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Metal binding by pyridine-2,6-bis(monothiocarboxylic acid), a biochelator produced by *Pseudomonas stutzeri* and *Pseudomonas putida*

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Abstract

Pyridine-2,6-bis(monothiocarboxylic acid) (pdtc), a natural metal chelator produced by *Pseudomonas stutzeri* and *Pseudomonas putida* that promotes the degradation of carbon tetrachloride, was synthesized and studied by potentiometric and spectrophotometric techniques. The first two stepwise protonation constants (pK) for successive proton addition to pdtc were found to be 5.48 and 2.58. The third stepwise protonation constant was estimated to be 1.3. The stability (affinity) constants for iron(III), nickel(II), and cobalt(III) were determined by potentiometric or spectrophotometric titration. The results show that pdtc has strong affinity for Fe(III) and comparable affinities for various other metals. The stability constants (log K) are 33.93 for $Co(pdtc)_2^{1-}$; 33.36 for $Fe(pdtc)_2^{1-}$; and 33.28 for $Ni(pdtc)_2^{2-}$. These protonation constants and high affinity constants show that over a physiological pH range the ferric pdtc complex has one of the highest effective stability constants for iron binding among known bacterial chelators.

Abbreviations: pdtc – pyridine-2,6-bis(monothiocarboxylic acid), K – metal-pdtc stability constant, K_{eff} – effective metal-ligand stability constant, CCl_4 – carbon tetrachloride

Introduction

The discovery of pyridine-2,6-bis(monothiocarboxylic acid) (pdtc) as a metabolic product of the bacterium *Pseudomonas putida* (Ockels et al. 1978) has led to much speculation about its physiological roles (Budzikiewicz 1993). Pdtc has been observed to form stable complexes with several metals (Hildebrand et al. 1984, Hildebrand 1988, Espinet et al. 1994). Moreover, the potential biotechnological value of pdtc increased when it was rediscovered in a CCl_4-degrading strain of Pseudomonas stutzeri (Lee et al. 1999, 2000; Lewis et al. 2001) and its copper complex was found to have the unique ability to hydrolyze carbon tetrachloride (CCl_4) to carbon dioxide and HCl. Therefore, a better characterization of the chelating properties of pdtc is important for understanding its catalytic activity toward CCl_4 and its role in bacterial physiology, for evaluating its potential for bioremediation of environments contaminated by chlorinated solvents, and in assessing its value in areas of clinical and commercial use.

Bacteria, in order to survive, must acquire iron and other metal micro-nutrients, including at least Cr, Co, Cu, Mn, Mo, Ni, Se, W, V, and Zn. Iron can be a limiting nutrient for many bacteria, due to its low solubility in aquatic environments (Neilands 1995). For this reason, many bacteria excrete low molecular weight biochelators, known as siderophores, to bind iron from the environment and transport it into the cell. The discovery that pdtc was excreted by *Pseudomonas stutzeri* strain KC under iron-stress conditions indicated that it might be a siderophore (Hildebrand et al. 1984). However, our recent studies

have found that the pdtc-iron complex is not readily taken into the pseudomonad's cells (Cortese et al. 2001). Therefore, increased knowledge of the relative metal-binding strengths of pdtc as compared to known siderophores, and its specificity toward iron as compared to other metals, is needed in order to define the natural physiological role of pdtc.

The usefulness of chelators in bioremediation efforts often depends on their ability to bind hazardous metal ions. Since most known microbial chelators have a high specificity toward iron, their value as bioremediation agents is limited due to the relatively high availability of iron compounds in many natural settings, which allows iron to outcompete other metals for binding to the chelator.

Pdtc is a unique chelator that has the exceptional property of promoting the degradation of CCl_4. The presence of metals in pdtc solutions has been shown to affect its ability to degrade CCl_4 (Lewis & Crawford 1995). CCl_4 degradation was shown to increase in the presence of copper, while cobalt, iron, and nickel inhibited the reaction (Lewis et al. 2001). A determination of the binding strengths of pdtc to these and other metals should help to explain these changes in reactivity.

Knowledge of the binding constants of pdtc to various metals may also have clinical and commercial implications. The success of a bacterial infection can depend on the ability of a microorganism to compete with a host for iron. Nutritional immunity is a medical approach that entails the use of chelators to compete with pathogenic bacteria for essential nutrients (Kochen 1977); pdtc might be useful in such a role. Also, chelators are often used for the removal or mobilization of toxic metal ions from the tissues of mammals (Bergeron et al. 1999). Pdtc could perhaps be used in this type of clinical application. Finally, in agricultural settings, many plants can suffer damage from the low availability of iron. Iron chelators, and perhaps pdtc, can be used to increase the availability of iron to plants and alleviate such conditions.

Pdtc can be produced not only biologically, but also synthetically through a relatively simple method (Hildebrand et al. 1983), allowing experiments to be performed with very pure preparations of the molecule and its metal complexes. However, no previous research has yielded quantitative data on the stability of these various pdtc-metal complexes. Hence, we focused our experiments on potentiometric and spectrophotometric studies of pdtc with several metals. Our goal was to determine stability constants and relative binding strengths for pdtc and several of the physiologically important metals that it binds.

Materials and methods

All the commercial reagents were of the highest purity available and were used without further purification. Inductively Coupled Plasma Emission (ICP) standard metal stock solutions (1 g l^{-1} per metal ion) of Cu(II), Fe(III), Ni(II), Zn(II), and Mn(II) were obtained from Fisher Scientific (Pittsburgh, PA). These standards contained 2% HNO_3 to keep the metals in solution. A Cr(III) stock solution was made with $Cr_2(SO_4)_3$ suspended in 2% HNO_3. Pdtc was synthesized using the method of Hildebrand et al. (1983). Pdtc stock solutions were prepared by weighing out 0.100 g of pdtc and diluting it volumetrically to 10 ml with dimethylformamide (DMF) (Aldrich Milwaukee, WI). Standardized 1N NaOH and HCl solutions (certified 1.005–0.995 N) were obtained from Fisher Scientific. These solutions degassed in argon were used for titrations and pH adjustments. The spectrophotometric competition studies were carried out with stock solutions of disodium ethylenediaminetetraacetate dihydrate (EDTA) (BioRad, Hercules CA), 2,6-pyridinedicarboxylic acid (DPA), $Cr_2(SO_4)_3$, and K_3FeCN_6 were obtained from (Aldrich Milwaukee, WI).

General Instruments. Absorption spectra were recorded on a Hewlett-Packard 8453 UV/Visible diode array spectrophotometer control by a HP Pentium-class computer. A Fisher Scientific Accument pH meter equipped with an Accument pH/ATC combination silver/silver chloride reference electrode was used for pH measurements. Volume-dependent titrations employed a 665 Dosimat Metrohm volume dispenser (Brinkman Instruments Inc. Westbury NY).

Potentiometric Titrations. All solutions were prepared with deionized distilled water of better than 18 megaohm-centimeters resistivity. All measurements were made at 25 °C. The ionic strength in the titration experiments was fixed at 0.1 M with $NaClO_4$ (Fisher Scientific). The electrode was calibrated to read pH using hydrogen ion activity standards solutions: 4, 7, and 10 (all $\pm$ 0.02 pH 25 °C). Potentiometric titration employed the use of the automatic dispenser and the pH meter. Argon-saturated solutions were titrated with 0.1 N NaOH. All samples were 20 ml in total

volume unless otherwise stated. Samples contained less then 0.3% volume DMF. Samples were allowed to equilibrate for at least 5 minutes and pH remained constant. The titrated sample was continually purged with argon to minimize interference of air-derived CO_2. Temperature was maintained at 25 °C.

Spectrophotometric Titrations. UV-visible absorption spectra were recorded using a 1.0 cm path length quartz cell. Spectra were analyzed on a Hewlett Packard computer running UV-visible Chemstation software (revision 52). Concentrations of metal ions and pdtc examined ranged from 10^{-4} to 10^{-5} M. All samples were 20 ml unless otherwise stated. Samples were allowed to equilibrate at least 20 minutes or until spectra remained constant. Preliminary experiments were done to ensure that equilibrium was approached within 20 minutes by monitoring the samples for several days. Equilibrium times were minimized in order to avoid acid or base catalyzed hydrolysis of pdtc.

Mass Spectrometry. A 0.2 mM sample solution was analyzed by negative or positive electrospray-ionization mass spectrometry (ES/MS) (Quattro II, Micromass Ltd., U.K.). Samples were delivered into the source at a flow rate of 5 μl/min using a syringe pump (Harvard Apparatus, South Natick, MA). A potential of 2.5–3 kV was applied to the electrospray needle. The sample cone voltage was maintained at 12 V. The counterelectrode, skimmer, and RF lens potentials were tuned to maximize the ion beam for the given solvent. Detector resolution was set at 15,000, and source temperature was kept constant at 80 °C. The instrument was calibrated using a poly(ethylene glycol) solution. All spectra were an average of 10–15 scans. The structures of complexes were confirmed using daughters' fragmentation (MS/MS). The hexapole collision cell was filled with argon gas.

Results

Ligand Protonation Constants. Pdtc has three protonation sites, one on the pyridine nitrogen and two on carbonyl sulfur atoms (Figure 1). They are denoted LH_1, LH_2, and LH_3, respectively. Precipitation will occur as the concentration of pdtc increases and pH approaches 2. DMF was used earlier to investigate redox properties of pdtc-nickel complexes (Kruger & Holm 1999). We found also that DMF or water:DMF (1:1) is an acceptable solvent for pdtc and its metal complexes.

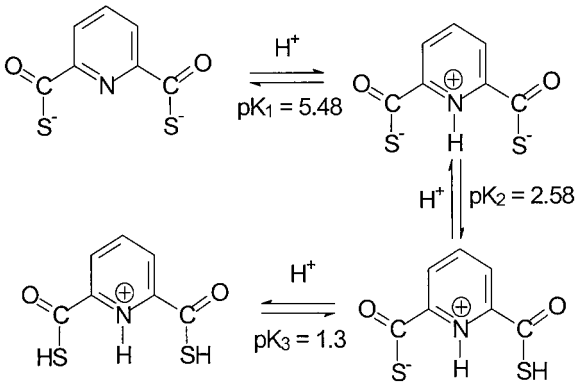

Figure 1. Stepwise protonation of pdtc. This amphoteric biochelator has three distinctive pKas. Depending on pH, the net charge of pdtc can change from negative to positive under strong acidic conditions. Protonation constants were determined by potentiometric titration as described in the text.

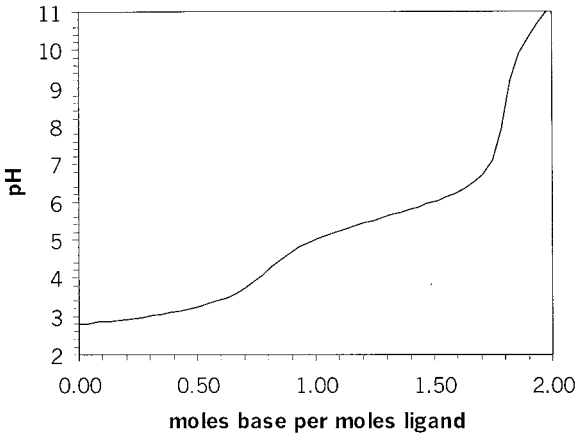

Figure 2. Potentiometric titration curve for pdtc titrated with a 0.1 N NaOH solution. [pdtc] = 0.05 M; T = 25 °C and I = 0.1 M ($NaClO_4$).

The protonation constants for pdtc were determined by potentiometric titration (Figure 2). As indicated by the titration, the pK_1 is 5.48 and pK_2 is 2.58, where:

$$K_n = \frac{LH_n}{[H^+][LH_{n-1}]}$$

These constants were obtained using the computer program BigBest, which gave a standard deviation of 0.055 (Martell & Motekaitis 1992). The third protonation constant pK_3 is estimated to be 1.3. Figure 2 shows the stepwise protonation of pdtc.

The results of potentiometric titration were confirmed by recording UV-vis absorption spectra of the pdtc solution at different pH values. Changes in absorption spectra during titration of pdtc by NaOH are presented in Figure 3. A color change near the first protonation constant (protonation of the nitrogen on

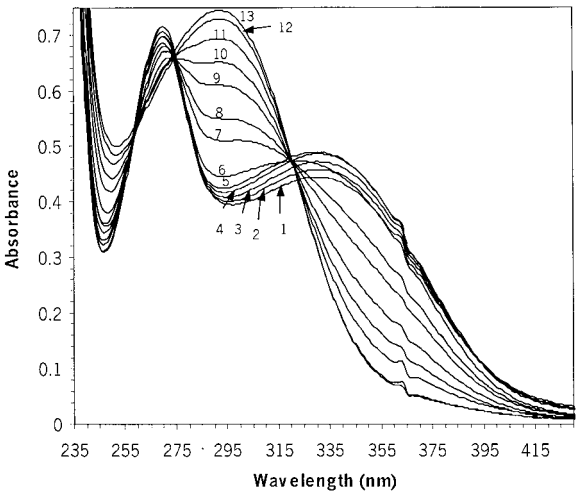

Figure 3. Spectral changes during titration of pdtc by NaOH. I = 0.1 N $NaClO_4$; T = 25.0 °C; l = 1.0 cm; [pdtc] = 0.275 mM, pH = (1) 1.00; (2) 1.21; (3) 1.51; (4) 2.08; (5) 2.98; (6) 4.11; (7) 4.75; (8) 5.00; (9) 5.35; (10) 5.63; (11) 5.96; (12) 6.82; (13) 12.5.

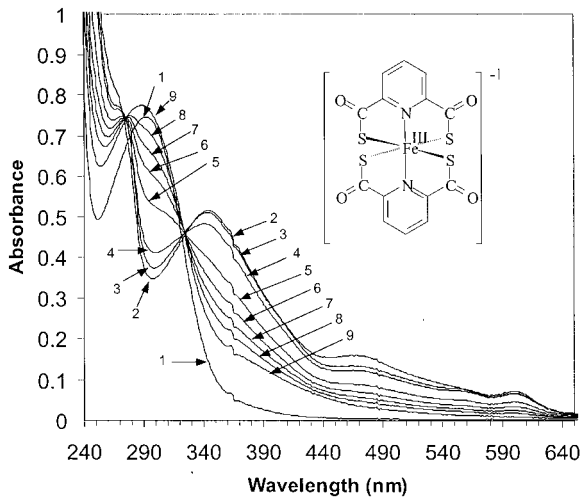

Figure 4. Spectral changes during titration of $Fe(pdtc)_2^{1-}$ by 1N NaOH. I = fixed with 0.1 N $NaClO_4$; T = 25.0 °C; l = 1.0 cm; [pdtc] = 0.275 mM; (1) Free pdtc at pH from 9 to 12; $Fe(pdtc)_2$ at pH = (2) 1.00 to 9.00; (3) 10.06; (4) 11.12; (5) 11.39; (6) 11.48; (7) 11.62; (8) 11.72; (9) 12.05.

the pyridine ring of pdtc) was observed. Pdtc absorption spectra from 230 to 430 nm were strongly affected as the solution pH changed from acidic to basic.

Ferric Complexes. The first analysis of the stability constant for the pdtc ferric complex was done using spectrophotometric ligand-ligand competition methods. Solutions of EDTA, dipicolinic acid (DPA), and K_3FeCN_6 were prepared for competitive studies. The addition of EDTA and DPA to solutions containing the pdtc ferric complex did not affect the spectra produced by the pdtc ferric complex. The addition of pdtc to K_3FeCN_6 yielded the spectra for free pdtc. The results of these experiments indicated that the complex had a stability constant greater than the stability constant of 10^{16} for DPA (Faucherre et al. 1966), yet a weaker stability constant than the overall stability constant of 10^{52} for cyanide (Martell & Smith 1974). The comparison of monodentate cyanide ligand to tridentate DPA and pdtc is less meaningful, but another tridentate chelator able to compete with pdtc for Fe(III) was not available.

A titration curve of the ferric pdtc complex yielded a reversible spectral change over the pH range 10 to 12 (Figure 4). With no other competing ligands present, hydroxide is the competing ligand causing a spectral change from the $Fe(pdtc)_2^{1-}$ spectrum to the spectrum of free $pdtc^{2-}$. Plotting the spectral change at a specific wavelength, fitting a curve, taking the derivative of this curve twice, and setting it equal to zero yields

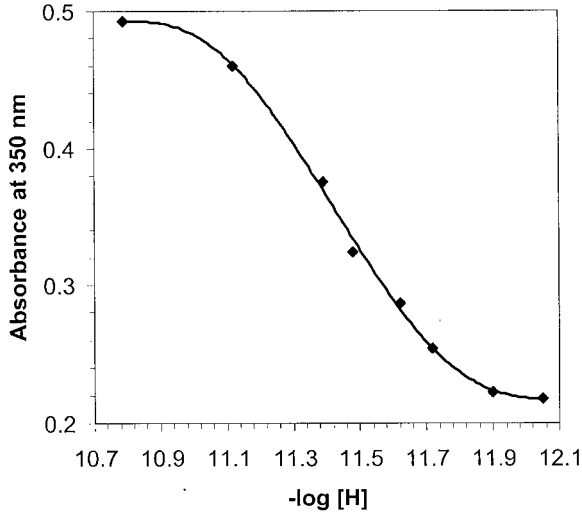

Figure 5. Graphical determination of inflection point of Fe(pdtc)2 titrated by 1 N NaOH. I = 0.1 N $NaClO_4$; T = 25.0 °C; l = 1.0 cm; [pdtc] = 0.275 mM; λ = 350 nm.

the point of inflection (Figure 5), found to occur at pH = 11.43.

At this pH, $Fe^{III} + 2\ pdtc^{2-} = Fe(pdtc)_2^{1-}$ (^{1}R). Iron and hydroxides are known to form the following complexes: $Fe(OH)^{2+}$, $Fe(OH)_2^{1+}$, $Fe(OH)_3$, $Fe(OH)_4^{1-}$, $Fe(OH)_5^{2-}$. To a negligible extent, they also form $Fe_2(OH)_2^{4+}$ and $Fe_3(OH)_4^{5+}$ (^{2}R). The total amount

of metal present is known and can be defined by the following relation:

$$\begin{aligned} Fe_T^{III} &= [Fe(pdtc)_2^{1-}] + [Fe(pdtc)^{1+}] + [Fe(OH)_1^{2+}] \\ &+[Fe(OH)_2^{1+}] + [Fe(OH)_3] + [Fe(OH)_4^{1-}] \\ &+[Fe(OH)_5^{2-}] + [Fe^{III}] \end{aligned} \quad (1)$$

Electrospray MS/MS confirmed the presence of $Fe(pdtc)_2^{-1}$. The absence of a corresponding peak for $Fe(pdtc)^{+1}$ indicates that this species does not form to a detectable extent. Hence, in the subsequent calculation $Fe(pdtc)^{1+}$ was not included as a competitive species.

The formation constants for iron hydroxides have been previously determined (Singley & Black 1967; Baes & Mesmer 1967).

$$\beta_{xyOH} = \frac{[Fe_x(OH)_y^{(3-y)+}][H^+]^y}{[Fe^{3+}]^x}. \quad (2)$$

With the substitution of the above equations:

$$\begin{aligned} Fe_T^{III} &= [Fe(pdtc)_2^{1-}] + \frac{\beta_{11OH}[Fe^{III}]}{[H^+]} + \frac{\beta_{12OH}[Fe^{III}]}{[H^+]^2} \\ &+\frac{\beta_{13OH}[Fe^{III}]}{[H^+]^3} + \frac{\beta_{14OH}[Fe^{III}]}{[H^+]^4} + [Fe^{III}]; \end{aligned} \quad (3)$$

and solving the above equation for the iron concentration:

$$[Fe^{III}] = \frac{(-Fe_T^{III} + [Fe(pdtc)_2^{1-}]) \times [H^+]^4}{\beta_{11OH}[H^+]^3 + \beta_{12OH}[H^+]^2 + \beta_{13OH}[H^+] + \beta_{14OH}[H^+]^4}; \quad (4)$$

and knowing that Fe(III) forms the following complexes with pdtc (Hildebrand et al. 1984; Cortese et al. 2001):

$$K_{11Fepdtc} = \frac{[Fe(pdtc)^{1+}]}{[Fe^{III}][pdtc^{2-}]} \quad (5)$$

and

$$K_{12Fepdtc} = \frac{[Fe(pdtc)^{1-}]}{[(Fepdtc)^{1+}][pdtc^{2-}]}; \quad (6)$$

or in terms of overall stability constant:

$$\beta_{Fe^{III}pdtc} = \frac{[Fe(pdtc)_2^{1-}]}{[Fe^{III}][pdtc^{2-}]^2}; \quad (7)$$

and with $[Fe(pdtc)_2^{1-}]$, $[pdtc^{2-}]$, and $[Fe^{III}]$ known, $\beta_{Fe^{III}pdtc}$ can now be calculated. Data from Figure 4 were used to calculate concentrations of $[Fe(pdtc)_2^{1-}]$ and $[pdtc^{2-}]$. During sodium hydroxide titration, the initial concentrations of added $[Fe^{III}]$ were also known. The binding constant for the ferric pdtc complex, log $\beta_{Fe^{III}pdtc}$ was calculated to be 33.93, but given the uncertainty in reported stability constants for iron hydroxide complexes, and since these values were used directly to compute constants for Fe(III):$(pdtc)_2$, the above number should be considered as an estimate rather than an exact value

Titrations with H_2SO_4. As shown in Equation (5), the determination of β_{12} depends on the concentration of $[pdtc^{2-}]$. The concentration of pdtc is also dependent upon the first, second, and third protonation constants:

$$K_n^H = \frac{[H_n pdtc]}{[H_{n-1}pdtc][H^+]}. \quad (8)$$

Therefore, the hydrogen ion competes with any metal for pdtc. With successive additions of acid, it becomes possible to find a concentration of protons that outcompetes the metal ion. Since the protonation constants were previously determined, it becomes possible to estimate the stability constant based on the proton concentration. The free Fe(III) concentration can be defined as:

$$[Fe^{III}] = Fe_T^{III} - [Fe(pdtc)_2^{1-}]. \quad (9)$$

A mass balance on the total ligand present yields, (leaving out the species $Fe(pdtc)_2^{1+}$ for reasons discussed above):

$$\begin{aligned} T_L &= 2 \times [Fe(pdtc)_2^{1-}] + [pdtc^{2-}] + [Hpdtc^{1-}] \\ &+[H_2pdtc] + [H_3pdtc^{1+}]. \end{aligned} \quad (10)$$

Using Equations (10) and (8) to solve for $[pdtc^{2-}]$:

$$[pdtc^{2-}] = \frac{-(-T_L + 2 \times [Fe(pdtc)_2^{-1}]) \times K_1^H \times K_2^H \times K_3^H}{(K_1^H \times K_2^H \times K_3^H + [H^+] \times K_2^H \times K_3^H + [H^+]^2 \times K_3^H + [H^+]^3}. \quad (11)$$

The concentration of $[Fe(pdtc)_2^{1-}]$ was determined spectrophotometrically using a calibration curve at a wavelength of 605 nm. Now all three components of Equation (5) are known from Equations (9) and (11) and the $[Fe(pdtc)_2^{1-}]$ calibration curve. The log β_{12}

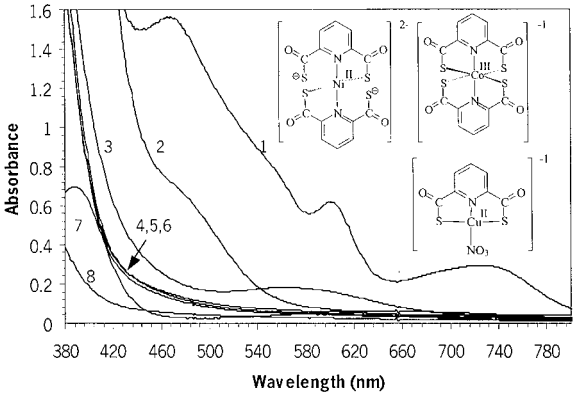

Figure 6. Visible spectra of various metal complexes and free pdtc. I = 0.1 N $NaClO_4$; T = 25.0 °C; l = 1.0 cm; [M] = 0.275 mM; and 2% HNO_3, (1) $[Fe^{III}(pdtc)_2]^{1-}$; (2) $[CoIII(pdtc)_2]^{1-}$; (3) $[NiII(pdtc)_2]^{2-}$; (4) $[MnIII(pdtc)_2]^{1-}$; (5) $[CrIII(pdtc)_2]^{1-}$; (6) $[pdtc]^{2-}$; (7) $[CuIIpdtcNO_3]^{1-}$; (8) $[ZnII(pdtc)]^{2-}$. Solutions of complexes were prepared by mixing a stechiometric amount of ICP standard of metals solutions with pdtc solution. More details about structures of metal:pdtc complexes are presented in Cortese et al. 2001.

was found to be 32.49, but this value has several limitations for use. In order to approach a point where hydrogen ion could compete with iron, a concentration of 7–8 molar H_2SO_4 had to be reached. At an ionic strength this high, and because pdtc is believed to undergo acid hydrolysis at this pH, the number has little value in the determination of a physiological stability constant. However, the number generated gives a fairly accurate estimate of the real stability constant at this extremely high ionic strength and can be considered valuable for comparing the relative strengths of pdtc with other metal compounds.

Fe(III)-Metal Competition. For a cross competition of Fe(III)/metal study, 0.5 mM of Fe(III), Co(III), Ni(III), and 0.5 mM pdtc in 2% HNO_3 were used. The spectra of other metal complexes were too similar to determine which metal complex was formed (Figure 6). For two-metal + pdtc mixtures the presence of both metal complexes was confirmed using electrospray mass spectrometry.

Metals with similar affinities for pdtc can be evaluated numerically for relative strengths. For metals with a 2:1 complex formation, the following relation can be used:

$$\beta_{\mathrm{M2pdtc}} = \beta_{\mathrm{M1pdtc}} \frac{[\mathrm{M}_1^{z_1}][\mathrm{M}_2(\mathrm{pdtc})_2^{z_2-4}]}{[\mathrm{M}_2^{z_2}][\mathrm{M}_1(\mathrm{pdtc})_2^{z_1-4}]}. \qquad (12)$$

In Equation (12) M^z represents free metal ion and $M(pdtc)_2$ represents metal:pdtc. This equation was used to determine the stability constant for the $[Co(pdtc)_2]^{1-}$ and $[Ni(pdtc)_2]^{-2}$ complexes. Since the $[Fe(pdtc)_2]^{1-}$ complex has a distinctive absorbance peak at 605 nm (Figure 6), the concentration of this species was measured with a calibration curve. A mass balance would then yield the following relation:

$$\mathrm{T}_{\mathrm{M}^z} = [\mathrm{M(pdtc)}_2^{z-4}] + [\mathrm{M}^z] \qquad (13)$$

Using Equation (12) to find each species concentration and then solving for β_{M2pdtc}, Equation (13) yields the overall stability constant. It was found that log β_{Copdtc} was 33.45 and log β_{Nipdtc} was 33.56.

Discussion

Potentiometric Titration. Results of the titration of pdtc show that pK_1 is 5.48 and that pK_2 is 2.58. The program BigBest minimizes the standard deviations of fit over the entire titration curve by variation of the protonation constants. The error associated with the fit was relatively low. The calculated standard deviation was ∓ 0.055. Error analysis of the third protonation constant was computed as 1.3. The determination of the third protonation constant for pdtc is an estimate at best, since titration methods are only valid at a pH range from 2 to 12, and the computation was out of the range of experimental data.

Spectrophotometric Titration. The challenge of measuring the overall equilibrium for pdtc was difficult, due to the nature of the proton dissociation constants and the high affinity of pdtc to the metals of interest. Competition studies for metabolic chelators often use EDTA as the competitor, even if EDTA has a lesser stability constant. This method relies on the fact that the complex has protonation sites with high affinities to the proton, and which also bind to the metal. As the pH is lowered, the apparent strength of the complex is weakened to a greater extent than is EDTA, so that at a certain pH, EDTA can outcompete the complex. If the pKa values of the ligand are low, and the stability constant is greater than that of EDTA, the ligand outcompetes EDTA at any pH. Thus, hydroxide was chosen as the competing ligand for measuring the stability constant.

Limitations on the accuracy of the determined stability constant included the fact that pdtc slowly undergoes base-catalyzed hydrolysis in a solution with

basic pH. For this reason the time allowed for equilibrium to be reached was less then 10 minutes. It was noted, however, that changes in the spectra occurred rapidly (less than 1 minute), and very little change occurred after the first minute, indicating that the amount of time for equilibrium was sufficient.

Figure 4 shows how, with the addition of hydroxide, the ferric pdtc spectra approaches the spectra of free pdtc. Our experimental data included spectra up to a pH of 12.05. At this pH, it was found that returning the pH below 9 resulted in the original spectra at that pH, showing complete reversibility. Increasing the pH beyond 12 further approached the spectra of free pdtc. However, increasing the pH beyond 12.05, and allowing the sample to equilibrate for 10 minutes resulted in irreversible spectral changes. Hence, base catalyzed hydrolysis was suspected beyond a pH of 12.05 and data did not include spectra produced at a pH greater than 12.05.

The method used was verified by measuring the stability constant for EDTA under the same method and conditions. Hydroxide was found to compete with EDTA over the pH range of 6.5 to 9. Under the same titration conditions as in the $Fe^{III}(pdtc)_2$ experiment, [EDTA] was found to equal [Fe^{III}EDTA] at a pH of 7.63. The effective metal ligand complex stability constant log K_{eff} for EDTA at this pH was calculated to be 14.9 (Perrin 1979). K_{eff} was calculated as defined by Crumbliss (1991). The experimental log K_{eff} of EDTA was found to be 15.1. The calculated error of the log $K_{eff\,FeEDTA}$ in our study was then found to be 1.5%.

The results of the titration with a copious amount of H_2SO_4 resulted in a log β_{Fepdtc} of 32.49 versus the log β_{Fepdtc} of 33.93. This 4% difference in log β_{Fepdtc} values is likely due to the different ionic strengths of the solutions used in the experiments and any possible error in the estimation of the third protonation constant.

Reported stability constants of metabolically produced chelators can be very large. For example, enterobactin has an estimated log K_{ML} of 52 (Harris et al. 1979). The overall formation constant quantifies the chelator's affinity for a metal in the absence of competing H^+ ions. The effective metal-ligand complex stability constant, K_{eff}, has been used to quantify the ligand's affinity for a metal in the competing ligand protonation reactions (Winkelmann 1991). Figure 7 shows how the chelating power of enterobactin is weakened as the pH is lowered. The K_{eff} for both Fe(III)enterobactin and Fe(III)pdtc were calculated using equations published earlier (Crumbliss 1991).

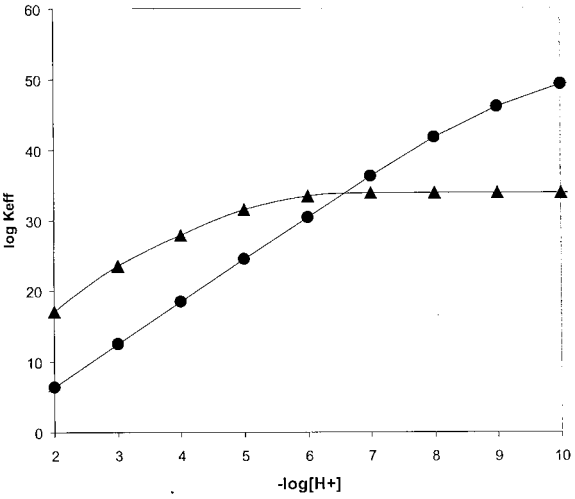

Figure 7. The effective Fe(III) binding constant of enterobactin and pdtc, K_{eff}, as a function of pH. ● enterobactin; ▲ pdtc; K_{eff} was calculated according to the CRC Handbook of Microbial Iron Chelates (Crumbliss 1991) and published constants for enterobactin (Harris et al. 1979).

Due to the low pKa values of pdtc, calculations show that pdtc will outcompete enterobactin below a pH of 6.6.

Our results thus show that at physiological pH ranges the stability constant of ferric:pdtc complex is among the highest known for a bacterial iron chelator. The stability constants for other metals such as Co(III) and Ni(II) are similarly high. These exceptional metal binding properties will undoubtedly be found to play significant roles in biological functions and environmental effects of pdtc.

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