Inorg. Chem. **2005**, *44*, 2706–2709

Release of Iron from Ferritin by Aceto- and Benzohydroxamic Acids

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Received August 23, 2004

The release of iron from ferritin by aceto- and benzohydroxamic acids was studied at two different iron chelator concentrations (100 and 10 mM), at two pH values (7.4 and 5.2), and in the presence or absence of urea. Collectively, the results demonstrate that both aceto- and benzohydroxamic acids remove iron from ferritin. Aceto- and benzohydroxamic acids penetrate the ferritin shell and react directly with the iron core of the ferritin cavity probably forming mono(hydroxamate) iron(III) complexes which exit ferritin and react with the excess hydroxamate in the solution to produce bis(hydroxamate) iron(III) complexes. The sizes of both the benzohydroxamic acid and the mono(benzohydroxamate) iron(III) complex, 6 and 7 Å, respectively, are larger than that of the ferritin channels which indicates the flexibility of the channels to allow the entry and exit of these molecules. The size of the hydroxamic acid influenced the effectiveness of the iron release from ferritin following the expected trend with smaller iron chelators showing greater effectiveness. Likewise, the percentage of iron removed from ferritin was pH-dependent; the percentage of iron removed at pH 5.2 was greater than that at pH 7.4. Finally, the presence of urea, capable of opening the ferritin channels, dramatically increased the effectiveness of the iron chelator in removing iron from ferritin, especially at pH 7.4.

Introduction

Iron is an essential element for living organisms, but it is highly toxic in excess. Living organisms store iron to provide an appropriate concentration and, at the same time, protect themselves from the toxic effects of an iron excess. The major intracellular storage form for iron is ferritin. The structure of ferritin is a spherical protein shell composed of 24 subunits surrounding an aqueous cavity with a diameter of about 8 nm which is capable of accommodating up to 4500 iron atoms as a ferrihydrite iron(III) core.[1−3] Channels are generated by the multisubunit construction of the ferritin shell. Eight hydrophilic channels of about 4 Å lead to the protein cavity.[1,2] Water, metallic cations, and molecules of appropriate size diffuse through these channels from the external solution to the cavity or from the cavity to the external solution.

The ferritin iron entry and exit processes are extraordinarily complex.[4] Much more is known about Fe(II) entry, oxidation, and mineral deposition in the ferritin cavity than about iron mobilization from ferritin.[4−9] Two mechanisms are chemically feasible for removing iron from ferritin: reduction followed by iron(II) chelation and direct iron(III) chelation. The first mechanism is thought to occur in vivo and is commonly used in the laboratory, frequently with thioglycolic acid as the reducing agent and 2,2′-bipyridyl or ferrozine as

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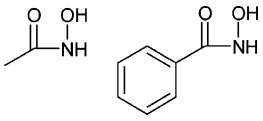
10.1021/ic048840s CCC: $30.25
Published on Web 03/15/2005

Scheme 1. Schematic Structure of Aceto- and Benzohydroxamic Acid



the iron(II) chelating agent, to produce apoferritin from ferritin.[10] The second mechanism, ferritin iron mobilization by direct iron(III) chelation, has traditionally been considered to be extremely slow.[11,12] The iron(III) chelating agent must be capable of penetrating the ferritin channels, reacting with the iron mineral, and forming the iron(III) complex which finally exits the ferritin. The second mechanism has been proposed for the release of iron from ferritin by a series of bidentate hydroxypyridinones.[12,13]

In this paper, we describe the reaction of ferritin with the aceto- and benzohydroxamic acid iron chelators (Scheme 1).

Iron(III) complexation of hydroxamate ligands has been extensively studied, mainly, because of the existence of natural hydroxamate-containing siderophores. Desferrioxamine B, a natural trihydroxamate-containing siderophore produced by *Streptomyces pylosus*, is currently used for iron chelation therapy.[14]

The aim of this study was to investigate the release of iron from ferritin through direct iron(III) chelation by hydroxamate molecules and to study how the size of the molecules and other parameters, such as pH or the presence of urea, affect their ability to remove iron from ferritin.

Experimental Section

Horse spleen ferritin (76 mg/mL, 2260 irons per ferritin) and aceto- and benzohydroxamic acids were purchased from Sigma-Aldrich. Aqueous solutions were prepared with water purified through the Milli-Q system. Ferritin (0.50 mg/mL, 1 mM in iron) was incubated with the chelator, aceto- or benzohydroxamic acid, at two different concentrations (100 and 10 mM) in 0.1 M buffer (acetate for pH 5.2 or TRIS for pH 7.4, 0.1 M NaCl) at room temperature. After 1 h, the resulting solutions were exhaustively dialyzed against several changes of water using a Spectra/Por Float-A-Lyzer with a molecular weight cutoff (MWCO) of 300 000 Da to separate the Fe(III) chelates from ferritin. The iron concentration of the dialyzed solutions was measured by atomic absorption in a Perkin-Elmer 5100 spectrometer. The same experiments were also performed in the presence of urea (10 mM) which was incubated with the ferritin for 1 h before the reaction with aceto- or benzohydroxamic acids was carried out. The experimental data were the averages of triplicate determinations. The ability of aceto- and benzohydroxamic acids to release iron from ferritin was expressed as the percentage of iron removed.

Full optimizations of the geometry of the aceto- and benzohydroxamic acids and their iron(III) complex models were done using MM2.[15] Once the global minimum energy structures were obtained, the diameter, surface area, and volume[16] were calculated using ChemProp Std Server software.[17] The bond distances and angles of the modelized structures compared well with those obtained by X-ray diffraction for related compounds.[18−22] The diameters obtained are shown in Table 1, and the modelized structures of the iron(III) complexes are shown in Figure 1.

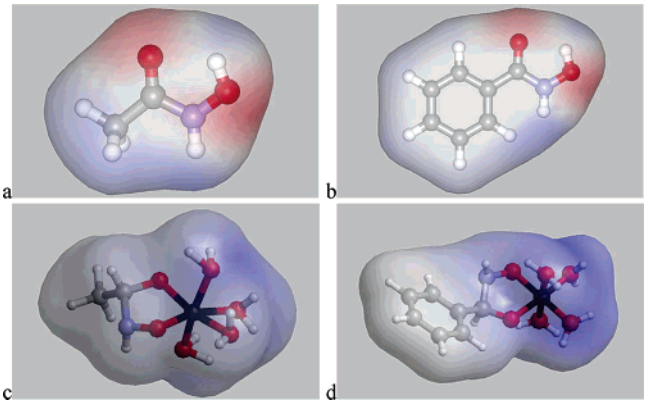


Figure 1. Modelized structures of (a) acetohydroxamic acid and (b) benzohydroxamic acid and the mono- and bis(hydroxamate) complexes, (c) [Fe(acetohydroxamate)$(H_2O)_4$] and (d) [Fe(benzohydroxamate)$(H_2O)_4$] showing the van der Waals surfaces.

Table 1. Diameters (Å) of the Modelized Structures

acetohydroxamic acid	3	benzohydroxamic acid	6
[Fe(acetohydroxamate)$(H_2O)_4$]	4	[Fe(benzohydroxamate)$(H_2O)_4$]	7

Results and Discussion

Bidentate aceto- and benzohydroxamate molecules form, depending on the ligand/iron(III) ratio, tris-, bis-, or mono-(hydroxamate) iron(III) complexes which can be identified by their UV−vis spectral characteristics: λ_{max} shifts from ∼430 to ∼460 and ∼500 nm.[23,24] The presence of the characteristic broad band of ferritin in the visible spectrum prevents spectrophotometric monitoring of the time-dependent release of iron from ferritin upon the addition of a hydroxamate ligand. The UV−vis spectra of the solutions, obtained after the ferritin and hydroxamic acid were incubated for 1 h, consisted of a broad band corresponding to the mixture of ferritin and the hydroxamate iron(III) complex (Figure 2-1) which disappeared after the solution was dialyzed resulting in the typical spectrum for ferritin with a

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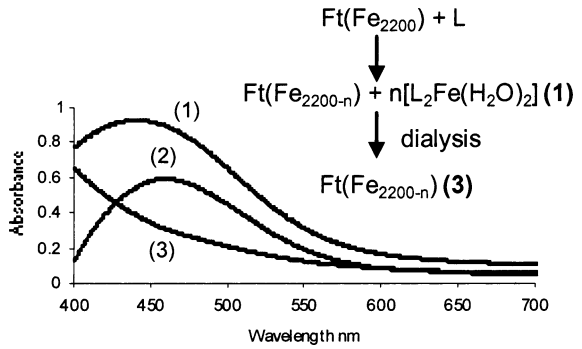

Figure 2. UV−vis spectra of (1) solution resulting from the ferritin + L (hydroxamic acid) reaction before dialyzing and (3) after dialyzing and (2) the difference between spectrum 1 and spectrum 3 with the general schematic reaction.

smaller amount of iron (Figure 2-3). Subtraction of the initial and final spectra (before and after dialyzing) produced a spectrum with a single absorption centered in the range 452−458 nm (Figure 2-2), depending on the hydroxamic acid used. Similar spectra were obtained in the dialysis reservoir.

These results are consistent with the suggestion made by Hider et al. for the release of iron from ferritin by bidentate hydroxypyridinone molecules. They proposed that the iron-chelating molecule penetrates the ferritin shell reacting directly with the iron core in the cavity to form the mono-(hydroxamate) iron(III) complex which leaves the ferritin and reacts with the excess hydroxamate in the solution to give the tris(hydroxamate) iron(III) complex.[12,13] However, the iron species we detected outside the ferritin absorbed in the range of 452−458 nm which is characteristic of bis- rather than tris(hydroxamate) iron(III).[24]

In principle, since the ferritin channels are only 4 Å in diameter, only hydrophilic molecules with a molecular cross section smaller than the channel diameter can enter the ferritin cavity. Thus, acetohydroxamic acid (diameter of 3 Å) and the monoacetohydroxamate iron(III) complex (diameter of 4 Å) (Table 1) were able to penetrate and leave, respectively, the ferritin cavity. However, several studies have demonstrated that molecules with a diameter considerably larger than 4 Å are able to enter the ferritin cavity;[25,26] this indicates that the ferritin channels are sufficiently flexible to permit the access of the larger molecules. The present study provides further evidence of this flexibility because benzohydroxamic acid (diameter of 6 Å) and the monobenzo-hydroxamate iron(III) complex (diameter of 7 Å) (Table 1) were also shown to be able to enter and exit the ferritin cavity.

The amount of iron removed from the ferritin depends on the hydroxamic acid used, its concentration, the pH, and the presence or absence of urea. Figure 3 shows the percentage of iron removed from ferritin by aceto- and benzohydroxamic acids at pH 5.4 and 7.4, at 100 and 10 mM, and in the presence and absence of urea.

As shown in Figure 3, the iron release from ferritin was dependent on the ligand concentration, although the increase

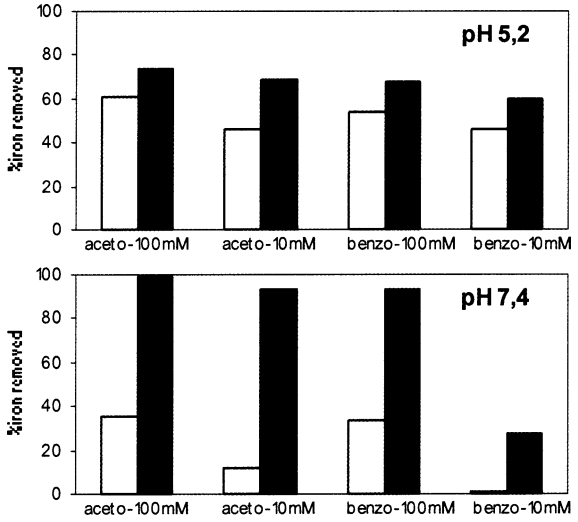

Figure 3. Comparison of percentage of iron removed from ferritin by aceto- and benzohydroxamic acids. The black bars correspond to the experiments carried out in the presence of urea.

was not linear with the concentration of hydroxamic acid. At the same concentration, more iron was removed from the ferritin by the acetohydroxamic acid than by the benzohydroxamic acid. This must be a consequence of the smaller size of the acetohydroxamic acid which increases its access to the ferritin cavity compared to that of benzohydroxamic acid. This trend was followed regardless of the pH and hydroxamic acid concentration used.

The efficiency of the release of iron from ferritin by the hydroxamate ligand was increased when the pH was lowered from 7.4 to 5.2 (Figure 3). A similar observation was made by Hider et al. for the release of iron from ferritin by bidentate hydroxypyridinone; they reported a more marked influence of pH with negatively charged ligands.[12] This behavior can be explained by the negative charge of the ferritin surface at high pH; it decreases in magnitude as the pH is reduced to the pI (4.6). This negative charge would repel the partially deprotonated fraction of the aceto- and benzohydroxamic acids, decreasing the amount of the iron chelator entering the ferritin cavity and therefore reducing the effectiveness of the iron release. The fact that the pH has a more marked effect on benzohydroxamic acid than it does on acetohydroxamic acid must be a consequence of its lower pK_a (8.90 for benzohydroxamic acid vs 9.20 for acetohydroxamic acid); this means that a larger amount of the benzohydroxamic acid is negatively charged at these pH values than of acetohydroxamic acid. The idea that only the neutral aceto- and benzohydroxamic molecules would penetrate the ferritin channels is consistent with the experiments carried out by Chasteen et al. on the diffusion into ferritin of radical molecules; they concluded that only the neutral and positively charged molecules could penetrate the ferritin channels, whereas the negative ones do not.[27,28]

The influence of the presence of urea on the iron release from ferritin by aceto- and benzohydroxamic acid was greater

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than that of the above parameters, especially at pH 7.4. The presence of urea (10 mM) dramatically increased the amount of iron removed from ferritin by either aceto- or benzohydroxamic acid at 100 or 10 mM (Figure 3).[25] As can be seen in Figure 3, the release of iron from ferritin was complete at 1 h in the presence of urea, even at a low concentration of acetohydroxamic acid. A dynamic structural model with a partial dissociation of the apoferritin could be envisioned to explain these findings. However, Theil et al. demonstrated that apoferritin does not dissociate into subunits, even in the presence of concentrations of urea higher than that used in the present work,[29] and therefore, this behavior must be a consequence of the great flexibility of the ferritin channels. Interestingly, the role of urea was more pronounced at pH 7.4 than it was at pH 5.2 so that the presence of urea had a greater influence than any other parameter analyzed, including the size of the hydroxamic acids and the pH. This may be caused by the higher negative charge of the ferritin surface at pH 7.4 than at pH 5.2 which would favor the ferritin channel−urea interaction; this hypothesis requires further exploration.

The present results show that the release of iron from ferritin by aceto- and benzohydroxamic acids is not as slow as that traditionally considered for direct iron chelation. Thus, under conditions of a 10-fold molar excess of acetohydroxamic acid (10 mM) with respect to ferritin (1 mM in iron), 60% of the iron is removed in 1 h at pH 5.2. Moreover, the presence of urea drastically enhances the rate of iron mobilization from ferritin so that complete iron release is achieved in some cases. Likewise, the results unambiguously show that the ferritin channels are flexible permitting the entry and exit of molecules with a cross section larger than the measured diameter of the ferritin channels.

Acknowledgment. Support from the Spanish Ministerio de Ciencia y Tecnología (MCT Project BQU2001/3221) and the European Community (MERG-CT-2004-508033) is gratefully acknowledged.

IC048840S

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