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# A mechanistic study of ferrioxamine B reduction by the biological reducing agent ascorbate in the presence of an iron(II) chelator

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## ABSTRACT

The iron overload drug desferal (desferrioxamine B) forms the stable iron complex ferrioxamine B. The reduction potential of ferrioxamine B ( $E^{\circ} = -482$  mV versus NHE pH 7) prohibits its reduction by biological reducing agents such as ascorbate, but it was found that the iron(II) chelator 2,2'-bipyridine (bipy) facilitates this reduction. Evidence is given to support the formation of a ternary complex between iron, bipy, and desferrioxamine B as the key step in facilitating the reduction. The equilibrium constant for the formation of the ternary complex was found to be  $8.9 \times 10^7$  and ternary complex formation is explained in terms of a three step mechanism. The mechanism for the reduction of ferrioxamine B is discussed in terms of rapidly established pre-equilibria which include ternary complex formation, ascorbic acid deprotonation, and encounter complex formation between ascorbate and the ternary complex. These equilibria are followed by rate limiting reduction of the ternary complex. Bipy was found to be a similar facilitator to sulfonated bathophenanthroline for the reduction of ferrioxamine B by ascorbate.

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## 1. Introduction

Severe anemias including  $\beta$ -thalassemia, myelodysplastic syndrome, and aplastic anemia require patients to receive repeated blood transfusions which leads to potentially fatal transfusional iron overload. The most common methods for iron removal from these patients involves chelation therapy utilizing desferal [1], the more recently FDA approved drug Exjade [2], or Deferiprone [3] which is approved for use in Europe and Asia. Desferal is the trade name for the mesylate salt of desferrioxamine B ( $H_4DFB$ , Fig. 1a), a naturally occurring siderophore isolated from *Actinomyces* [4], *Nocardia*, and *Streptomyces* [5]. Desferal exhibits an extremely strong and selective affinity for iron(III) ( $\log \beta_{Fe(III)} = 30.6$ ) [6]. Studies have demonstrated that desferal chelation therapy increases survival rate [7], forestalls and possibly reverses cardiac disease [8], and reduces serum ferritin levels in the liver of iron-overload patients [9].

Vitamin C ( $H_2A$ ), or ascorbic acid, deficiency results from an increase in the conversion of ascorbate ( $HA^-$ , Fig. 1b) to oxalate [10], and is often associated with iron-overload [11]. Ascorbate increases the levels of chelatable iron, by delaying the transfer of iron from ferritin to insoluble hemosiderin [12,13]. Combination therapy with desferal and ascorbate for iron overload treatment results in an increase in urinary iron excretion [14]. Unfortunately, large doses of ascorbate in iron-overload cases increase cell and organ

damage and ultimately lead to cardiac arrest [15]. These effects are also observed when iron overloaded patients having normal ascorbate levels are dosed with both ascorbate and desferal. The increase in side effects for patients with above average ascorbate levels may be due to increased amounts of iron available for Fenton chemistry and free radical generation.

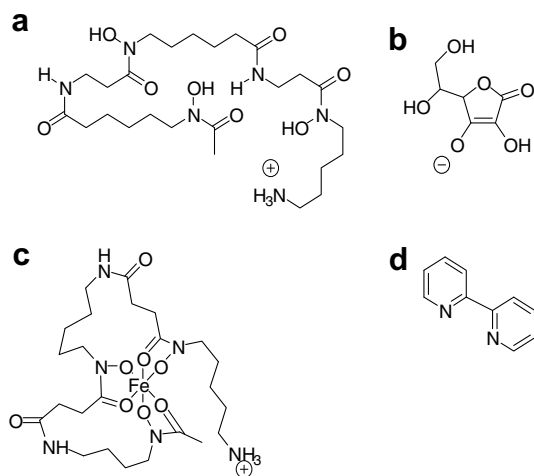
Ferrioxamine B ( $Fe(HDFB)^+$ , Fig. 1c), the iron complex of desferrioxamine B, is commonly believed to be outside the range of biological reducing agents due to its reduction potential ( $-482$  mV versus NHE at pH 7) [16], which prevents it from contributing to Fenton Chemistry and free radical generation [17–19]. This is true when only ferrioxamine B and the biological reducing agents are in solution, but *in vivo* there are many chemicals that can potentially facilitate the reduction of ferrioxamine B. Iron(II) chelators, such as porphyrins and histidine residues, will create a positive shift in the effective reduction potential of ferrioxamine B and could potentially allow for the facile reduction of iron by biological reducing agents at neutral pH values.

In previous work, it has been demonstrated that ferrioxamine B can be reduced by the biologically relevant molecules glutathione and ascorbate in the presence of the iron(II) chelator bathophenanthroline [20]. This reduction allowed for the removal of iron from the thermodynamically stable ferrioxamine B complex.

In the present work, we expand upon the mechanism of ferrioxamine B reduction and demonstrate that other iron(II) chelators can facilitate this reaction. The results present a reasonable method for iron reduction *in vivo* from extremely stable complexes and provide a possible explanation for the increased side effects observed in patients with elevated levels of iron and ascorbate.

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**Fig. 1.** Structures of (a) desferrioxamine B (H<sub>4</sub>DFB<sup>+</sup>), (b) ascorbate (HA<sup>-</sup>), (c) ferrioxamine B (Fe(HDFB)<sup>+</sup>), and (d) bipyridine (bipy).

## 2. Experimental

### 2.1. Materials

Ascorbic acid (H<sub>2</sub>A; Acros Organics (99%)), NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> (Fisher Scientific (99.4%)), NaNO<sub>3</sub> (Carolina Biological), and 2,2'-bipyridine (bipy; Fischer Scientific) were used as purchased. Ferrioxamine B (Fe(HDFB)<sup>+</sup>) aqueous solutions were prepared by dilution of a 27.3 mM Fe(HDFB)ClO<sub>4</sub> stock solution prepared from Fe(ClO<sub>4</sub>)<sub>3</sub> (Sigma–Aldrich) and desferrioxamine B mesylate (Sigma (95%)) as previously described [21]. 2-(*N*-Morpholino)ethanesulfonic acid (MES, Acros Organics) was used as a buffer and the pH was adjusted with either NaOH (Fisher Scientific) or HClO<sub>4</sub> (J.T. Baker (60–62%)). All solutions were aqueous and made with deionized water.

### 2.2. Methods

pH measurements were carried out using an Accumet pH Meter 910 equipped with an Accumet pH electrode filled with 3.0 M NaCl and standardized with three buffer solutions. Kinetic runs were measured using an Agilent 8453 spectrophotometer and analyzed with the biochemical analysis software for Agilent ChemStation.

### 2.3. Ternary complex formation

The equilibrium constant and the stoichiometry of ternary complex formation, Fe(H<sub>1+y</sub>DFB)(bipy)<sub>x</sub><sup>1+y</sup>, was determined from the spectrophotometric titration of Fe(HDFB)<sup>+</sup> and bipy. To a 10 mL solution containing 0.25 mM Fe(HDFB)<sup>+</sup>, 9.9 mM bipy, 0.1 M NaNO<sub>3</sub>, and 0.05 M NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub> a series of 0.025 mL additions of HClO<sub>4</sub> was used to lower the pH from 5.56 to 4.92. There was a 15 min wait between additions to allow for equilibrium to occur. Before each addition the solution was monitored spectrophotometrically from 350 to 700 nm. The experiment was repeated four times and the data analyzed at six wavelengths per trial (328–338 nm).

The kinetics of ternary complex formation were monitored under pseudo-first-order conditions, where equal volumes of a solution containing 0.28 mM Fe(HDFB)<sup>+</sup> and another solution containing bipy (1.40–28.0 mM) were mixed and the absorbance increase was monitored at 515 nm. Both solutions were made with a 0.1 M MES buffer at identical pH (5.65–6.35). All kinetic runs were made at 25 °C and *I* = 0.1 M MES. Observed rate constants represent an average of 3–7 independent trials.

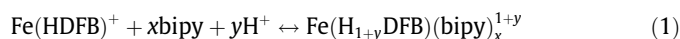
### 2.4. Reduction kinetics

The kinetics of iron reduction was monitored under pseudo-first-order conditions, where equal volumes (0.500 mL) of three solutions were mixed and the absorbance increase was monitored at 520 nm. The first of the three solutions contained 0.28 mM Fe(HDFB)<sup>+</sup>, the second solution contained ascorbate (0.121–1.21 M), and the third contained bipy (0–28.0 mM). Each solution was buffered (pH 5.65–6.35) in 0.1 M MES. All kinetic runs were made at 25 °C and *I* = 0.1 M MES. Observed rate constants represent an average of 3–7 independent trials.

## 3. Results

### 3.1. Thermodynamics of the reaction between ferrioxamine B and bipy

Desferrioxamine B forms a stable complex with Fe(III) and shows minimal reaction with ascorbate over a 24 h time period. Immediately upon addition of the iron(II) chelator 2,2'-bipyridine (bipy, Fig. 1d) a visible change in color is observed. The λ<sub>max</sub> shifts from the characteristic band of Fe(HDFB)<sup>+</sup> at 428–526 nm, which corresponds to the presence of Fe(bipy)<sub>3</sub><sup>2+</sup>. To determine how bipy acts to facilitate the reduction of the stable ferrioxamine B complex, the interaction between ferrioxamine B and bipy in the absence of ascorbate was monitored. This reaction resulted in a small increase in absorbance between 350 and 700 nm.<sup>1</sup> Since an observable change was seen in the visible spectrum, it was assumed that the inner coordination sphere of the iron(III) ion was changing, with the most likely possibility that one or more bipys were replacing hydroxamate groups from H<sub>4</sub>DFB<sup>+</sup> to form a ternary complex (Reaction (1)).



Spectrophotometric titrations were performed to determine the stoichiometry of the reaction and the structure of the ternary complex.<sup>1</sup> If there are only two light absorbing species, the equilibrium constant from Reaction (1) can be combined with Beer's Law to produce Eq. (2) [22]; where *A*<sub>obs</sub> is the equilibrium absorbance measured at each pH, *A*<sub>FeHDFB</sub> is the absorbance when the iron is all in the form of Fe(HDFB)<sup>+</sup>, and *A*<sub>ternary</sub> is the absorbance when all the iron has been converted to the ternary complex, Fe(H<sub>1+y</sub>DFB)(bipy)<sub>x</sub><sup>1+y</sup>.

$$A_{\text{obs}} = \frac{(A_{\text{FeHDFB}} - A_{\text{obs}})}{K[\text{bipy}]^x[\text{H}^+]^y} + A_{\text{ternary}} \quad (2)$$

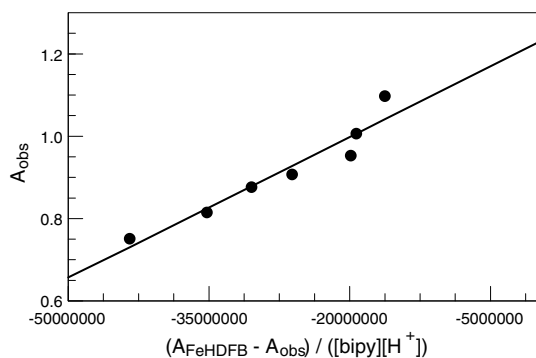
Eq. (2) represents a linear relationship between *A*<sub>obs</sub> and (A<sub>FeHDFB</sub> - A<sub>obs</sub>)/[bipy]<sup>x</sup>[H<sup>+</sup>]<sup>y</sup>. After a series of plots where *x* and *y* were varied between 0, 1, 2, and 3 it was found that a linear relationship existed only when both *x* and *y* equaled 1 (Fig. 2). It was concluded that the structure of the ternary complex was Fe(H<sub>2</sub>DFB)(bipy)<sub>2</sub><sup>2+</sup> and an average value of log *K* (Eq. (3)) was determined to be 7.95(±0.10). The value is slightly greater than the literature results for phenanthroline based ternary complexes of Fe(HDFB)<sup>+</sup> [20,23].

$$K = [\text{Fe(H}_2\text{DFB)(bipy)}_2^{2+}]/[\text{Fe(HDFB)}^+][\text{H}^+][\text{bipy}] \quad (3)$$

### 3.2. Kinetics of the reaction between ferrioxamine B and bipy

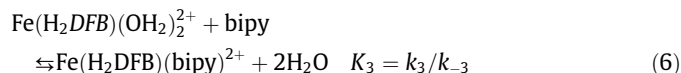
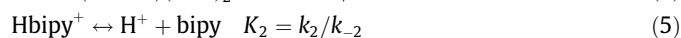
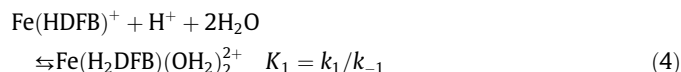
Once the nature of the ternary complex was determined, the mechanism of its formation was examined through a series of reactions between bipy and Fe(HDFB)<sup>+</sup> under pseudo-first-order condi-

<sup>1</sup> Data contained in Supplementary material.



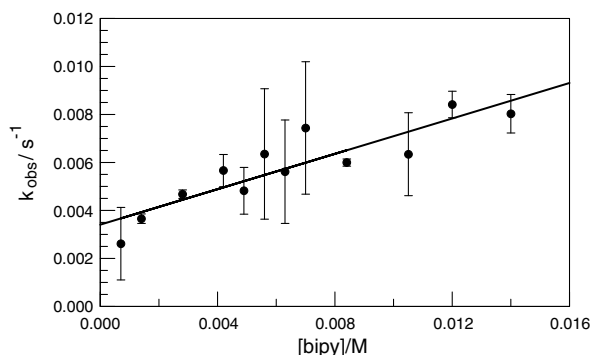
**Fig. 2.** Spectrophotometric pH titration of ferrioxamine B and bipy. Conditions:  $[\text{Fe}(\text{HDFB})^+] = 0.25 \text{ mM}$ ,  $[\text{bipy}] = 9.9 \text{ mM}$ ,  $[\text{NaNO}_3] = 0.1 \text{ M}$ ,  $[\text{NaC}_2\text{H}_3\text{O}_2] = 0.05 \text{ M}$ , pH 4.92, 5.04, 5.09, 5.23, 5.31, 5.42, 5.56,  $T = 24 \text{ }^\circ\text{C}$ ,  $\lambda = 330 \text{ nm}$ . Solid line represents a linear fit of the data with a slope of  $1.1(\pm 0.4) \times 10^{-8}$  and intercept of  $1.22(\pm 0.11) \text{ M}^{-2}$ .

tions with bipy in excess. For this reaction, observation of the absorbance at a single wavelength over time resulted in a saturation profile which could be fit to a single exponential.<sup>1</sup> Due to the small change in absorbance for these reactions the error bars are relatively large. A linear relationship was observed for the plot of  $k_{\text{obs}}$ , the observed first-order rate constant, as a function of the total bipy concentration (Fig. 3). The proposed mechanism described in reactions (4)–(6) is consistent with all collected data. The mechanism consists of partial opening of the  $\text{Fe}(\text{HDFB})^+$  complex (Reaction (4)), deprotonation of bipy (Reaction (5)), and reaction of the deprotonated bipy with the partially opened ferrioxamine B complex to produce the ternary complex (Reaction (6)). If relaxation kinetics are assumed for the rate determining step (Reaction (6)) and that the other reactions are rapidly established pre-equilibrium, then Eq. (7) may be derived for  $k_{\text{obs}}$ .

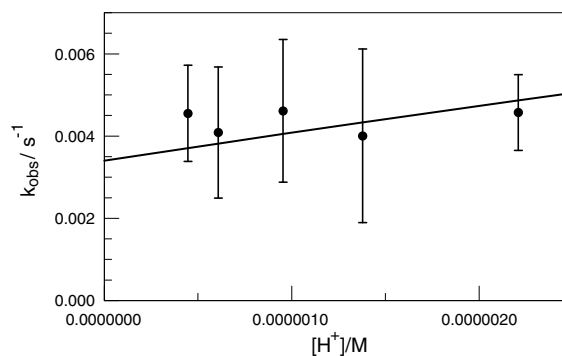


$$k_{\text{obs}} = \frac{k_3 K_2 [\text{H}^+] [\text{bipy}]_{\text{tot}}}{(K_2 + [\text{H}^+] + K_1^{-1} [\text{H}^+] + K_2 K_1^{-1}) [\text{H}^+] + K_2 K_1^{-1}} + k_{-3} \quad (7)$$

The microscopic rate constants  $k_3$  and  $k_{-3}$  were determined from the data in Figs. 3 and 4. Both data sets were fit to Eq. (7) and used

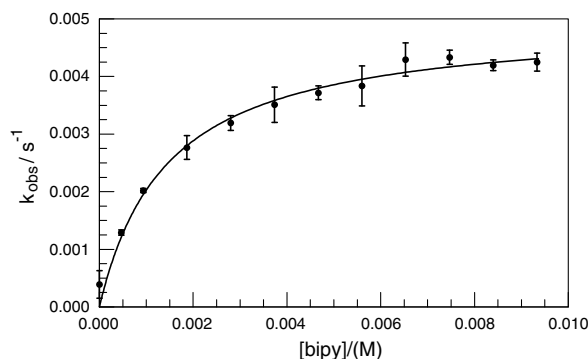


**Fig. 3.** Plot of the pseudo-first-order rate constant ( $k_{\text{obs}}$ ) for ternary complex formation as a function of bipy concentration. Conditions:  $[\text{Fe}(\text{HDFB})^+] = 0.14 \text{ mM}$ ,  $[\text{bipy}] = 0.70\text{--}14 \text{ mM}$ ,  $[\text{MES}] = 0.1 \text{ M}$ , pH 5.96,  $T = 25 \text{ }^\circ\text{C}$ ,  $\lambda = 515 \text{ nm}$ . The solid line represents the linear best fit of the data, with a slope of  $0.37(\pm 0.1) \text{ s}^{-1} \text{ M}^{-1}$  and an intercept of  $3.0(\pm 1.0) \times 10^{-3} \text{ s}^{-1}$ . The error bars represent the standard deviation in  $k_{\text{obs}}$  values.

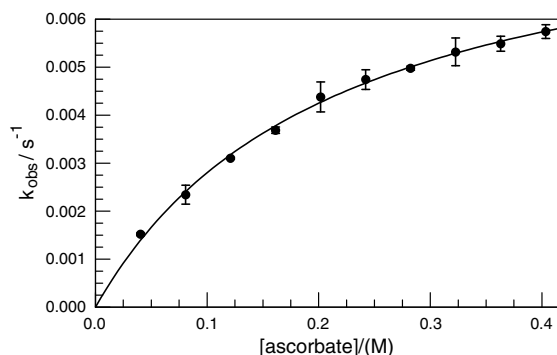


**Fig. 4.** Plot of the pseudo-first-order rate constant ( $k_{\text{obs}}$ ) for ternary complex formation as a function of acid concentration. Conditions:  $[\text{Fe}(\text{HDFB})^+] = 0.14 \text{ mM}$ ,  $[\text{bipy}] = 5.5 \text{ mM}$ ,  $[\text{MES}] = 0.1 \text{ M}$ , pH 5.65–6.35,  $T = 25 \text{ }^\circ\text{C}$ ,  $\lambda = 515 \text{ nm}$ . The solid line represents the fit of the data to  $k_{\text{obs}} = (a[\text{H}^+]/(20.9 + 4.6 \times 10^5[\text{H}^+])) + 3.4 \times 10^{-3}$ , where  $a = 1.5(\pm 1.2) \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$ . The error bars represent the standard deviation in  $k_{\text{obs}}$  values.

the previously determined values of  $K_1$  [24] and  $K_2$  [25]. Both fits produced similar values, and the average values were  $2.0(\pm 1.4) \times 10^4 \text{ s}^{-1} \text{ M}^{-1}$  and  $3.9(\pm 1.0) \times 10^{-3} \text{ s}^{-1}$  for  $k_3$  and  $k_{-3}$ , respectively. From the data in Fig. 3,  $\log K_3$  was found to be  $7.06(\pm 0.11)$ . The values for  $k_3$ ,  $k_{-3}$ , and  $K_3$  are consistent with previous literature values [20,23].



**Fig. 5.** Plot of the pseudo-first-order rate constant ( $k_{\text{obs}}$ ) for the reduction reaction as a function of bipyridine concentration. Conditions:  $[\text{Fe}(\text{HDFB})^+] = 0.095 \text{ mM}$ ,  $[\text{HA}^-] = 0.100 \text{ M}$ ,  $[\text{bipy}] = 0\text{--}9.33 \text{ mM}$ ,  $[\text{MES}] = 0.1 \text{ M}$ , pH 5.96,  $T = 25 \text{ }^\circ\text{C}$ ,  $\lambda = 520 \text{ nm}$ . The equation  $k_{\text{obs}} = a[\text{bipy}]/(b + [\text{bipy}])$  was fit to the data giving the solid line shown and values of  $a = 5.0(\pm 0.2) \times 10^{-3} \text{ s}^{-1}$  and  $b = 1.5(\pm 0.2) \times 10^{-3} \text{ M}$ . Error bars represent the standard deviation in  $k_{\text{obs}}$  values.

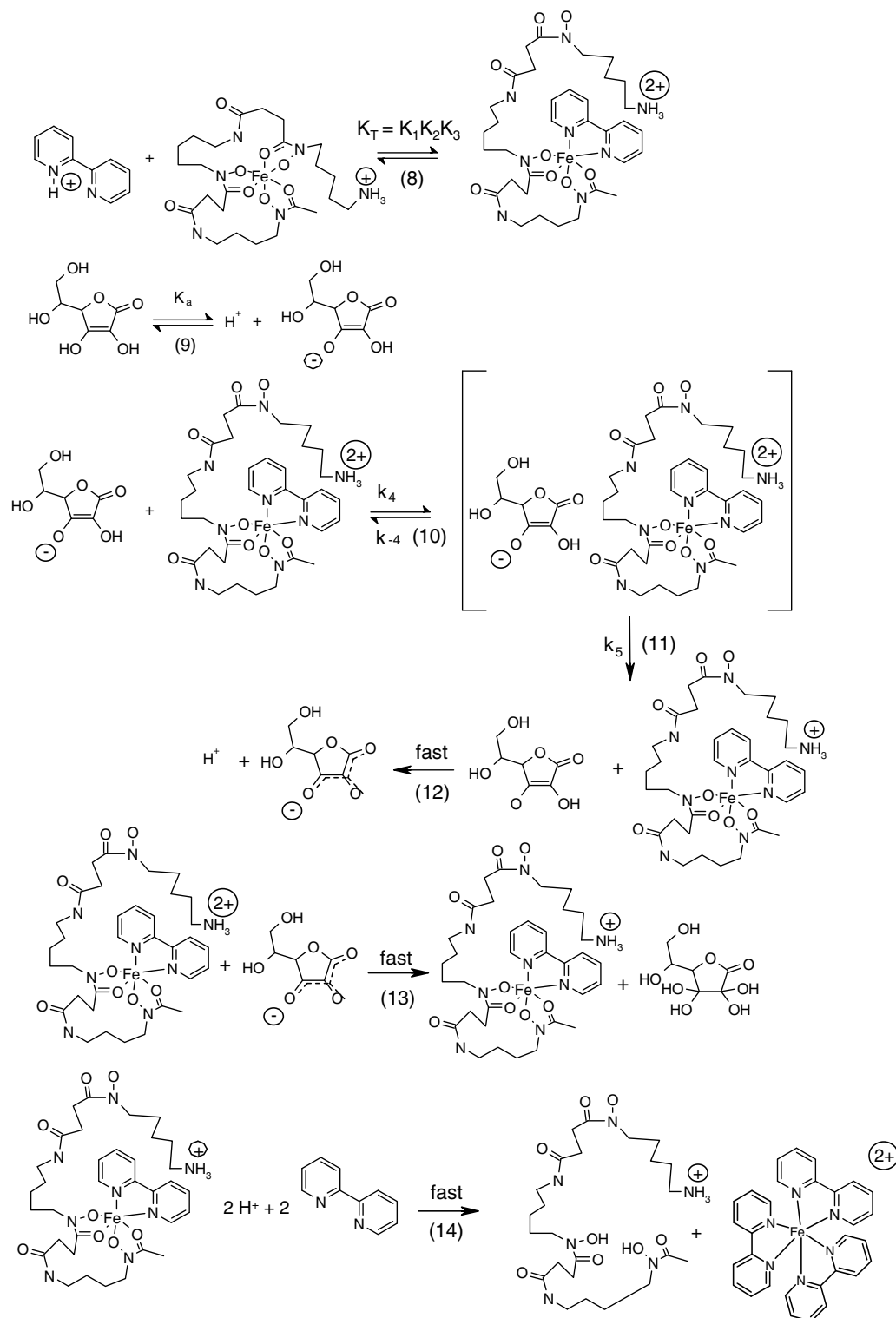


**Fig. 6.** Plot of the pseudo-first-order rate constant ( $k_{\text{obs}}$ ) for the reduction reaction as a function of ascorbate concentration. Conditions:  $[\text{Fe}(\text{HDFB})^+] = 0.095 \text{ mM}$ ,  $[\text{HA}^-] = 0.0403\text{--}0.403 \text{ M}$ ,  $[\text{bipy}] = 9.33 \text{ mM}$ ,  $[\text{MES}] = 0.1 \text{ M}$ , pH 5.96,  $T = 25 \text{ }^\circ\text{C}$ ,  $\lambda = 520 \text{ nm}$ . The equation  $k_{\text{obs}} = a[\text{ascorbate}]/(b + [\text{ascorbate}])$  was fit to the data giving the solid line shown and values of  $a = 8.8(\pm 0.5) \times 10^{-3} \text{ s}^{-1}$  and  $b = 0.21(\pm 0.2) \text{ M}$ . Error bars represent the standard deviation in  $k_{\text{obs}}$  values.

### 3.3. Kinetics of the reaction between ferrioxamine B, bipy, and ascorbate

The reaction of  $\text{Fe}(\text{HDFB})^+$ , ascorbate ( $\text{HA}^-$ ), and bipy produced an easily observable change in the UV–Vis spectrum with an isosbestic point at 585 nm and the largest change in absorbance at roughly 520 nm.<sup>1</sup> Pseudo-first-order kinetic experiments were monitored at 520 nm, with bipy and  $\text{HA}^-$  in excess, to determine

the mechanism of the reaction. The plots of  $k_{\text{obs}}$  as a function of the total bipy concentration and as a function of total ascorbate concentration reveal saturation dependence (Figs. 5 and 6). The observed rate constant did not vary over the pH range of 5.65–6.35 (average value of  $2.4(\pm 0.2) \times 10^{-3} \text{ s}^{-1}$ ).<sup>1</sup> The data is consistent with the mechanism described in Scheme 1. The mechanism includes three rapidly established pre-equilibria, the formation of the ternary complex (Reaction (8)), the acid dissociation of ascorbic



**Scheme 1.** Structural depiction of the proposed mechanism of ferrioxamine B reduction by ascorbate in the presence of bipy.

acid (Reaction (9)), and the formation of an encounter complex between ascorbate and the ternary complex (Reaction (10)). If the electron transfer (Reaction (11)) is the rate limiting step and Reactions (12)–(14) occur rapidly, then  $k_{\text{obs}}$  can be represented by Eqs. (15)–(17). For the mechanism an overall stoichiometry of two metal centers are reduced by one ascorbate molecule, which is consistent with other ascorbate reduction reactions reported in the literature [26].

$$k_{\text{obs}} = \frac{k_5 K_4 K_T [\text{HA}^-] [\text{Hbipy}^+]}{1 + K_4 K_T [\text{HA}^-] [\text{Hbipy}^+]} \quad (15)$$

$$[\text{Hbipy}^+] = \frac{[\text{bipy}]_{\text{total}}}{1 + K_2 [\text{H}^+]^{-1}} \quad (16)$$

$$[\text{HA}^-] = \frac{[\text{H}_2\text{A}]_{\text{total}}}{1 + [\text{H}^+] K_a^{-1}} \quad (17)$$

The data in Figs. 5 and 6 were fit to Eq. (8) to determine the values of  $K_4$  and  $k_5$ . Using the value of  $K_3$  determined above and the  $K_a$  value of  $\text{H}_2\text{A}$ , the value of  $K_4$  was found to be  $49(\pm 9)$ . The equilibrium constant for the encounter complex ( $K_4$ ) is consistent with reported values for ascorbate and metal complexes [27]. To determine the nature of the encounter complex the Fuoss Eq. (18) and the Debye-Hukel interionic potential (Eqs. (19) and (20)) were used to estimate the value of  $K_4$  [28].

$$K_4 = \frac{4\pi N a^3}{3000} e^{-U(a')/k_B T} \quad (18)$$

$$U(a') = \frac{z_1 z_2 e^2}{a' D} - \frac{z_1 z_2 e^2 \kappa}{D(1 + \kappa a')} \quad (19)$$

$$\kappa = \frac{8\pi N e^2 \mu}{1000 D k_B T} \quad (20)$$

This model assumes a strictly ionic interaction to form an outer-sphere association complex analogous to the Eigen-Wilkens model for complex formation [29]; where  $N$  is Avogadro's number,  $a$  is the center-to-center distance of closest approach between the two ions in cm,  $a'$  is the distance between the center of the two charges in cm,  $k_B$  is the Boltzmann constant in erg,  $e$  is the charge of an electron in esu,  $D$  is the dielectric constant,  $\mu$  is the ionic strength, and  $z_1$  and  $z_2$  are the charges of the two ions. Using the ARGUSLAB 4.0.1 [30] modeling software  $a$  and  $a'$  were estimated to be 3.8 Å and 9.3 Å, respectively. Using these values and conditions analogous to our experiments, the value of  $K_4$  was estimated from Eq. (11) to be 0.4. The calculated value is smaller than the experimental value and it appears that more than just ionic attraction is involved in the process.

From the fits of Figs. 5 and 6,  $k_5$  was determined to be  $6.9(\pm 0.5) \times 10^{-3} \text{ s}^{-1}$ . This is slightly faster than the rate of reduction of the ternary complex formed between ferrioxamine B and sulfonated bathophenanthroline ( $k = 2.1 \times 10^{-3} \text{ s}^{-1}$ ) [20]. Similar mechanisms for metal reduction by ascorbate have been reported and the rate constant for the reduction of the ternary complex by  $\text{HA}^-$  is significantly slower than for the reduction of other iron containing complexes [31].

#### 4. Discussion

The addition of an Fe(II) chelator (bipy) was shown to facilitate the reduction of ferrioxamine B ( $E_{1/2} = -482 \text{ mV}$  versus NHE at pH 7) [16]. This is a similar mechanism to previous results for bathophenanthroline, with bipy being more efficient at binding  $\text{Fe}(\text{H}_2\text{DFB})(\text{OH}_2)_2^{2+}$  (Reaction (6)) [20]. Ternary complex formation most likely facilitates the reduction by shifting the reduction potential of the Fe(III) center to a more positive value. A positive shift in reduction potential can be rationalized by Eq. (21) [16], where  $E_{\text{aq}}^{\circ}$  is the reduction potential of  $\text{Fe}(\text{OH}_2)_6^{3+/2+}$ ,  $\beta_{\text{Fe(III)}}$  is the Fe(III)

binding affinity for the inner-coordination sphere, and  $\beta_{\text{Fe(II)}}$  is the Fe(II) binding affinity of the same inner-coordination sphere.

$$E_{\text{complex}}^{\circ} = E_{\text{aq}}^{\circ} - 59.15 \text{ mV} \log \frac{\beta_{\text{Fe(III)}}}{\beta_{\text{Fe(II)}}} \quad (21)$$

In this case, we are comparing the inner-coordination sphere of the three hydroxamate groups of  $\text{Fe}(\text{HDFB})^+$  with the inner-coordination sphere of the ternary complex,  $\text{Fe}(\text{H}_2\text{DFB})(\text{bipy})^{2+}$ , where one hydroxamate group has been replaced by bipy. Inclusion of two soft nitrogen atoms from bipy in place of two hard oxygen atoms from a hydroxamate group in  $\text{Fe}(\text{HDFB})^+$  within the inner-coordination sphere of iron should increase the ability of the inner-coordination sphere to bind Fe(II) ( $\beta_{\text{Fe(II)}}$ ) and decrease its ability to bind Fe(III) ( $\beta_{\text{Fe(III)}}$ ). The net affect is a positive shift in the reduction potential for the ternary complex relative to  $\text{Fe}(\text{HDFB})^+$ , effectively tuning the reduction potential of the iron center into the range of NADH and other biologically relevant molecules. Although bipy is not a biologically important molecule there are *in vivo* many molecules with the capability to act as Fe(II) chelators and which may form ternary complexes with  $\text{Fe}(\text{HDFB})^+$ . There is evidence supporting the existence of ternary iron complexes in biological systems [32], most notable being the crystal structure of a membrane receptor, iron, and the siderophore pyochelin [33].

These results may help to explain some of the results found in the medical literature, where small amounts of ascorbate administered with desferal treatment increases the excretion of iron [14] and larger doses of ascorbate results in numerous toxic effects [15]. Ascorbate has been shown to promote iron uptake, induce iron release from ferritin, and prevent the degradation of ferritin [34]. Therefore, as ascorbate reaches normal levels more iron is available for chelation and an increase in iron excretion is expected and observed. Our results indicate that as ascorbate concentrations increase past normal levels a situation may occur where Fe(III) even in very stable complexes is reduced. It is possible that a catalytic cycle exists where ternary complexes with ferrioxamine B and/or iron carriers with labile iron sites in cells are reduced by ascorbate and reoxidized by oxygen, or other *in vivo* oxidizing agents, producing reactive oxygen species (ROS) and cell damage. This could help to explain the toxic effects observed when administering large doses of ascorbate to iron-overloaded patients. These Fenton like reactions could potentially be shut down at very high reducing agent levels as the iron is stabilized into the Fe(II) state and no longer reoxidizes to the Fe(III) state. This is consistent with the anti-oxidant effects observed at very high ascorbate levels [35].

These results in addition to being relevant to the treatment of iron-overload may also help explain how iron is released from siderophores, such as  $\text{H}_4\text{DFB}^+$ . Siderophores form iron complexes with large binding constants and extremely negative reduction potentials [36], which appears to eliminate iron reduction as a mechanism of release. These results demonstrate that iron can be released from ferrioxamine B through reduction by ascorbate on a reasonable time scale through a mechanism involving ternary complex formation. In summary, the reduction of ferrioxamine B can occur biologically and may be responsible for the side-effects related to the co-administration of ascorbate and desferal for iron-overload, and a ternary complex facilitated mechanism for the release of iron from thermodynamically and kinetically stable ferric complexes was demonstrated.

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