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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^o = −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 8.9×10^7 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 β-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 *Actinomycetes* [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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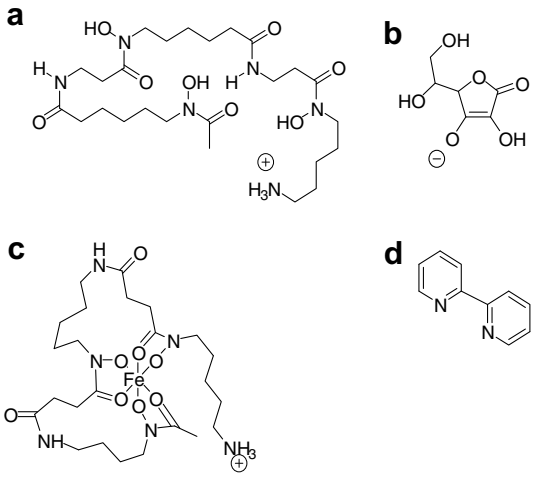

Fig. 1. Structures of (a) desferrioxamine B (H_4DFB^+), (b) ascorbate (HA^-), (c) ferrioxamine B ($Fe(HDFB)^+$), and (d) bipyridine (bipy).

2. Experimental

2.1. Materials

Ascorbic acid (H_2A; Acros Organics (99%)), $NaC_2H_3O_2$ (Fisher Scientific (99.4%)), $NaNO_3$ (Carolina Biological), and 2,2′-bipyridine (bipy; Fischer Scientific) were used as purchased. Ferrioxamine B ($Fe(HDFB)^+$) aqueous solutions were prepared by dilution of a 27.3 mM $Fe(HDFB)ClO_4$ stock solution prepared from $Fe(ClO_4)_3$ (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 $HClO_4$ (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, $Fe(H_{y+1}DFB)(bipy)_x^{y+1}$, was determined from the spectrophotometric titration of $Fe(HDFB)^+$ and bipy. To a 10 mL solution containing 0.25 mM $Fe(HDFB)^+$, 9.9 mM bipy, 0.1 M $NaNO_3$, and 0.05 M $NaC_2H_3O_2$ a series of 0.025 mL additions of $HClO_4$ 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 $Fe(HDFB)^+$ 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 $Fe(HDFB)^+$, 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_{max} shifts from the characteristic band of $Fe(HDFB)^+$ at 428–526 nm, which corresponds to the presence of $Fe(bipy)_3^{2+}$. 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.[1] 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_4DFB^+ to form a ternary complex (Reaction (1)).

$$Fe(HDFB)^+ + x\text{bipy} + yH^+ \leftrightarrow Fe(H_{1+y}DFB)(bipy)_x^{1+y} \quad (1)$$

Spectrophotometric titrations were performed to determine the stoichiometry of the reaction and the structure of the ternary complex.[1] 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_{obs} is the equilibrium absorbance measured at each pH, A_{FeHDFB} is the absorbance when the iron is all in the form of $Fe(HDFB)^+$, and $A_{ternary}$ is the absorbance when all the iron has been converted to the ternary complex, $Fe(H_{1+y}DFB)(bipy)_x^{1+y}$.

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

Eq. (2) represents a linear relationship between A_{obs} and $(A_{FeDFB} - A_{obs})/[bipy]^x[H^+]^y$. 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 $Fe(H_2DFB)(bipy)^{2+}$ 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 $Fe(HDFB)^+$ [20,23].

$$K = [Fe(H_2DFB)(bipy)^{2+}]/[Fe(HDFB)^+][H^+][bipy] \quad (3)$$

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 $Fe(HDFB)^+$ under pseudo-first-order condi-

[1] Data contained in Supplementary material.