Biodegradation **14:** 73–82, 2003.

The determination of the stability constant for the iron(II) complex of the biochelator pyridine-2,6-bis(monothiocarboxylic acid)

M. Scott Brandon[1,*], Andrzej J. Paszczynski[2,3], Roger Korus[1] & Ronald L. Crawford[2,3]
[1]*Department of Chemical Engineering, University of Idaho, Moscow, ID 83844-1021, USA (*author for correspondence: e-mail: andrzej@uidaho.edu);* [2]*Environmental Biotechnology Institute, University of Idaho, Moscow, ID 83844-1052, USA;* [3]*Department of Microbiology, Molecular Biology & Biochemistry, University of Idaho, Moscow, ID 83844-3052, USA*

Accepted 27 February 2003

Key words: metal chelation, pdtc, pyridine-2,6-bis(monothiocarboxylic acid), pyridine-2,6-dithiocarboxylic acid, stability constant

Abstract

Pyridine-2,6-bis(monothiocarboxylic acid), also known as pyridine-2,6-dithiocarboxylic acid (pdtc), is a unique and powerful metal chelator produced by *Pseudomonas stutzeri* and *Pseudomonas putida*. The actual physiological roles of pdtc in these pseudomonads are not known with certainty, though it is likely that the compound acts as a siderophore, an antibiotic, or both. The stability constant of $Fe^{III}(pdtc)_2^{2-}$ was determined in previous work to be $10^{33.36}$. Here we determined that the stability constant of $Fe^{II}(pdtc)_2^{2-}$ is 10^{12}. We determined this stability constant through potentiometric and spectrophotometric measurements of a ligand-ligand competition study using 2,6-pyridine dicarboxylic acid as the competitor for iron. Comparing the stability constant for $Fe^{II}(pdtc)_2^{2-}$ to the constant for $Fe^{III}(pdtc)_2^{2-}$ shows that the stability constant of $Fe^{II}(pdtc)_2^{2-}$ is approximately 21 orders of magnitude smaller. This represents a very significant decrease in the binding strength of pdtc toward iron. Thus, if the host cell produces pdtc as a siderophore for sequestering Fe(III), it is likely that a second metabolite or a membrane protein of the host cell is used for reduction of the chelated iron at or near the cell membrane in order to facilitate its release from pdtc for cellular use.

Abbreviations: pdtc – pyridine-2,6-bis(monothiocarboxylic acid), pyridine-2,6-dithiocarboxylic acid; dpa – pyridine-2,6-dicarboxylic acid, dipicolinic acid; ICP – inductively coupled plasma emission; K – stepwise and/or protonation constant; β – metal-ligand stability constant; K_{eff} – effective stability constant; DMF – dimethylformamide; ISFET – ion sensitive field effect transistor; ES/MS – electrospray-ionization mass spectrometry; MS/MS – tandem mass spectrometry

Introduction

Iron(III) is a micro-nutritional metal required for many metabolic pathways in living systems. Iron in the aerobic environment, however, is not readily available in solution for use by these living systems. Although iron ranks fourth in abundance of all elements on earth, the availability of iron(III) in solution is extremely low. Ferric hydroxide has a solubility product constant of 10^{-38}, resulting in a free ferric ion concentration of 10^{-18} M at a pH of 7.4 (Neilands & Nakamura 1991). Iron(II) as a free ferrous ion is much more abundant with a concentration of up to 100 mM, but often it is only available to organisms in anaerobic environments. In aerobic conditions, many bacteria have overcome this deficiency in iron(III) availability by producing low molecular weight metabolites, or siderophores, to bind iron and keep it in solution (Neilands 1995).

Pyridine-2,6-bis(monothiocarboxylic acid) or pyridine-2,6-dithiocarboxylic acid (pdtc), a unique siderophore produced by *Pseudomonas stutzeri* and *Pseudo-*

monas putida, binds to iron(III) resulting in a brown-colored solution. The addition of sodium dithionite ($Na_2S_2O_4$) causes the solution to turn blue and then back to brown when the solution is exposed to air (Ockels et al. 1978). Further studies showed that *P. putida* and related species produce pdtc under iron deficient conditions (Budzikiewicz 1993). This suggests that these pdtc-producing bacterial strains produce pdtc as a siderophore to be used as a means to obtain iron(III) from their environment for nutritional purposes.

Pdtc shows some properties consistent with an antibiotic and other properties consistent with a cellular protection agent. For example, pdtc forms soluble complexes with metals such as Au, Bi, Co, Cr, Cu, Fe, Mn, Nd, Ni, Pb, Pd, and Zn, many of which are essential nutrient metals. Insoluble pdtc complexes are formed with toxic metals such as As, Cd, Hg, Pb, Se, Sn, Te, and Tl causing these metals to precipitate out of solution (Cortese et al. 2002). The growth of pdtc-producing *P. stutzeri* strain KC was greatly enhanced at a pdtc concentration of 32 μM. In competition studies with *E. coli*, pdtc produced by *P. stutzeri* significantly suppressed *E. coli* growth, but the addition of Fe relieved pdtc inhibition, as did the addition of Co and Cu. The addition of Zn increased pdtc inhibition of *E. coli* growth, possibly due to the increased toxicity of Zn once it is complexed with pdtc (Sebat et al. 2001).

In determining the metabolic route by which pdtc is produced, it has been suggested that the production of pdtc comes from the sulfination of pyridine-2,6-dicarboxylic acid (dpa). Studies have been performed to determine the intermediates formed in the production of pdtc (Hildebrand et al. 1985, 1986; Hübner et al. 1990) leading to the idea that pdtc is a derivative of dpa. This is difficult to prove. Through the chemical synthesis of pure pdtc based on the process of Hildebrand et al. (1983), it was found that the storage of pdtc in water results in its abiotic hydrolysis to dpa (Cortese et al., 2002). Thus, metabolite isolation studies alone are insufficient to establish a biosynthetic relationship between the two molecules.

Stability constants determine with which metal(s) pdtc is most likely to bind within a particular environment and thus can help us deduce its role in bacterial metabolism. Recent work has shown that pdtc will bind to iron(III) with an extraordinary stability constant of $10^{33.36}$ (Stolworthy et al. 2002). The release of this tightly bound iron(III) from pdtc might be brought about by decreasing of the stability constant of iron-pdtc through the reduction of the metal to iron(II).

Many methods have been employed in the determination of stability constants of organic-metal complexes. Of particular value for efforts reported here is the use of methods that determine stability constants through competition for metal binding with a metal-complexing agent of known binding affinity and known protonation constants (Dimmock et al. 1995; Crumbliss 1991; Martell & Motekaitis 1992). As with any method, care must be taken in determining the limitations of the methods employed. For instance, a pH between 2 and 12 is generally the range recognized to be acceptable for stability constant determination (Martell & Motekaitis 1992). The ionic strength of the solution must also be tightly controlled since a high ionic strength can cause interruption of the metal-ligand competition reaction especially if it is higher than 0.1 to 0.5 M (Colston & Robinson 1997).

Sodium dithionite ($Na_2S_2O_4$) causes the reduction of the $Fe^{III}(pdtc)_2^{1-}$ to $Fe^{II}(pdtc)_2^{2-}$ accompanied by a change in solution color from brown to blue (Ockels et al. 1978; Hildebrand et al. 1984; Cortese et al. 2002). We used $Na_2S_2O_4$ because other readily available reductants have too low a redox potential to reduce ferric iron to ferrous in pdtc complex. The use of sodium dithionite, however, has several additional limitations that must be monitored. Sulfur dioxide, a decomposition component of sodium dithionite, is a strong reducing agent that will readily absorb O2 from the air causing further decomposition of sodium dithionite (Lambeth & Palmer 1973). High temperatures (e.g., 45 °C) increase the degradation of sodium dithionite as does low pH (Holman & Bennet 1994). Degradation significantly decreases as pH increases (Shaikh & Javaid Zaidi 1993). The half-life of a dithionite solution was found to be 2616 minutes at a pH of 12.98 (Kilroy 1980). Thus, experiments using dithionite should be conducted under anaerobic conditions and at room temperature to reduce the degradation of dithionite.

To perform ligand-ligand based competition experiments with pdtc, the competing ligand must bind in the same characteristic fashion as does pdtc as a tridentate ligand. The ligand dpa is a tridentate ligand, and its stability constant with iron(II) is $10^{10.36}$ (Anderegg 1960; Smith et al. 2001). Molar addition experiments revealed that the metal-ligand complex has the structure of a 1:2 complex of iron with dpa, $Fe^{III}(dpa)_2^{1-}$ (Figure 1 includes mass spectra.) (Morimoto & Satô 1963). Protonation constants of dpa are

$10^{4.66}$ and $10^{2.07}$ (Faucherre et al. 1966; Smith et al. 2001).

The work reported here is an attempt to determine the stability constant of $Fe^{II}(pdtc)_2^{2-}$ through ligand-ligand competition of a known metal-ligand complex of $Fe^{II}(dpa)_2^{2-}$.

Theory to determining the stability constant for $Fe^{II}(pdtc)_2^{2-}$

The determination of the stability constant for $Fe^{II}(pdtc)_2^{2-}$ begins with setting up equations for the complex equilibrium (Crumbliss 1991; Martell & Motekaitis 1992). The stability constant (Equation (1)) for $Fe^{II}(pdtc)_2^{2-}$ is as follows:

$$\beta_{Fe^{II}(pdtc)_2}\frac{[Fe^{II}(pdtc)_2^{2-}]}{[Fe^{II}]\times[pdtc^{2-}]^2} \tag{1}$$

The stability constant (Equation (2)) for $Fe^{II}(dpa)_2^{2-}$ is as follows:

$$\beta_{Fe^{II}(dpa)_2}\frac{[Fe^{II}(dpa)_2^{2-}]}{[Fe^{II}]\times[dpa^{2-}]^2} \tag{2}$$

Combining Equations (1) and (2) will result in an equation to solve for the stability constant of $Fe^{II}(pdtc)_2^{2-}$ (Equation (3)).

$$\beta_{Fe^{II}(pdtc)_2}=\beta_{Fe^{II}(dpa)_2}*\frac{[Fe^{II}(pdtc)_2^{2-}]\times[dpa^{2-}]^2}{[Fe^{II}]\times[dpa_2^{2-}]\times[pdtc^{2-}]^2} \tag{3}$$

The three-step protonation of pdtc using the protonation constants written in terms of the concentration of a protonated species of pdtc (Equations (4)–(6)) is as follows:

$$K_{H(pdtc)}=\frac{[H(pdtc)^{1-}]}{[H^+]\times[pdtc^{2-}]} \tag{4}$$

$$K_{H_2(pdtc)}=\frac{[H_2(pdtc)]}{[H^+]\times[H(pdtc)^{1-}]} \tag{5}$$

$$K_{H_3(pdtc)}=\frac{[H_3(pdtc)^{1+}]}{[H^+]\times[H_2(pdtc)]} \tag{6}$$

The two-step protonation of dpa using the protonation constants written in terms of the concentration of a protonated species of dpa (Equations (7) and (8)) is as follows:

$$K_{H(dpa)}=\frac{[H(dpa)^{1-}]}{[H^+]\times[dpa^{2-}]} \tag{7}$$

$$K_{H_2(dpa)}=\frac{[H_2(dpa)]}{[H^+]\times[H(dpa)^{1-}]} \tag{8}$$

Using mass spectroscopy for verification, it can be deduced that the species $[Fe^{II}(pdtc)]$ and $[Fe^{II}(dpa)]$ will not form in any appreciable amount. Additionally, due to questions of validity of the use of the 3rd protonation constant of pdtc (Equation (6)), the term $[H_3(pdtc)^{1+}]$ will be dropped (Stolworthy et al. 2002). Finally, an excess concentration of ligands (both pdtc and dpa) compared to the molar equivalence of iron(II) will result, and it can be assumed that negligible free iron will form, therefore $[Fe^{II}] \sim 0$. The expression for the concentration of total pdtc in terms of all pdtc species, with the protonated species substituted by Equations (4)–(6) written in terms of the concentration of free pdtc (Equations (9), (10)) is as follows:

$$\begin{aligned}T_{pdtc} &= [pdtc^{2-}]+2\times[Fe^{II}(pdtc)_2^{2-}]\\ &\quad +[H(pdtc)^{1-}]+[H_2(pdtc)]\end{aligned} \tag{9}$$

$$[pdtc^2]=\frac{T_{pdtc}-2\times[Fe^{II}(pdtc)_2^{2-}]}{1+K_{H(pdtc)}\times[H^+]+K_{H(pdtc)}\times K_{H_2(pdtc)}\times[H^+]^2} \tag{10}$$

The expression for the concentration of total dpa in terms of all dpa species, with the protonated species substituted by Equations (7) and (8) written in terms of the concentration of free dpa (Equation (11)) is as follows:

$$[dpa^{2-}]=\frac{T_{dpa}-2\times[Fe^{II}(dpa)_2^{2-}]}{1+K_{H(dpa)}\times[H^+]+K_{H(dpa)}\times K_{H_2(dpa)}\times[H^+]^2} \tag{11}$$

The expression for the concentration of total iron(II) in terms of all iron(II) species written in terms of the concentration of free iron (Equation (12)) is as follows:

$$[Fe^{II}]=T_{Fe^{II}}-[Fe^{II}(pdtc)_2^{2-}]-[Fe^{II}(dpa)_2^{2-}]=0 \tag{12}$$

From Equation (12), it is now possible to determine the concentration of $Fe^{II}(dpa)_2^{2-}$ by measuring the

concentration of $Fe^{II}(pdtc)_2^{2-}$, and Equation (12) can be rewritten as follows:

$$[Fe^{II}(dpa)_2^{2-}] = T_{Fe^{II}} - [Fe^{II}(pdtc)_2^{2-}] \quad (13)$$

$$[dpa^{2-}] = \frac{T_{dpa} - 2 \times T_{Fe^{II}} + 2 \times [Fe^{II}(pdtc)_2^{2-}]}{1 + K_{H(dpa)} \times [H^+] + K_{H(dpa)} \times K_{H_2(dpa)} \times [H^+]^2} \quad (14)$$

Combining Equations (10), (12), and (14) with Equation (3), there is now a complex equilibrium expression to determine the stability constant of $Fe^{II}(pdtc)_2^{2-}$ (Equation (15)).

$$\beta_{Fe^{II}(pdtc)_2} = \beta_{Fe^{II}(dpa)_2} \times \frac{[Fe^{II}(pdtc)_2^{2-}] \times a}{(T_{Fe^{II}} - [Fe^{II}(pdtc)_2^{2-}]) \times b} \quad (15)$$

$$a = \left(\frac{T_{dpa} - 2 \times T_{Fe^{II}} + 2 \times [Fe^{II}(pdtc)_2^{2-}]}{1 + K_{H(dpa)} \times [H^+] + K_{H(dpa)} \times K_{H_2(dpa)} \times [H^+]^2} \right)^2$$

$$b = \left(\frac{T_{pdtc} - 2 \times [Fe^{II}(pdtc)_2^{2-}]}{1 + K_{H(pdtc)} \times [H^+] + K_{H(pdtc)} \times K_{H_2(pdtc)} \times [H^+]^2} \right)^2$$

Hydrogen concentration is determined through pH measurements of the ligand-ligand competition experiment. The concentration of $Fe^{II}(pdtc)_2^{2-}$ is determined spectrophotometrically. Total iron concentration is known from the amount of $Fe^{II}(dpa)^{2-}$-tetraethylammonium added to the experimental solution. Protonation constants for pdtc (Stolworthy et al. 2002) along with protonation constants of dpa and the overall stability constant of $Fe^{II}(dpa)_2^{2-}$ (Anderegg 1960; Faucherre et al. 1967; Smith et al. 2001) are as follows:

$$K_{H(pdtc)} = 10^{5.48} \qquad K_{H(dpa)} = 10^{4.66}$$

$$K_{H_2(pdtc)} = 10^{2.58} \qquad K_{H_2(dpa)} = 10^{2.07}$$

$$\beta_{Fe^{II}(dpa)_2} = 10^{10/36}$$

From a given concentration of a known metal-ligand complex and addition of an unknown ligand, the stability constant of the unknown ligand will vary with the addition of the unknown ligand. To determine the stability constant of the metal to the unknown ligand, the amount of total competing ligand at which the concentration of $Fe^{II}(pdtc)_2^{2-}$ equals the concentration of the known metal-ligand complex $Fe^{II}(dpa)_2^{2-}$ must be found. From spectrophotometric measurements, the concentration of $Fe^{II}(pdtc)_2^{2-}$ will increase slowly followed by a more rapid and steady increase. Finally, the concentration of $Fe^{II}(pdtc)_2^{2-}$ will become constant. From this characteristic, a graph of $Fe^{II}(pdtc)_2^{2-}$ absorbance vs. total pdtc can be made. This titration curve can be fitted to a three-point polynomial equation, taking the derivative twice, and setting it equal to zero. This will result in an inflection point on the graph giving the total pdtc and absorbance of $Fe^{II}(pdtc)_2^{2-}$ where $[Fe^{II}(pdtc)_2^{2-}] = [Fe^{II}(dpa)_2^{2-}]$. This data can be placed into Equation (15) and will result in a stability constant for $Fe^{II}(pdtc)_2^{2-}$.

Materials and methods

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 to 10 ml with dimethylformamide (DMF) (Aldrich Milwaukee, WI). $Fe^{III}(dpa)_2^{2-}$ was synthesized in the form of a tetraethyl ammonium salt from a modification of the Hildebrand et al. (1984) method by substituting dpa (Aldrich Milwaukee, WI) for pdtc. The product's structure was verified by electrospray mass spectroscopy (Figure 1). Iron(III) solutions were obtained from Fisher Scientific (Pittsburgh, PA) in the form of inductively coupled plasma emission (ICP) standard metal stock solutions (1 g/L per metal ion and containing 2% HNO_3 to keep metals in solution). $Fe^{III}(dpa)_2^{2-}$ stock solutions were prepared by weighing out 0.100 g of $Fe^{III}(dpa)^{2-}$, tetraethylammonium salt and diluting with 10 ml DMF. NaOH (1N and 10N) and HCl (1N) solutions were obtained from Fisher Scientific (Pittsburgh, PA). Sodium dithionite ($Na_2S_2O_4$; Aldrich) solution was prepared as a 1 M solution made fresh daily. Sodium perchlorate ($NaClO_4$; Fisher Scientific) was prepared as a 2.5 M solution. All solutions were prepared using deionized distilled water of better than 18 megaohm-centimeters of resistivity. All experiments were performed in an anaerobic chamber containing 4% hydrogen and 96% nitrogen. All stock solutions, acids, bases, and other support solutions also were purged, stored, and prepared in an anaerobic chamber. All measurements were taken at 25°C.

The ionic strength in each experiment was fixed at 0.1 M with $NaClO_4$. Each experiment was fixed at 0.1 M with $Na_2S_2O_4$ to maintain reduced conditions.

General instruments

Absorption spectra were recorded and analyzed on a Hewlett-Packard 8453 UV/Visible diode array spectrophotometer controlled by a HP Pentium-class computer running UV-Visible Chemstation software (revision 5.2). An IQ Scientific Instruments pH meter equipped with an ion sensitive field effect transistor (ISFET) pH sensor electrode using KCl gel and a temperature sensor was used for pH measurements.

Spectrophotometric titrations

UV-Visible absorption spectra were recorded using a 1.0-cm path length quartz cell with a threaded cap and silicon septum. A 10-ml sample containing 0.1 M $Na_2S_2O_4$, 0.1 M $NaClO_4$, and 0.118 and 0.130 mM $Fe^{II}(dpa)_2^{2-}$ was prepared under anaerobic conditions. A 10-ml blank containing 0.1 M $Na_2S_2O_4$ and 0.1 M $NaClO_4$ was also prepared under anaerobic conditions. A 1.25-ml aliquot of the sample and blank were placed into separate, capped quartz cells with equal light path length. The spectrophotometer was blanked each time before the working sample was analyzed. Once a starting background point was established, pdtc was added in constant aliquots to both the blank and the working sample in each capped quartz cell and allowed to sit for 10 minutes to equilibrate before a blank and a spectrum were taken again. The spectrum peak of interest was that of $Fe^{II}(pdtc)_2^{2-}$ found at 687 nm with an extinction coefficient (ε_{687}) of 8520 M^{-1} cm^{-1}.

Potentiometric titrations

The electrode was calibrated to read pH using hydrogen ion activity standard solutions: 4, 7, and 10 (all +/− 0.02 pH @ 25 °C). Potentiometric titrations were made by preparing enough sample to make 1.25-ml aliquots of sample for each spectra analyzed. The ionic strength in the titration experiments was fixed at 0.1 M with $NaClO_4$ (Fisher Scientific). Each aliquot was placed into a 2-ml scintillation vial, and each aliquot received the appropriate addition of pdtc according to its spectra as described previously. These aliquots, all prepared and sealed under anaerobic conditions, were allowed to equilibrate for 10 minutes before pH measurements were taken.

Mass spectrometry

A 0.2-mM sample solution was analyzed by using a 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 counter electrode, 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 represent a combined average of 10–15 scans. The structures of complexes were confirmed using daughters' fragmentation tandem mass spectrometry (MS/MS). The hexapole collision cell was filled with argon gas, which was used to generate daughters' fragments.

Results

The extinction coefficient (ε_{687}) for $Fe^{II}(pdtc)_2^{2-}$ was found to be 8520 M^{-1} cm^{-1}. Through qualitative experimentation, it was found that a minimum of 0.1 M $Na_2S_2O_4$ was needed to keep the iron(II)-pdtc complex in a reduced state. Lower concentrations would slowly revert the complex to an oxidized state, turning the solution from blue to brown within one hour of mixing. A 0.1-M $Na_2S_2O_4$ solution revealed a reduced state for at least 12 hours, providing more than enough time to run a single experiment.

The pH of the starting working solution was about 6. As pdtc was added, the absorbance peak at 687 nm increased (Figure 2). This series of increasing absorbance vs. pdtc was plotted (Figure 3) and an inflection point found. The resulting inflection point was placed back into Equation 15 to determine the stability constant of $Fe^{II}(pdtc)_2^{2-}$. A total pdtc concentration of 0.134 mM revealed a stability constant of 1011.84. This experiment was repeated using data points from past experiments. Using data points corresponding to a pH of 8 to 9, a spectral graph and an inflection point curve showed similar results as seen in the previous experiment. A total pdtc concentration of 0.141 mM revealed a stability constant of $10^{12.13}$.

A solution of $Fe^{II}(pdtc)_2^{2-}$ was prepared (Hildebrand et al. 1983) and was manipulated by acid and

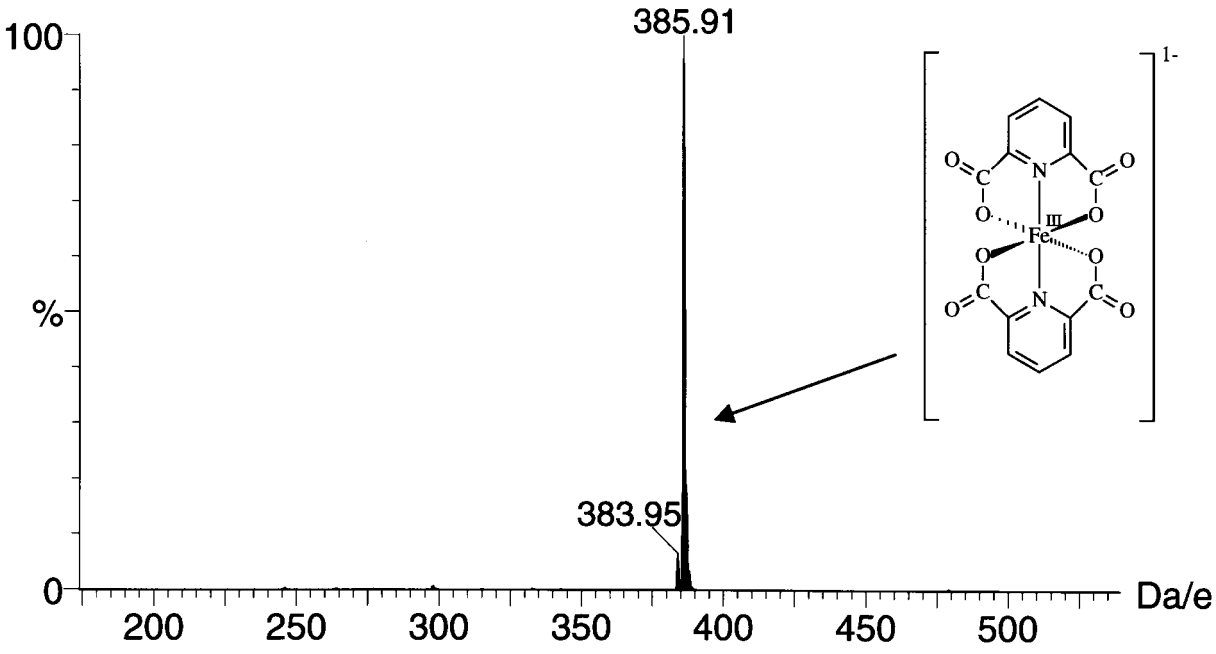

Figure 1. Electrospray mass spectrum and structure of $Fe^{(III)}(dpa)_2^{-1}$ complex.

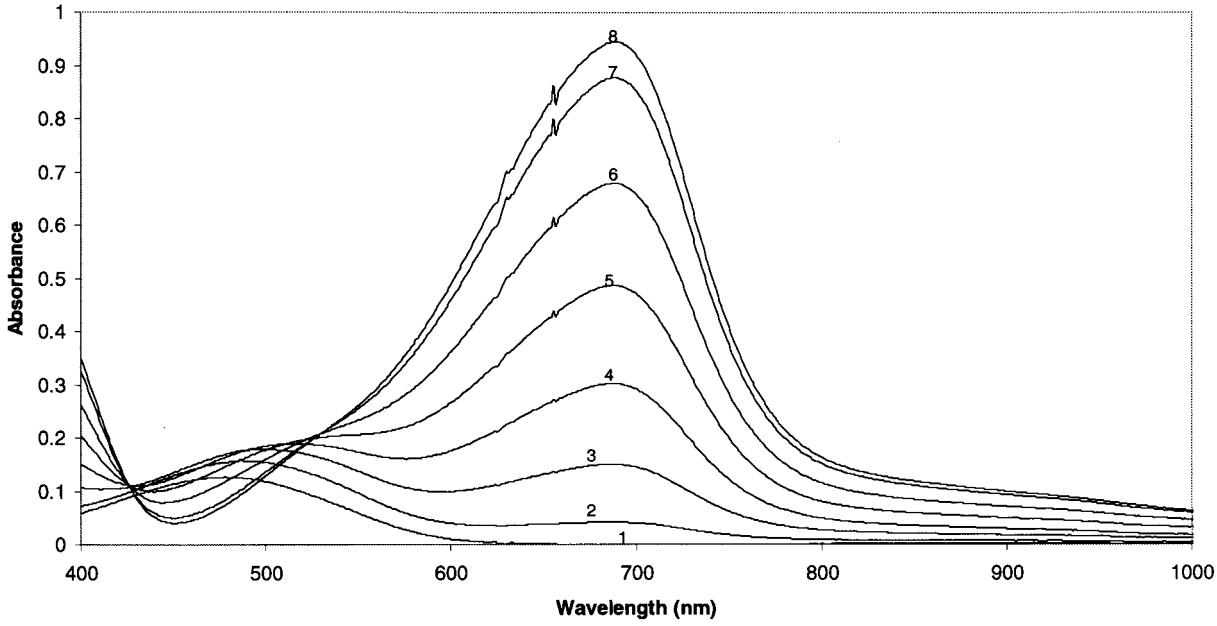

Figure 2. Spectral Changes During Titration of $Fe^{II}(dpa)_2^{2-}$ by Pdtc. I = 0.1 M $NaClO_4$; $[Na_2S_2O_4]$ = 0.1 M; T = 25.0 °C; l = 1.0 cm; $[Fe^{II}(dpa)_2^{2-}]$ = 0.130 mM. Total pdtc = (1) 0; (2) 0.0339; (3) 0.0677; (4) 0.102; (5) 0.135; (6) 0.169; (7) 0.203; (8) 0.237 mM.

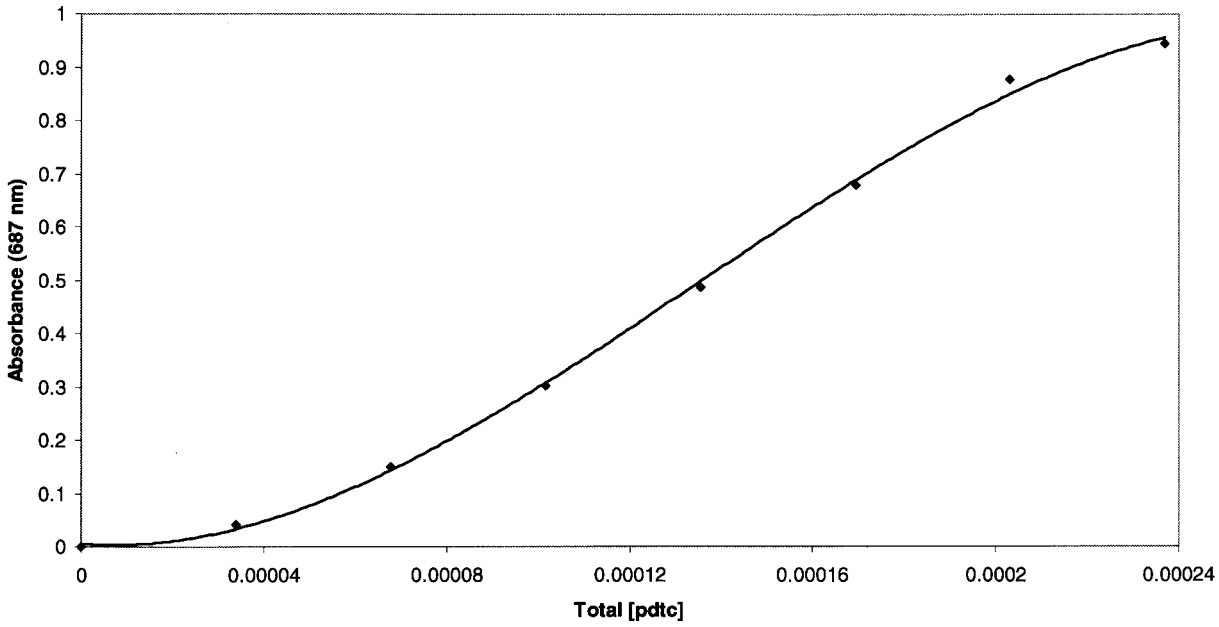

Figure 3. Graphical Determination of Inflection Point of $Fe^{II}(pdtc)_2^{2-}$. I = 0.1 M $NaClO_4$; $[Na_2S_2O_4]$ = 0.1 M; T = 25.0 °C; l = 1.0 cm; $[Fe^{II}(dpa)_2^{2-}]$ = 0.130 mM; λ = 687 nm. At a total pdtc concentration of 0.134 mM, an absorbance of 0.4926 reveals a stability constant for $Fe^{II}(pdtc)_2^{2-}$ of $10^{11.84}$.

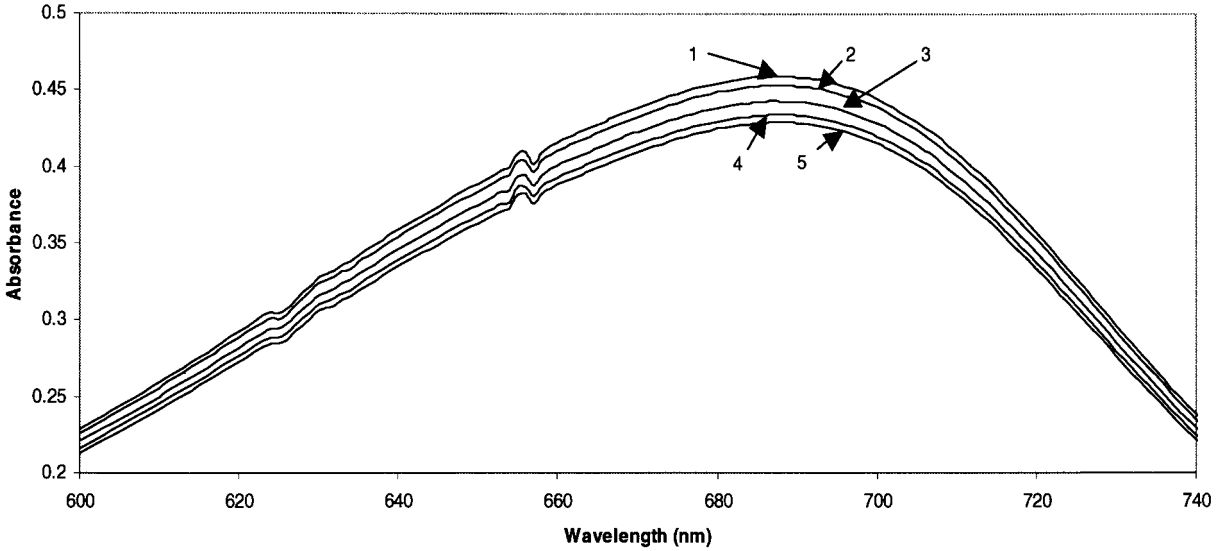

Figure 4. Spectral Changes During Titration of $Fe^{II}(dpa)_2^{2-}$ by HCl. I = 0.1 M NaClO4; $[Na_2S_2O_4]$ = 0.1 M; T = 25.0 °C; l = 1.0 cm; $[Fe^{II}(pdtc)_2^{2-}]$ = 0.539 mM. pH = (1) 6.15; (2) 6.02; (3) 5.83; (4) 5.56; (5) 5.05.

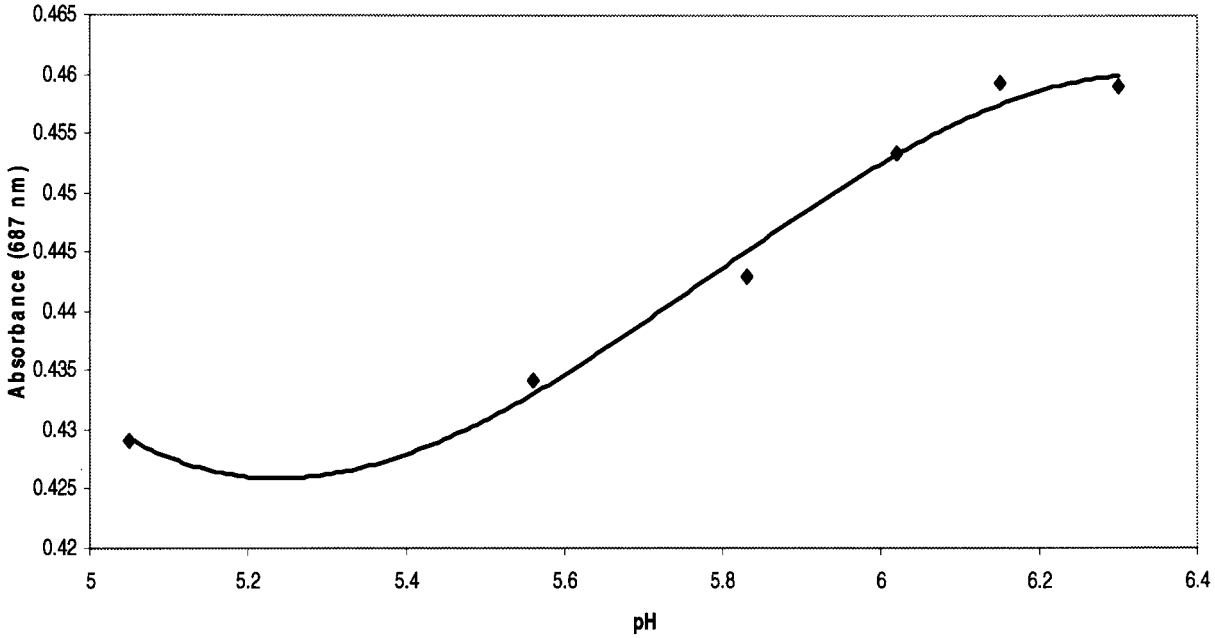

Figure 5. Graphical Determination of Inflection Point of $Fe^{II}(pdtc)_2^{2-}$. I = 0.1 M $NaClO_4$; $[Na_2S_2O_4]$ = 0.1 M; T = 25.0 °C; l = 1.0 cm; $[Fe^{II}(pdtc)_2^{2-}]$ = 0.0539 mM; λ = 687 nm. At a pH of 5.79, an absorbance of 0.4430 revealed a stability constant for $Fe^{II}(pdtc)_2^{2-}$ of $10^{12.26}$. A data point at pH 6.3 was added to force inflection of curve.

base titrations. The solution started with a pH of around 6. As pH was increased using NaOH, the absorbance peak at 687 nm indicated little to no change. At a pH of 11.4, the absorbance peak began to decrease. Beyond a pH of 12, the absorbance decreased sharply. In another experiment where pH was decreased using HCl, a slight decrease in absorbance occurred (Figure 4), and then absorbance became constant up to a pH of 5. After this, absorbance dropped sharply and approached zero absorbance at a pH of 2. The slight curve of inflection was plotted (Figure 5), and an inflection point was found. A data point was added at a pH of 6.3 to force an inflection of the curve. The stability constant was determined by using Equation (1) where Equation (10) was used for [pdtc], and [FeII] was found by stating total iron is equal to free iron(II) and $Fe^{II}(pdtc)_2^{2-}$, a modification of Equation (14) since no $Fe^{II}(dpa)_2^{2-}$ was present. At the inflection point, [FeII] + 2[pdtc] = $[Fe^{II}(pdtc)_2^{2-}]$. A pH of 5.79 revealed a stability constant of $10^{12.26}$.

Discussion

Addition of pdtc to $Fe^{II}(dpa)_2^{2-}$ in the working sample showed a slight increase in $Fe^{II}(pdtc)_2^{2-}$ concentrations. As additional pdtc was titrated, a steady increase in $Fe^{II}(pdtc)_2^{2-}$ concentrations was noted and further addition of pdtc resulted in $Fe^{II}(pdtc)_2^{2-}$ concentrations becoming constant. These measurements were used as the data point series for inflection point calculations resulting in a stability constant. The addition of dpa to $Fe^{II}(pdtc)_2^{2-}$ should result in a decrease of $Fe^{II}(pdtc)_2^{2-}$ concentrations, indicating a reversible equilibrium reaction. This experiment resulted in a linear concentration drop of $Fe^{II}(pdtc)_2^{2-}$ but did not reveal a titration curve. A reaction was occurring with $Fe^{II}(pdtc)_2^{2-}$ beyond simple volume dilution.

To confirm the stability constant obtained from $Fe^{II}(dpa)_2^{2-}$ with pdtc titrations, a $Fe^{II}(pdtc)_2^{2-}$ solution was titrated with acid and base using methods similar to those of Stolworthy et al. (2002). Adding base to the $Fe^{II}(pdtc)_2^{2-}$ solution showed little to no change in absorbance as pH was increased using NaOH. As pH was increased further, a decrease in $Fe^{II}(pdtc)_2^{2-}$ concentrations was noted followed by a dramatic decrease in concentration after a pH of 12; concentrations continued to decrease until a pH of 12.7 was reached. Since no indication of a titration curve was evident after a pH of 12.7, base titration was halted at this point due to the possibility of base-catalyzed hydrolysis of pdtc.

The acid titration showed a slight inflection curve at a pH around 5.79. Below a pH of 5, $Fe^{II}(pdtc)_2^{2-}$ concentration dropped off dramatically to almost zero at a pH around 2. The acid experiment should show reversibility when base is added to the solution. Within the inflection point data series, the addition of a base would result in the increase of absorbance, but not to initial experimental levels. It is also possible that $Fe^{II}(pdtc)_2^{2-}$ was being oxidized due to the degradation of sodium dithionite in low pH environments. The addition of sodium dithionite, however, did not increase absorbance to initial experimental levels. This indicates that a reaction was occurring with $Fe^{II}(pdtc)_2^{2-}$ and HCl, along with simultaneous degradation of sodium dithionite. In retrospect, acidic environments caused the rapid decomposition of sodium dithionite causing absorbance peak irreversibility due to the oxidation of iron in the pdtc complex.

The acid run, although resulting in a stability constant of 10^{12}, has limitations as well. As acid was added, concentration did decrease beyond simple dilution, but dithionite was also being degraded at the same time. It is also noted that the inflection point did not reveal a situation where [FeII] and 2[$pdtc^{2-}$] equals [$Fe^{II}(pdtc)_2^{2-}$].

Conclusion

The stability constant of $Fe^{II}(pdtc)_2^{2-}$ was determined to be 10^{12}. Comparing this to the stability constant for $Fe^{III}(pdtc)_2^{2-}$ of $10^{33.36}$, the stability constant of $Fe^{II}(pdtc)_2^{2-}$ is approximately 21 orders of magnitude smaller; a significant decrease in the binding strength of pdtc to iron. If the host cell produces pdtc as a siderophore, this would show that a second metabolite from the host cell could be used in the reduction of iron in order to facilitate the release of iron from pdtc for cell use. This would also allow multiple use of pdtc as a cell metabolite. As a potential antibiotic, pdtc shows a much stronger affinity to iron(III) than to iron(II), thus pdtc could significantly reduce the amount of iron(III) available to competing bacteria and give cells producing pdtc an advantage.

Acknowledgments

This work was funded in part by the Department of Chemical Engineering, the Environmental Biotechnology Institute at the University of Idaho, Moscow, ID, and the U.S. Department of Agriculture grant IDA 01228

References

Anderegg G (1960) Pyridinderivate als Komplexbildner I. Pyridincarbonsäuren. Helv. Chem. Acta 43: 414–424

Budzikiewicz H (1993) Secondary Metabolites from Fluorescent *Pseudomonads*. FEMS Microbiol. Rev. 104: 209–228

Colston BJ & Robinson VJ (1997) Use of metal ion indicators to determine complex stability constants: the method of competitive equilibration. Analyst, November, 122: 1451–1455

Cortese MS, Paszczynski A, Lewis TA, Sebat JL, Borek V, & Crawford RL (2002) Metal chelating properties of pyridine-2,6-bis(thiocarboxylic acid) produced by *Pseudomonas* spp. and the biological activities of the formed complexes. Biometals 15: 103–120

Crumbliss AL (1991) Aqueous solution equilibrium and kinetic studies of iron siderophore and model siderophore complexes. In: Winkelmann G (Ed) Handbook of Microbial Iron Chelates. CRC Press. Boca Raton, Florida: 177–233

Dimmock PW, Warwick P, & Robbins RA (1995) Approaches to predicting stability constants. Analyst, August, 120: 2159–2170

Faucherre J, Petitfaux C, & Charlier B (1966) Sur la structure et la stabilité des chélates cuivriques de l'acide dipicolique. Bull. Chem. Soc. France 3: 1091–1098

Hildebrand U, Lex J, Taraz K, Winkler S, Ockels W, & Budzikiewicz H (1984) Untersuchugen zum Redox-System bis-(Pyridin-2,6-dicarbothioato)-ferrat(II)/-ferrat(III). Z. Naturforsch 39b: 1607–1613

Hildebrand U, Ockels W, Lex J, & Budzikiewicz H (1983) Zur Struktur eines 1:1-Adduktes von Pyridin-2,6-dicarbothiosäure und Pyridin. Phosphor. Sulf. 16: 361–364

Hildebrand U, Taraz K, & Budzikiewicz H (1985) [(Methoxythio)carbonyl]pyridine derivatives – A new class of sulfur compounds. Tetrahed. Let. 26: 4349–4350

Hildebrand U, Taraz K, & Budzikiewicz H (1986) 6-(Hydroxythio)carbonylpyridin-2-carbonsäure und Pyridin-2-carbonsäure-6-onothicarbonsäure als biosynthetische Zwischenstufen bei der Bildung von Pyridin-2,6-di(monothicarbonsäure) aus Pyridin-2,6-dicarbonsäure. Z. Naturforsch 41c: 691–694

Holman DA & Bennet DW (1994) A multicomponent kinetics study of the anaerobic decompostition of aqueous sodium dithionite. J. Phys. Chem. 98: 13300–13307

Hübner J, Taraz K, & Budzikiewicz H (1990) Acylsufenic acids. Phosphor. Sulf. Silicon 47: 367–374

Kilroy W (1980) Anaerobic decompostion of sodium dithionite in alkaline solution. J. Inorg. Nuc. Chem. 42: 1071–1073

Lambeth DO & Palmer G (1973) The kinetics and mechanism of reduction of electron transfer protiens and other compounds of biological interest by dithionite. J. Biol. Chem. 248: 6095–6103

Martell AE & Motekaitis RJ (1992) Determination and Use of Stability Constants. VCH Publishers Inc., Berlin

Morimoto I & Satô T (1963) Spectrophotometric studies of the nature of several iron-pyridinecarboxylic acid complexes. Bull. Chem. Soc. Japan 36: 605–610

Neilands JB (1995) Siderophores: Structure and function of microbial iron transport compounds. J. Biol. Chem. 207: 26723–26726

Neilands JB & Nakamura K (1991) Detection, determination, isolation, characterization and regulation of microbial iron chelates.

In: Winkelmann G (Ed) Handbook of Microbial Iron Chelates. CRC Press. Boca Raton, Florida: pp 1–14

Ockels W, Römer A, & Budzikiewicz H (1978) An Fe(II) complex of pyridine-2,6-di(monothiocarboxylic acid)-A novel bacterial metabolic product. Tetrahed. Let. 36: 3341–3342

Sebat JL, Paszczynski AJ, Cortese MS, & Crawford RL (2001) Antimicrobial properties of pyridine-2,6-dithiocarboxylic acid, a metal chelator produced by *Pseudomonas* spp. Appl. Environ. Microbiol. 67: 3943–3942

Shaikh A & Javaid Zaidi SM (1993) Kinetics of oxygen absorption in aqueous sodium dithionite solutions. J. Chem. Technol. Biotechnol. 56: 139–145

Smith RM, Martell AE, & Motekaitis RJ (2001) NIST Critically Selected Stability Constants of Metal Complexes Database. Version 6.0 for Windows. U.S. Department of Commerce Technology Administration, National Institute of Standards and Technology

Stolworthy JC, Paszczynski A, Korus R, & Crawford RL (2002) Metal binding by pyridine-2,6-bis(monothicarboxylic acid), a biochelator produced by *Pseudomonas stutzeri* and *Psuedomonas putida*. Biodegrad. 12: 411–418.