121.
(9) Morgan, E. H. *Biochim. Biophys. Acta* **1971**, *244*, 103.
(10) Harris, D. C.; Aisen, P. In *Iron Carriers and Iron Proteins*, Physical Bioinorganic Chemistry Series; Loehr, T. M., Ed.; VCH Publishers: New York, 1989; Vol. 5, pp 239–351.
(11) Baker, E. N.; Lindley, P. F. *J. Inorg. Biochem.* **1992**, *47*, 147.
(12) Kretchmar, S. A.; Craig, A.; Raymond, K. N. *J. Am. Chem. Soc.* **1993**, *115*, 6758.

0002-7863/94/1516-0840$04.50/0

from transferrin but have relatively low rates of iron removal even at high ligand concentrations. These results provide the background for new efforts in developing more effective sequestering agents for the treatment of iron overload.

We have synthesized a series of DFO derivatives by attaching catecholate and hydroxypyridinonate groups to the free amine of DFO.[13,14] The structures of these derivatives, DFOMTA, DFOCAM, DFOCAMC, and DFOHOPO, are shown in Figure 1. All three incorporated binding groups, catechol, hydroxamic acid, and hydroxypyridinone, are found in siderophores. Typically they bind Fe^{3+} through six oxygen atoms with considerable thermodynamic stability.[4,8] The success of these DFO derivatives has been demonstrated both *in vivo* and *in vitro*. It has been shown that the addition of a single catecholate moiety to DFO increases the rate of iron removal from transferrin more than 100 times.[13] In *in vivo* iron removal studies, DFOHOPO and DFOCAMC reduced the iron content of the liver and spleen as much as DFO but required much lower dosages to do so.[15]

These octadentate ligands are also potential plutonium and thorium removal agents.[16] Recent results of the plutonium decorporation experiments using DFOCAMC, DFOHOPO, and DFOMTA in mice have shown that these ligands are extremely effective in complexing and removing plutonium from mammalian tissues.[14,17-19] These results suggest that the DFO derivatives are potentially useful medicinal chelating agents. This paper presents a detailed solution thermodynamic study of these ligands, including characterization of the metal ligand complex species formed in solution and their relative stabilities as a function of pH.

Experimental Section

Solution Thermodynamics. General Methods. All solutions were prepared using distilled water that was further purified by passing through a Millipore Milli-Q cartridge system (resistivity = 18 MΩ-cm) and then degassed by boiling for 60 min while being purged by argon gas. Once prepared, solutions were protected from the ingress of oxygen and carbon dioxide by storing under a slight positive pressure of argon, which was purified by passing through Ridox (Fisher) and Ascarite II (A. H. Thomas) scrubbers.

A solution of 0.100 M KCl was prepared from 99.99% KCl (Fisher Scientific) and was used to maintain constant ionic strength during all titrations. Carbonate-free 0.1 M KOH was prepared from Baker Dilut-It analytic concentrate KOH and was standardized against potassium hydrogen phthalate to a phenolphthalein endpoint. Solutions of 0.1 M HCl were similarly prepared and were standardized against the 0.1 M KOH solution to phenolphthalein endpoint. Ferric solutions (0.1 M in ca. 0.1 M HCl) were prepared from ferric chloride hexahydrate (Aldrich, analytical grade) and standardized by EDTA titration with variamine blue B as an indicator. For all titrations, the observed pH was measured as $-\log[H^+]$. The glass electrode was calibrated in hydrogen ion concentration units by titrating 2.000 mL of standardized HCl diluted in 50.00 mL of 0.100 M KCl, with 4.200 mL of standardized KOH. The calibration titration data were analyzed by a nonlinear least-squares program.[20]

Potentiometric pH Titrations. The apparatus and method used for potentiometric pH titrations have been described elsewhere.[21] In this study ligands and metal complexes of ca. 0.001 M were titrated at 25.0 °C. The thermodynamic reversibility of each potentiometric titration was checked by cycling the titration from low to high pH and back to low pH (or the reverse). The titration data were refined by the nonlinear least-squares refinement program BETA90.[22]

Spectrophotometric pH Titrations. The apparatus and method for spectrophotometric titrations have been described in detail elswehwere.[23] The four DFO derivatives of ca. 0.1 mM were titrated to determine the protonation constants of the functional groups in each of the derivatives and to examine the spectral properties of these ligands. In addition, in order to determine the protonation constants of the ferric complexes about 0.1 mM of each ligand was also titrated spectrophotometrically in the presence of equimolar concentration of Fe^{3+}; 50–80 spectra were collected and then refined by the programs BETA90 to give protonation constants. The UV/vis spectra generated by the spectrophotometric titrations were further analyzed by the method of Schwarzenbach.[24]

Spectrophotometric Competition Titrations. The apparatus and programs used for the competition titrations of the ferric DFOMTA, DFOCAMC, and DFOHOPO were the same as those used for spectrophotometric pH titrations. Solutions of the ligands (ca. 0.1 mM), Fe(III) (ca. 0.1 mM), and EDTA (from 1.0 to 4.0 mM depending on the ligand) were titrated from pH 5 to 6 at 25.0 °C. Three sets of data, including spectra at wavelengths between 402 and 650 nm, pH values, and respective volumes of solutions, were refined to determine the overall formation constant for each of the complexes by using the factor analysis and nonlinear least-squares refinement program, REFSPEC.[21]

The formation constant of the ferric DFOCAM complex was determined by competition against *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA). Five solutions were prepared containing DFOCAM (ca. 0.18 mM), CDTA (ca. 10 mM), and Fe(III) (ca. 0.13 mM). The pH was adjusted to 7.0–8.0. The solutions were maintained at 25.0 °C, until no further change in the visible spectrum was seen; then the final pH and UV/vis spectrum (HP 8450 spectrometer; 1-cm quartz cell) of each solution were measured. The pH values, absorbance spectra, and protonation constants of the free ligand, and CDTA were accumulated to calculate the overall formation constant.

Proton NMR Titrations of DFOCAMC and Its Ga^{3+} Complex. Proton NMR titrations of DFOCAMC and its Ga^{3+} complex were performed with D_2O solutions (ca. 0.01 M) at room temperature. Data were collected on a 400 MHz Bruker AM-400 spectrometer. Deuterium oxide was degassed and stored under an inert atmosphere. The electrode was calibrated in water with proton concentration units as described in the general section above. The corrected values of pD were calculated according to the relationship pD = pH + 0.4;[25] pD was adjusted with fresh D_2O solutions of 10% NaOD or 20% DCl.

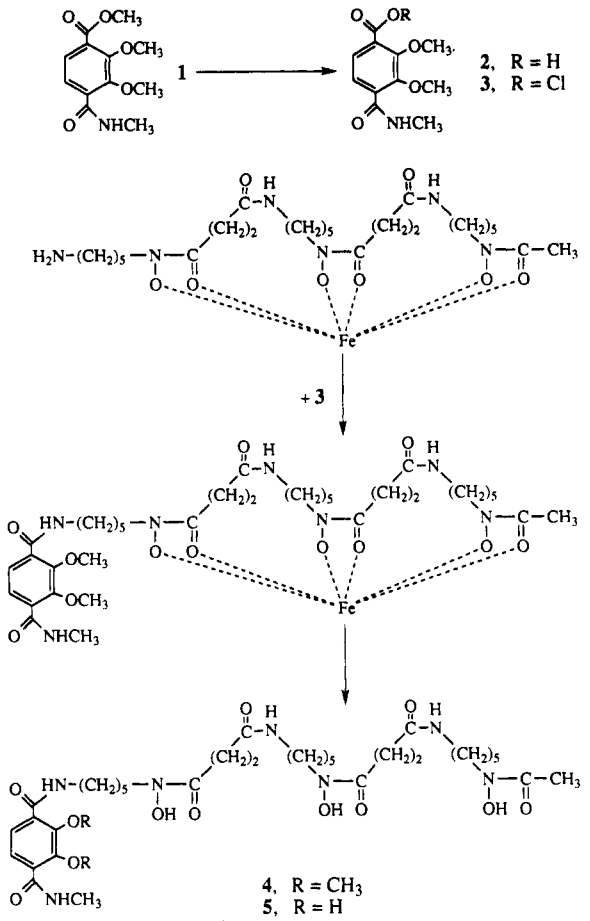
Syntheses of DFO Derivatives. Samples of DFOCAM, DFOCAMC, and DFOHOPO were synthesized and characterized according to the previously described methods.[13,14] DFOMTA was prepared according to Scheme 1.

Methyl-2,3-dimethoxy-4-(methylcarbamoyl)benzoate (1). Sodium methyl-2,3-dimethoxyterephthalate[26] (5.0 g, 20 mmol) was dissolved in 20 mL of thionyl chloride with stirring at 0 °C. After 2 h the escess thionyl chloride was removed under vacuum to give a light yellow solid. The solid was added into methylamine (1.0 g, 32 mmol) solution in 40 mL of THF and stirred at room temperature for 1 h and filtered. The filtrate was evaporated to dryness and then loaded on 40 g of silica gel in a 50 × 50 mm (d/h) column with CH_2Cl_2 as eluting solvent. A white crystalline product was obtained after removing the solvent, yield: 4.5 g (89%); ^1H NMR (250 MHz, $CDCl_3$) δ 3.0 (d, 3H, methyl), 3.93 (s, 6H, methoxy), 3.97 (s, 3H, methoxy), 7.6 (d, 1H, arom), 7.6 (d, 1H, arom).

2,3-Dimethoxy-4-(methylcarbamoyl)benzoic Acid (2). Methyl-2,3-dimethoxy-4-(methylcarbamoyl)benzoate (4.1 g, 16 mmol) was added

(13) Rodgers, S. J.; Raymond, K. N. *J. Med. Chem.* **1983**, *26*, 439.
(14) White, D. L.; Durbin, P. W.; Jeung, N.; Raymond, K. N. *J. Med. Chem.* **1988**, *31*, 11.
(15) Rodgers, S. J. Ph.D. Dissertation, University of California at Berkeley, 1985, pp 37–42.
(16) Raymond, K. N. In *Environmental Inorganic Chemistry*; Irgolic, K. J., Martell, A. E., Eds.; Proceedings, U.S.–Italy International Workshop on Environmental Inorganic Chemistry, San Miniato, Italy, June 5–10, 1983, VCH: Deerfield Beach, FL, 1985; pp 331–347.
(17) Durbin, P. W.; Jeung, N.; Rodgers, S. J.; Turowski, P. N.; Weitl, F. L.; White, D. L.; Raymond, K. N. *Rad. Prot. Dos.* **1989**, *26*, 351.
(18) Stradling, G. N.; Gray, S. A.; Moody, J. C.; Hodgson, A.; Raymond, K. N.; Durbin, P. W.; White, D. L.; Turowski, P. N. *Int. J. Radiat. Biol.* **1990**, *59*, 1269.
(19) Test Results of DFOMTA: unpublished results of Dr. P. Durbin.
(20) Martell, A. E.; Motekaitis, R. M. *Determination and Use of Stability Constants*; VCH: New York, 1988, 48–50.
(21) (a) Scarrow, R. C. Ph.D. Dissertation, University of California at Berkeley, 1985. (b) Turowski, P. N.; Rodgers, S. J.; Scarrow, R. C.; Raymond, K. N. *Inorg. Chem.* **1988**, *27*, 474.
(22) Franczyk, T. S. Ph.D. Dissertation, University of California at Berkeley, 1991; pp 77–78 and 112.
(23) Garrett, T. M.; McMurry, T. J.; Hosseini, M. W.; Reyes, Z. E.; Hahn, F. E.; Raymond, K. N. *J. Am. Chem. Soc.* **1991**, *113*, 2965.
(24) For a simple protonation equilibrium, such as $A + nH^+ = H_nA$, in which the two species have different extinction coefficient at certain λ, an equation can be derived as $A_{obs} = (A_0 - A_{obs})/(K_{H_nA}\cdot[H^+]^n + C$, where A_{obs} is the absorbencies at the λ at each pH, A_0 is the initial absorbence at the λ, and C is constant. The protonation constant K_{H_nA} might be calculated from the slope of a linear correlation of A_{obs} vs $(A_0-A_{obs})/[H^+]^n$. For more detailed information, see: Anderegg, G.; L'Eplattenier, F.; Schwarzenbach, G. *Helv. Chim. Acta* **1963**, *46*, 1409.
(25) Bates, R. G. *Determination of pH, Theory and Practice*; John Wiley & Sons: New York, 1964.

Scheme 1



to an aqueous NaOH solution (4.0 M, 50 mL). The mixture was stirred at room temperature for 4 h. The resultant solution was acidified to pH 2 with 6.0 M HCl to give an off-white preciptate. It was recrystallized from hot water to give 3.2 g (14 mmol, 85%) of colorless crystals: ^{1}H NMR (250MHz, $CDCl_3$) δ 3.0 (d, 3H, methyl), 3.97, 4.09 (s + s, 6H, methoxy), 7.75 (br s, 1H, amide), 7.91 (d, 1H, arom), 7.98 (d, 1H, arom). Anal. Calcd (found) for $C_{11}H_{13}NO_5$: C, 55.31 (55.23); H, 5.54 (5.47); N, 5.86, (5.85).

DFOMTA(Me)$_2$ (4). **2** was suspended in 10 mL of thionyl chloride with stirring at 0 °C. After 2 h, the mixture was taken to dryness to give a light yellow 2,3-dimethoxy-4-(methylcarbamoyl)benzoyl chloride (**3**), which was directly used for the following synthetic step. Desferal, the mesylate salt of DFO (6.6 g, 10 mmol) and $FeCl_3$ (1.7 g, 10 mmol) were dissolved in water (140 mL) in a 1-L 3-necked flask equipped with two addition funnels and an overhead stirrer. To the red solution was added a NaOH solution (0.50 M, 75 mL) at 0 °C and stirred vigorously for 10 min. Following this, a solution of **3** in 25 mL of ether and 25 mL of sodium hydroxide solution (0.50 M) was added simultaneously to the mixture with stirring during a 30-min period. Over the next 1.5 h, 15 mL of a 0.50 M of NaOH solution were added to keep the pH above 9. The separated ether layer was washed twice with 75 mL of water. The combined aqueous layers were reduced in volume by about one-half, and the pH was raised to 12 with 4.0 M NaOH solution. The resulting ferric hydroxide was removed by filtration. Acidification with 6.0 M HCl to pH 2 yielded a white precipitate, which was recrystallized from MeOH to give 4.5 g (5.75 mmol, 57.5%) of off-white powder: ^{1}H NMR (250 MHz, DMSO-d_6) δ 1.1–1.16 (m, 18H, CH_2), 1.95 (s, 3H, CH_3CO), 2.2 (t, 4H, CH_2), 2.5 (t, 4H, CH_2), 2.7 (d, 3H, $NHCH_3$), 2.9 (m, 4H, CH_2), 3.2 (m, 2H, CH_2), 3.4 (t, 6H, $NOHCH_2$), 3.8 (d, 6H, OCH_3), 7.2 (q, 2H, arom H), 7.7 (br t, 2H, amide), 8.4 (m, 2H, amide) , 9.6 (m, 3H, NOH); +FAB-MS *m/e* 782.5 $(MH)^+$. Anal. Calcd (found) for $C_{36}H_{59}N_7O_{12}$: C, 55.30 (55.44); H, 7.60 (7.41), N, 12.53 (12.60).

DFOMTA (5). Neat BBr_3 (2 mL, ca. 20 mmol) was carefully added to **4** (1.0 g, 1.28 mmol) suspended in 20 mL of CH_2Cl_2 with vigorous stirring at room temperature. The reaction mixture was allowed to stir for 1 week. The solvent and excess BBr_3 were removed under vacuum and the residue was hydrolyzed with water (25 mL). After 36 h of stirring, an off-white solid was collected by filtration. It was dissolved in 4.0 M NH_4OH solution and filtered to remove the insoluble impurities. The filtrate was acidified with 4.0 M HCl to give a white precipitate, which was collected and washed with water and cold methanol, and then dried in a vacuum oven at 50 °C overnight to give 0.56 g (0.74 mmol, 58%) of product: ^{1}H NMR (250 MHz, DMSO-d_6): δ 1.1–1.6 (m, 18H, CH_2), 1.96 (s, 3H, CH_3CO), 2.2 (t, 4H, CH_2), 2.5 (t, 4H, CH_2), 2.8 (d, 3H, $NHCH_3$), 3.0 (q, 4H, CH_2), 3.2 (t, 2H, CH_2), 3.4 (t, 6H, CH_2NOH), 7.7 (q, 2H, arom H), 8.8 (br t, 2H, amide), 9.6 (m, 3H, NOH), 12.8 (br s, 2H, phenol); +FAB-MS *m/e* 754.5 $(MH)^+$, 776 $(MNa)^+$. Anal. Calcd (found) for $C_{34}H_{55}N_7O_{12}\cdot H_2O$: C, 52.90 (53.13); H, 7.43 (7.14); N, 12.68 (12.60).

Table 1. Protonation and Stability Constants with Fe^{3+} of DFO and Its Derivatives

	DFOMTA	DFOCAM	DFOCAMC	DFOHOPO	DFO[a]
log K_1	11.1 (1)	12.1[b]	13.0[b]	9.71 (3)	9.70
log K_2	9.71 (3)	9.74 (3)	9.83 (3)	9.18 (3)	9.03
log K_3	9.24 (3)	9.21 (3)	9.31 (2)	8.50 (3)	8.39
log K_4	8.85 (3)	8.59 (3)	8.78 (2)	4.92 (3)	
log K_5	6.22 (3)	7.22 (3)	7.83 (3)		
log K_6			3.24 (4)		
log K_{FeLH}	9.21 (6)	9.47 (9)	9.71 (6)	7.3 (2)	
			9.5[d]	7.2[c]	
log K_{FeLH_2}	4.0 (2)	5.56 (9)	7.71 (2)		
	3.8[c]	5.8[c]	7.7[c]		
log K_{FeLH_3}			2.94 (9)		
log β_{110}	34.8 (4)	35.4 (4)	34.9 (2)	30.7 (3)	30.6
pM[e]	28.5	28.2	26.7	26.7	26.6

[a] Reference 35. [b] Estimate. [c] Determined by spectrophotometric titrations. [d] Determined by proton NMR titrations. [e] pM = –log $[Fe^{3+}]$ at pH 7.4 with total ligand concentration = 10^{-5} M and total iron concentration = 10^{-6} M.

Results and Discussion

Ligand Protonation Constants and UV/vis Spectra. The ligands DFOMTA, DFOCAM, DFOCAMC, and DFOHOPO are, respectively, pentaprotic (H_5L), pentaprotic (H_5L), hexaprotic (H_6L), and tetraprotic (H_4L) acids. Their protonation constants were determined by potentiometric titrations, and the results are tablulated as log K_n values in Table 1, where

$$H_{n-1}L + H^+ \rightleftharpoons H_nL \qquad K_n = \frac{[H_nL]}{[H^+][H_{n-1}L]} \qquad (1)$$

For each of the ligands the protonation constants were assigned by comparing them with the known protonation constants of DFO and the appropriate catecholate or hydroxypyridinonate groups. For example, the constants $K_2 - K_4$ of DFOMTA were assigned to the DFO moiety, while the constants K_1 and K_5 were assigned to the catecholamide binding group. $K_2 - K_4$ are almost identical to the protonation constants of DFO,[27] and K_1 and K_5 are near the protonation constants of 2,3-dihydroxyterephthalamides.[28] Similar assignments were made for DFOCAM and DFOCAMC. The latter has an extra protonation constant, K_6, which is similar to that of 1,2-dihydroxybenzoic acid[29] and was assigned to the ligand carboxylic acid. The ligand DFOHOPO has four protonation constants, of which K_1–K_3 are the protonation constants of the DFO moiety, while K_4 is associated with the protonation of the hydroxypyridinonate group (it is similar to the protonation constant of *N*,*N*-dimethyl-1-hydroxy-2(1*H*)-pyridinone-6-carboxamide[30]).

To confirm the assignment of the protonation constants the UV/vis spectra of these ligands, whose major absorption bands

(26) Weitl, F. L.; Raymond, K. N.; Durbin, P. W. *J. Med. Chem.* **1981**, *24*, 203.
(27) Borgias, B.; Hugi, A. D.; Raymond, K. N. *Inorg. Chem.* **1989**, *28*, 3538.
(28) Garrett, T. M.; Miller, P. W.; Raymond, K. N. *Inorg. Chem.* **1989**, *28*, 128.
(29) Avdeef, A.; Sofen, S. R.; Bregante, T. L.; Raymond, K. N. *J. Am. Chem. Soc.* **1978**, *100*, 5362.
(30) Scarrow, R. C.; Riley, P. E.; Abu-Dari, K.; White, D. L.; Raymond, K. N. *Inorg. Chem.* **1985**, *24*, 954.

Table 2. Characteristic UV/vis Spectra of the Four DFO Derivatives and Their Ferric Complexes[a]

compound	condition	DFOMTA	DFOCAM	DFOCAMC	DFOHOPO
ligand	doubly protonated	336 (2.7)	309 (2.8)	337 (2.8)	306 (6.3)
	singly protonated	358 (4.0)	330 (4.6)	360 (3.7)	345 (4.6)
ferric ion	low pH region	336 (4.8)	310 (4.7)	332 (4.9)	303 (4.6)
complex		430 (3.4)	428 (3.5)	430 (2.7)	418 (3.1)
	high pH region	361 (6.5)	328 (7.2)	344 (4.9)	348 (5.7)
		424 (4.3)	389 (3.4)	416 (2.9)	426 (2.8)
		516 (3.1)	510 (3.4)	522 (2.1)	

[a] Wavelength at the absorption maximum, nm (molar extinction, 10^3 M^{-1} cm^{-1}).

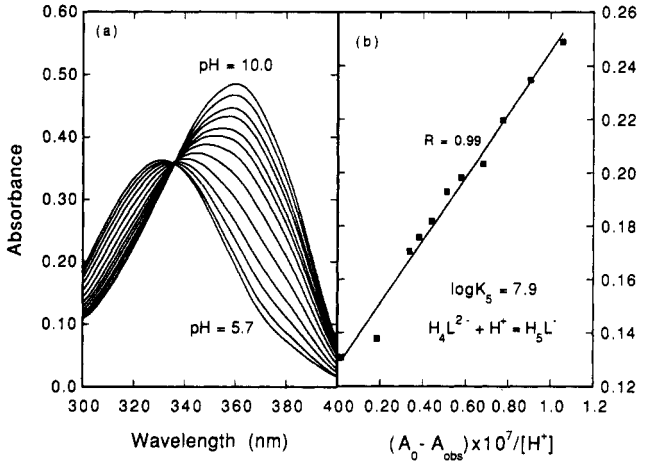


Figure 2. (a) UV spectra of DFOCAMC as a function of pH, as generated by spectrophotometric titration ([DFOCAMC] = 0.146 mM; [KCl] = 0.100 M; 25.0 °C). (b) Schwarzenbach plot for DFOCAMC from pH 6.0–7.0, where A_0 is the average absorbance between 358 and 362 nm at pH 10.0, and A_{obs} is the average apparent absorbance at wavelength 358–362 nm.

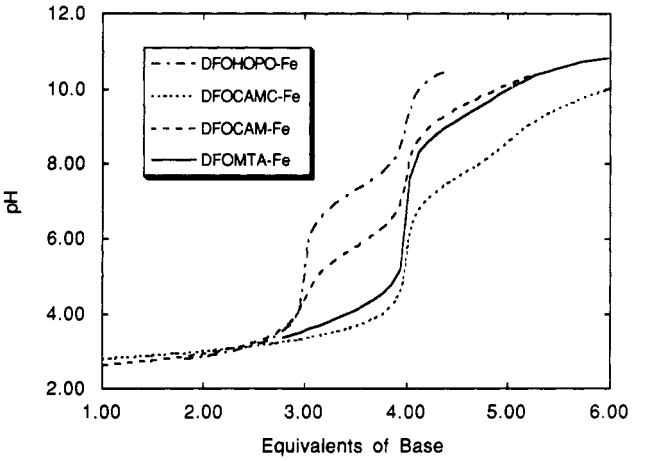


Figure 3. Potentiometric titration curves for 1:1 solutions of ferric ion and ligands. All solutions were at 25.0 °C, μ = 0.100 M (KCl): [DFOMTA-Fe] = 1.12 mM, [DFOCAM-Fe] = 1.08 mM, [DFOCAMC-Fe] = 1.24 mM, and [DFOHOPO-Fe] = 1.40 mM.

are due to the attached functional groups, were measured and analyzed by the method of Schwarzenbach.[24] For example, the family of spectra of DFOCAMC shown in Figure 2 was measured over the pH range 5.7 to 10.0. As the pH decreases from 10.0, the transition at 360 nm (ϵ = 3700 M^{-1} cm^{-1}) shifts to higher energy at 337 nm (ϵ = 2800 M^{-1} cm^{-1}), forming a sharp isosbestic point at 335 nm. This spectral change is ascribed to the protonation of the more acidic phenolic oxygen. A Schwarzenbach plot of the data between pH 6 and 7 yielded a log K of 7.9 (Figure 2), which is almost identical to log K_5 determined by potentiometric titration, confirming the assignment of K_5 as the protonation constant of the more acidic phenolic oxygen. Similar results were obtained for DFOCAM, DFOCAMC, and DFOHOPO; they are tabulated in Tables 1 and 2.

Equilibria of Ferric Complexes. DFOMTA, DFOCAM, DFOCAMC, and DFOHOPO are all octadentate ligands incorporating four bidentate binding groups. Since the high-spin Fe^{3+} is six-coordinate, there is a competition between the functional groups for coordination. To probe the coordination chemistry of these DFO derivatives, potentiometric, spectrophotometric, and ^1H NMR titrations were carried out.

Potentiometric Titrations of Ferric Complexes. Potentiometric titrations of the ferric complexes were performed for 1:1 solutions of ferric ion and ligand from pH 2 to 11. Figure 3 shows the potentiometric titration curves of the four ferric complexes. Based on these data, the reaction equilibria and respective protonation constants were calculated; they are listed in Table 1 with the following definition:

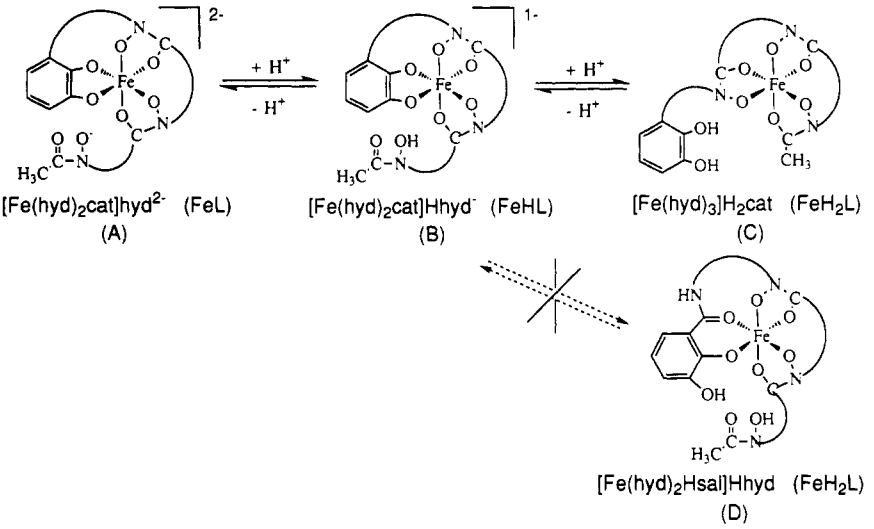
$$FeLH_{n-1} + H^+ \rightleftharpoons FeLH_n \qquad K_{FeLHn} = \frac{[FeLH_n]}{[H^+][FeLH_{n-1}]} \tag{2}$$

Among the three catecholate derivatives DFOMTA and DFOCAM have two one-proton association steps, which link three diferent species, FeL, FeLH, and $FeLH_2$, from high pH to low pH as shown in Table 1. The ligand DFOCAMC behaves similarly to DFOMTA and DFOCAM, but has one more protonation step, which is due to the protonation of the phenol carboxylic group. Since the catecholate group is a stronger ligand than hydroxamate above neutral pH, the high pH species (FeL) is assigned as a monocatecholate bishydroxamate ferric complex with an unbound hydroxamate group ([Fe(hyd)$_2$cat]hyd) (A in Scheme 2). This assignment is in agreement with the fact that the first protonation constants of the three ferric complexes are consistently close to the protonation constants of the hydroxamate groups in the free ligands, implying that one of the three hydroxamate binding groups does not bind the ferric ion. Thus the second species (FeLH) is assigned as a monocatecholate bishydroxamate ferric complex with an uncoordinated, protonated hydroxamate group ([Fe(hyd)$_2$cat]Hhyd) (B in Scheme 2). Below pH 4 the titration curves (Figure 3) show that three protons are released upon ligand binding to ferric ion (for DFOCAMC, an additional proton is released from the carboxylic acid group), suggesting that all three catecholate derivatives form six-coordinate ferric complexes. From the stoichiometry this hexa-coordinate species ($FeLH_2$) could be either a trishydroxamate ferric complex (Fe(hyd)$_3$H$_2$cat, C in Scheme 2) or a bishydroxamate monosalicylate ferric complex ([Fe(hyd)$_2$Hsal]Hhyd, D in Scheme 2). The spectra indicate that species D does not form (*vide infra*), as denoted by the crossing out mark (X) in the scheme.

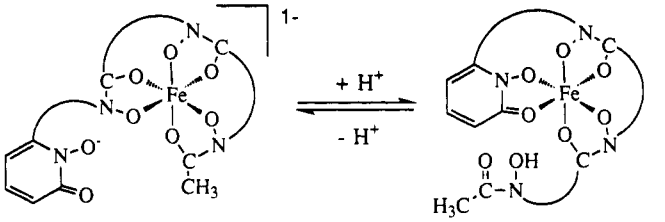
The hydroxypyridinonate derivative, DFOHOPO, has only one protonation equilibrium over the pH range 2–10. Since the hydroxypyridinonate group is more acidic than the hydroxamate group, the equilibrium is assigned (Scheme 3) as a trishydroxamate ferric complex (FeL or [Fe(hyd)$_3$(hopo)]), above neutral pH that converts to a bishydroxamate monohydroxypyridinonate ferric complex (FeLH or [Fe(hyd)$_2$hopo)Hhyd)]) at low pH.

Spectrophotometric Titrations of Ferric Complexes. To test the equilibria of the ferric complexes obtained from the potentiometric titrations, spectrophotometric titrations of the complexes

Scheme 2



Scheme 3



were carried out over the pH range 2–10. The families of spectra for the ferric complexes of DFOMTA, DFOCAM, and DFOHOPO are shown in Figures 4 (The spectra of the ferric DFOCAMC are very similar to that of the ferric DFOCAM.). The four ferric complexes exhibit two common features, which are in agreement with the equilibria shown in Schemes 2 and 3. First, the visible spectral shifts occur at a fairly high pH, where no dissociation due to the competition of protons is possible for the ferric complexes of the DFO derivatives. The spectral change is therefore a direct result of the change in the coordination sphere around the metal center. Second, all spectra show isosbestic points (In the DFOMTA-Fe system, isosbestic points are observed in the first-derivative plot of the spectra (Figure 5).). Analyzing the spectra over appropriate pH ranges by Schwarzenbach plots gives the equilibrium constants for the binding group exchange equilibria (Figure 4), which are almost identical to those independently determined by potentiometric titrations (Table 1).

For the three catecholate derivatives, at low pH both C and D (Scheme 2) conform to the observed stoichiometry. The ferric complex of DFOMTA shows two UV/vis spectral bands at 430 nm (ϵ = 3400 M^{-1} cm^{-1}) and 336 nm (ϵ = 4800 M^{-1} cm^{-1}). The lower energy band at 430 nm is the typical absorbance of a trishydroxamate ferric complex,[31] while the other is at exactly the same position as the transition of the fully protonated catecholate group of the free DFOMTA (Table 2). This result shows that the ferric ion is coordinated by three hydroxamate binding groups at low pH, while the catecholate group is unbound and fully protonated. Similar results were found for the other two catecholate derivatives, DFOCAM and DFOCAMC. It is clear that, for the three catecholate derivatives, the dominant species is the trishydroxamate complex at low pH (C in Scheme 2). As the pH is increased, an intramolecular substitution occurs, in which the bound hydroxamate group is replaced by the free catecholate group. As the pH is increased further, a second proton is released from the unbound hydroxamate group, which does not change the coordination environment of the ferric ion (Scheme 2). Thus no spectral change is involved in this equilibrium, which explains the observed isosbestic points in the spectrophotometric titrations.

The spectrophotometric titration of the ferric DFOHOPO complex also confirms the equilibrium derived from the potentiometric titrations (Scheme 3). In contrast to the catecholate derivatives, the trishydroxamate complex forms at high pH and exhibits two absorbance bands; the one at 426 nm is assigned to the ligand-to-metal-charge-transfer transition of the trishydroxamate ferric complex, while the one at 348 nm is the transition of the unbound and deprotonated hydroxypyridinone group (Table 2). As the pH decreases, a bishydroxamate monohydroxypyridinonate ferric complex [Fe(hyd)$_2$hopo] forms with a UV/vis spectrum characteristic of this coordination (Figure 4). These transformations (observed for both DFOHOPO and the catecholate derivatives) parallel the releative acidity of the ligand functional groups: hydroxypyridinonate > hydroxamate > catecholate.

Proton NMR Titrations of DFOCAMC and Its Ga^{3+} Complex. From the potentiometric and spectrophotometric titrations, the pH-dependent coordination modes of the four ferric complexes can be drawn as in Schemes 2 and 3. One question that remains is which one of the three hydroxamate groups is replaced by a catecholate or hydroxypyridinonate binding group in the species which have mixed coordination environments ([Fe(hyd)$_2$cat] (A and B in Scheme 2) and [Fe(hyd)$_2$hopo] (B in Scheme 3)). To address this a ^{1}H NMR titration of DFOCAMC was undertaken because of its relatively high solubility below neutral pD. Ferric ion was replaced with Ga^{3+} in the metal DFOCAMC complex because of their similar chemistry[32,33] and the diamagnetism of Ga^{3+}. The signal of the terminal methyl group of DFOCAMC is a singlet, and its chemical shift depends on the coordination and protonation of the terminal hydroxamate group. Thus this group was used to monitor whether or not the terminal hydroxamate group was coordinated to the metal.

In considering the coordination of the three hydroxamate groups in the species [Fe(hyd)$_2$cat], there are three possible structural isomers (Scheme 4). The species [Fe(hyd)$_2$cat] may be one of the three isomers or a mixture of them. The results of NMR pD titration of DFOCAMC and its Ga^{3+} complex in D_2O are presented in Figure 6. At high pD there is only one signal (peak b in Figure 6b) with a chemical shift at 2.01 ppm, which is almost identical to that of the free ligand's methyl protons. As the pD decreases this signal shifts downfield to 2.11 ppm in a manner similar to that of the free ligand (Figure 6a) and shows a gradual

(31) Konetschny-Rapp, S.; Jung, G.; Raymond, K. N.; Meiwes, J.; Zähner, H. *J. Am. Chem. Soc.* **1992**, *114*, 2224.

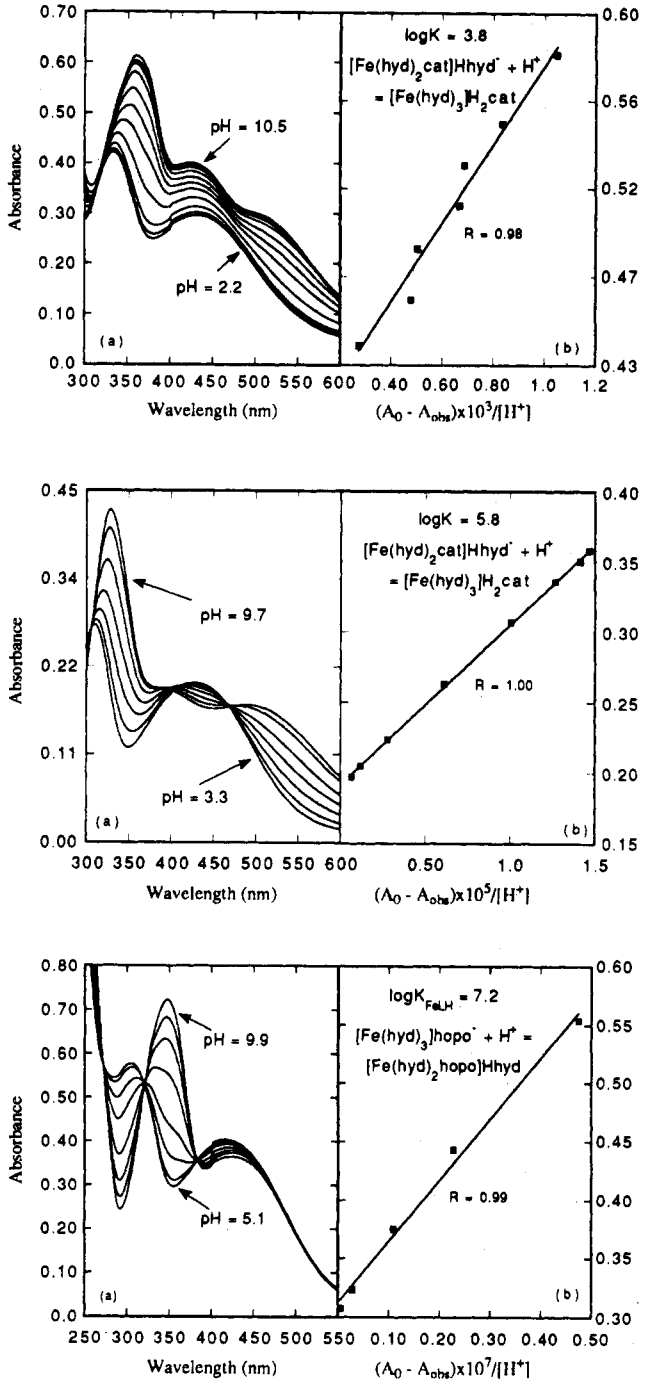


Figure 4. (a) UV/vis spectra of 1:1 solutions of ferric ion and ligand as a function of pH, μ = 0.100 M (KCl), T = 25.0 °C: [DFOMTA-Fe] = 0.0987 mM, [DFOCAM-Fe] = 0.0592 mM, and [DFOHOPO-Fe] = 0.127 mM. (b) Schwarzenbach plots as generated for DFOMTA, DFOCAM, and DFOHOPO within the respective pH ranges, 3.0–4.5, 4.4–6.3 and 5.0–7.5, where A_0 is the average absorbance between 359 and 362 nm at pH 10.5 for DFOMTA, 328 and 330 nm at pH 9.7 for DFOCAM, 343 and 347 nm at pH 9.9 for DFOHOPO, and A_{obs} is the average apparent absorbance at the same respective wavelength ranges.

decrease in intensity. These results clearly indicate that the species [Fe(hyd)$_2$cat] is isomer A, in which the terminal hydroxamate group is not bound and thus has a chemical shift similar to that of free DFOCAMC. At pD 9 another signal (peak a in Figure 6b) appears at 2.14 ppm and its intensity increases as the pD decreases. This new species is assigned to a trishydroxamate ferric complex, based on the species distribution of the DFOCAMC ferric complex generated from the potentiometric titration (Figure 7). As the pD decreases from 9 the concentration of the trishydroxamate complex starts to increase and the concentration of isomer A decreases.

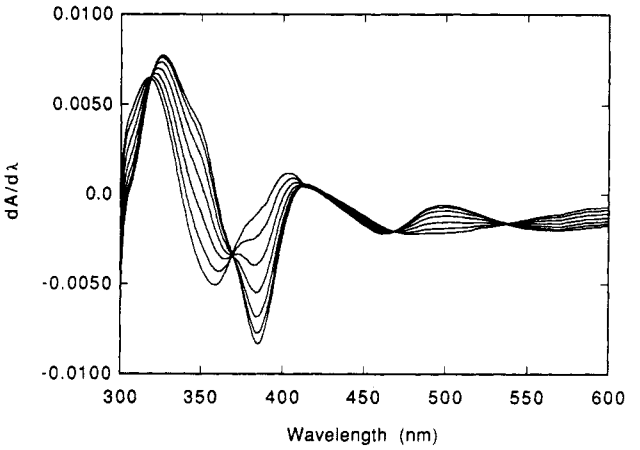


Figure 5. First-derivative plot, $dA/d\lambda$ vs λ, of the UV/vis spectra of a 1:1 ferric ion and DFOMTA solution from pH 3.0 to 4.5. Conditions are given in Figure 4a.

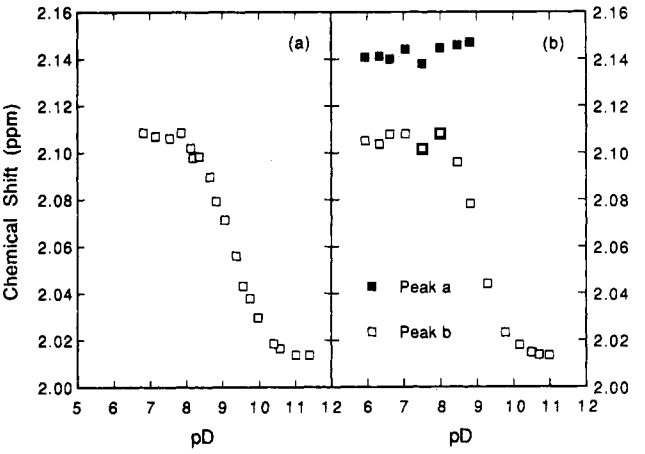
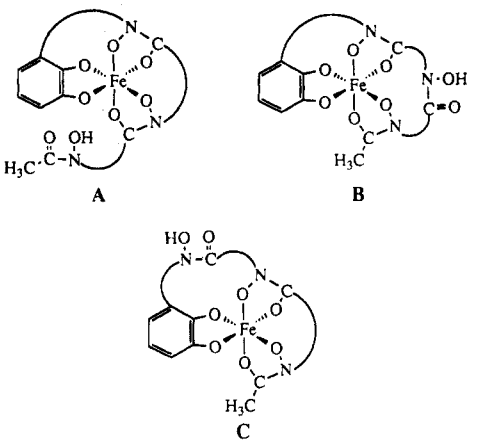


Figure 6. Proton NMR titrations of ca. 0.010 M of DFOCAMC and DFOCAMC-Ga in D_2O at 25.0 °C: (a) DFOCAMC and (b) DFOCAMC-Ga.

Scheme 4



Ferric Ion Binding Stability of the DFO Derivatives. The conventional metal–ligand binding constant is defined by the following equilibrium:

$$Fe^{3+} + L^{n-} \rightleftharpoons FeL^{(n-3)-} \qquad \beta_{110} = \frac{[FeL^{(n-3)-}]}{[Fe^{3+}][L^{n-}]} \quad (3)$$

Spectrophotometric titrations have demonstrated that the ferric complexes of the DFO derivatives remain six-coordinate without significant dissociation even at pH 2. Thus the stability constants of the ferric complexes of the four DFO derivatives were determined by spectrophotometric competition experiments.

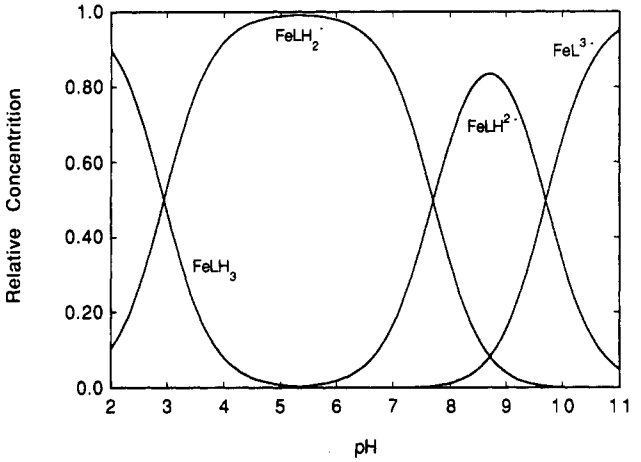


Figure 7. Species distribution plot of the ferric DFOCAMC complex ([DFOCAMC-Fe] = 0.1 mM).

The stability constants of the ferric DFOMTA, DFOCAMC, and DFOHOPO were determined by competition against EDTA over the pH range 5–6. The actual competition equilibrium can be expressed by the following equations:

$$FeY^- + LIGAND + nH^+ \rightleftharpoons FeLH_n + EDTA \quad (4)$$

$$K_{comp} = \frac{[FeLH_n][EDTA]}{[FeY^-][LIGAND][H^+]^n} = \frac{\beta_{11n}\,\alpha^{L^{m-}}}{\beta'_{110}\,\alpha^{Y^{4-}}} \quad (5)$$

where EDTA and LIGAND refer to all forms of unbound EDTA and ligand, thus

$$[EDTA] = [Y^{4-}] + [HY^{3-}] + \cdots + [H_6Y^{2+}] \quad \text{and}$$

$$[LIGAND] = [L^{m-}] + [HL^{1-m}] + \cdots + [H_mL]$$

α^{Y4-} and α^{Lm-} are the fractions of species[34] in the form of Y^{4-} and L^{m-} for EDTA and ligand. In eq 4 the FeY^- represents the ferric EDTA complex; $FeLH_n$ (n = 1 or 2) represents the protonated species of the complex, whose concentration can be calculated from the observed spectra. The concentration of the other three species in eq 5 and thus the K_{comp} were calculated by using pH and mass balance equations. With the known formation constants of ferric EDTA (β'_{110}),[35] β_{11n} and then β_{110} were calculated from the protonation constants K_{FeLH_n} of the ferric complexes. The stability constants of the ferric DFOMTA, DFOCAMC, and DFOHOPO are presented in Table 1.

Because of the low solubility of DFOCAM below neutral pH, the stability constant of its ferric complex was determined over a pH range 7.0–8.0 by competition against the stronger ligand CDTA.[35] Log β_{110} of the ferric DFOCAM was determined to be 35.4 (Table 1).

Of the four DFO derivatives, DFOCAM has the highest stability constant, and DFOHOPO has the lowest one. The stability constant of DFOHOPO is very close to that of DFO, indicating no significant intramolecular interaction among the binding groups. Since proton competition for weak acid ligands depends on the relative acidity and pH, the pM value is a more direct measure of the relative ligand complexing strength under physiological conditions.[8] Calculated pM values of the four DFO derivatives are presented in Table 1. At physiological pH DFOMTA and DFOCAM are more effective than DFO as iron chelators, while DFOCAMC and DFOHOPO have the same chelating ability as DFO. Although DFOMTA does not have the highest iron stability constant among the four derivatives, it is the most powerful chelator at pH 7.4.

The stability constants of the catecholate derivatives represent the total binding strength of two hydroxamate and one catecholate binding groups for Fe(III). They are the first examples of thermodynamically characterized iron complexes where the metal ion is coordinated by two hydroxamate and one catecholate binding groups. These species exhibit similar UV/vis spectra characteristic of this coordination (Figure 4). Although hydroxamate and catecholate groups are the two major functional groups found in siderophores, ligands incorporating both binding groups are unusual. Recently a new family of chromopeptide siderophores (pyoverdins) have attracted attention because of their fluorescence properties and antiparasitic effect on the growth of plants.[36,37] Preliminary results indicate that these compounds bind Fe^{3+} by means of two hydroxamate and one catecholate groups as $[Fe(hyd)_2cat]$.[37,38] Thus the catecholate derivatives in the present study provide good models for studying pyoverdins, and they are probably very similar in stability and UV/vis spectra.

Summary

Four potentially octadentate DFO derivatives, DFOMTA, DFOCAM, DFOCAMC, and DFOHOPO, have been studied as ferric ion complexing agents by potentiometric, spectrophotometric, and proton NMR titrations. Since the Fe(III) cation can only be six-coordinate there is a competition between the functional groups for coordination. The pH-dependent structures of the ferric complexes have been characterized as the following: (1) For the catecholate derivatives, the trishydroxamate ferric complex forms at low pH. As the pH increases, the terminal hydroxamate binding group is replaced by the catecholate group to form a bishydroxamate monocatecholate ferric complex (Scheme 2). (2) For DFOHOPO, the trishydroxamate ferric complex forms at high pH. As the pH decreases, one hydroxamate binding group is replaced by the more acidic hydroxypyridinonate binding group (Scheme 3). Stability constants of the four ferric complexes have been determined by spectrophotometric competition titrations. The stability constants of three catecholate derivatives represent the first examples of formation constants determined for bishydroxamate monocatecholate ferric complexes $[Fe(hyd)_2cat]$. With the characterization of their spectral properties, the species $[Fe(hyd)_2cat]$ provides a reference for studying a new family of siderophores, pyoverdins. The calculated pM values of the ferric complexes indicate two of the four DFO derivatives are more effective than their parent compound, DFO, as iron chelating agents at physiological pH.

Acknowledgment. We thank Dr. David L. White and the late Dr. Steven J. Rodgers for the synthesis of DFOCAM, DFOCAMC, and DFOHOPO. This research was supported by NIH Grants AI 11744 and DK 32999.

(32) Borgias, B. A.; Barclay, S. J.; Raymond, K. N. *J. Coord. Chem.* **1986**, *15*, 109.
(33) Cotton, F. A., Wilkinson, G. *Advanced Inorganic Chemistry*, 5th ed.; John Wiley & Sons: New York, 1988; pp 1288–1289.
(34) Harris, D. C. *Quantitative Chemical Analysis*, 2nd ed.; Freeman, W. H. & Company: New York, 1987; pp 257–267.
(35) Martell, A. E.; Smith, R. M. *Critical Stability Constants*; Plenum Press: New York, 1974–1981; Vol. 1–5.
(36) Deweger, L. A.; Schippers, B.; Lugtenberg, B. In *Iron Transport in Microbes, Plants and Animals*; Winkelmann, G., van der Helm, D.; Neilands, J. B.; Eds.; VCH: Weinheim, 1987.
(37) Demange, P.; Wendenbaum, S.; Bateman, A.; Dell, A.; Abdallah, M. A. In *Iron Transport in Microbes, Plants and Animals*; Winkelmann, G., van der Helm, D., Neilands, J. B., Eds.; VCH: Weinheim, 1987; pp 167–187.
(38) Demange, P.; Bateman, A.; Dell, A.; Piemont, Y.; Abdallah, M. A. *Biochemistry* **1990**, *29*, 1104.