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Comparison of the kinetics of iron release from a marine (*Trichodesmium erythraeum*) Dps protein and mammalian ferritin in the presence and absence of ligands

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This paper is dedicated to the memory of Edward I. Stiefel.

Abstract

The ferritin superfamily of iron storage proteins includes ferritin proper and Dps (DNA binding protein from starved cells) along with bacterioferritin. We examined the release of Fe from the Dps of *Trichodesmium erythraeum* (Dps_{tery}) and compared it to the release of Fe from horse spleen ferritin (HoSF) under various conditions. Both desferrioxamine B (DFB), a Fe(III) chelator, and ascorbic acid were able to mobilize Fe from Dps_{tery} at rates comparable to those observed for HoSF. The initial Fe release rate from both proteins increased linearly with the concentration of DFB, suggesting that the chelator binds to Fe in the protein. A small but significant rate obtained by extrapolation to zero concentration of DFB implies that Dps_{tery} and HoSF might release Fe(III) spontaneously. A similar result was observed for HoSF in the presence of sulfoxine. In a different experiment, Fe(III) was transferred from holoferritin to apotransferrin across a dialysis membrane in the absence of chelator or reducing agent. The apparent spontaneous release of Fe from HoSF and Dps_{tery} brings forth the hypothesis that the Fe core in Fe storage proteins might be continuously dissolving and re-precipitating *in vivo*, thus maintaining it in a highly reactive and bioavailable form.
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1. Introduction

Iron storage proteins play a crucial role in cellular iron homeostasis as they provide the cell with a temporary or a longer-term store for excess intracellular iron. The superfamily of iron storage proteins formally known as ferritins include non-heme ferritins from plants, animals, archea, and bacteria, heme containing bacterioferritin, and Dps proteins (DNA binding protein from starved cells) from bacteria and archea. While distantly related in terms of sequence, these three types of iron storage proteins preserve many structural and functional similarities. Ferritin and bacterioferritin are composed of 24 subunits that form a spherical protein shell with a central cavity capable of storing a maximum of 4500 Fe(III) atoms for ferritin and 2000 Fe atoms in bacterioferritin [1–3]. The protein shell of Dps comprises 12 subunits with a central cavity that has a lower storage capacity (500 Fe(III) atoms) [4–6]. The 24 subunits assemble into a 4-3-2 symmetry with channels formed at the fourfold and threefold axes [1,7]. Two

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types of channels are present; eight are primarily composed of hydrophilic amino acid residues (threefold axis) and six are lined with hydrophobic residues (fourfold axis) [1,8]. It has been postulated that the entry and exit paths for Fe atoms occur though these protein channels [8,9]. Mutations targeting the threefold pore and the presence of chaotropes have been shown to have a dramatic effect on the rate of Fe release suggesting a dynamic pore [9–11].

For ferritin and bacterioferritin the uptake of Fe involves the oxidation of ferrous iron by O_2 at the binuclear ferroxidase center. The di-iron center is located with helix bundles that form the subunit [1,8]. The resultant Fe(III) ions then migrate to the protein cavity and become incorporated into the mineral core. The structure of the Fe core appears to be related to the mineral ferrihydrite [2]. Unlike ferritin or bacterioferritin, the ferroxidase center for Dps proteins is located at the interface of adjacent subunits [12,13]. The Dpr/Dps (Dpr, Dps-like peroxide resistance protein) protein from *Streptococcus suis* incorporates ferrous iron through four negatively charged hydrophilic pores found in the protein [14]. In *S. suis*, the oxidation of Fe(II) by molecular oxygen occurs at the ferroxidase center [14]. In *Escherichia coli*, H_2O_2 has been shown to be a more effective oxidant for Fe(II) than molecular oxygen [15]. The Fe core for Dps proteins has not been studied in detail but XANES (X-ray absorption near edge structure) of Dps_{tery} (Dps from *Trichodesmium erythraeum)* indicates that the Fe core is composed of octahedrally coordinated Fe(III) as in ferritin [16].

The recovery of Fe from ferritin in the cell is crucial for biosynthesis and is also of great interest to medical research because the safe removal of Fe from ferritin is a goal of chelation therapies. The process involved in the dissolution of the Fe core *in vivo* remains unsolved, however. *In vitro* experiments show that Fe mobilization can occur in the presence of Fe(III) chelators or, more effectively, in the presence of reductants [17–19]. Whether reductants and chelators penetrate the protein shell has been a matter of some controversy. It appears that the charge of the molecule may be important in this process [20–22].

Iron mobilization has been as extensively studied in ferritin but not in Dps. Here we compare the mobilization of Fe from Dps_{tery} and from HoSF in the presence of desferrioxamine B (DFB) and ascorbic acid as well as in the absence of any chelator or reducing agent.

2. Materials and methods

Bovine apotransferrin, apo- and holo- horse spleen ferritin, desferroximane B, sulfoxine, and L-ascorbic acid were obtained from Sigma. The purity of these proteins was checked by native and SDS protein gels. The recombinant *T. erythraeum* IM101 Dps protein (Dps_{tery}) was prepared and purified as described in Castruita et al. [16]. Fe loading was carried out by incubating Dps_{tery} (100 μg/ml) in a solution with 500 μM freshly prepared iron (II) ammonium sulfate hexahydrate and the preparation was left at room temperature for 18 h. The protein–iron solution was washed with a solution of 50 mM EDTA, 100 mM oxalate (pH 7.0) to remove nonspecifically bound Fe [23]. The EDTA–oxalate solution (250 μl) was added to the protein solution followed by 10 min of incubation and then the EDTA–oxalate solution was removed by centrifugation at 3000*g* for 30 min in a Centricon 10 tube (Amicon). It has been shown that, in such treatment, EDTA and oxalate act synergistically to rapidly sequester precipitated or adsorbed Fe [23]. This treatment should be very effective to remove Fe bound to the outside of the protein; it may also remove some of the Fe from the protein core but this is of no consequence for our experiments. To remove all soluble Fe complexes from the supernatant, 1 ml of 50 mM Tris–HCl (pH 7.0) buffer was added and centrifuged for an additional 30 min (repeated twice). Horse spleen ferritin was washed with the EDTA–oxalate as explained for Dps_{tery} to remove nonspecifically bound Fe.

To quantify Fe, the method reported by Stookey was used and modified for protein use [24]. Protein (10 μl) was added to trace-metal clean 1M HCl (50 μl) and incubated at room temperature for 1 h then the solution was neutralized by adding 50 μl trace-metal clean NaOH. To this solution then 500 μl of 2 M sodium dithionite was added and incubated for 30 min. Finally, 250 μl of 4 mM ferrozine and 250 μl of 0.5 M sodium acetate were added and incubated for 18 h. The absorption of the Fe–ferrozine bond at 562 nm was used to quantify Fe.

2.1. Dissolution experiments

Iron release from ferritin and Dps_{tery} in the presence of desferrioxamine B was followed at the wavelength 493 nm. In 50 mM Tris–HCl buffer (pH 7.0), Dps_{tery} or ferritin was incubated at 25 °C with the appropriate concentration of desferrioxamine B (50 μM, 100 μM, 200 μM, and 1 mM). The complexation of Fe(III) by the chelator was monitored by a UV–visible spectrophotometer. The final protein concentration in the Dps_{tery} experiment was ~70 nM, corresponding to 20 μM Fe in the core (ca. 280 Fe atoms/protein). For ferritin, the final protein concentration was 16 nM, corresponding to 33 μM Fe (ca. 2000 Fe atoms/protein). The reduction and Fe release from Dps_{tery} was measured spectrophotometrically at 562 nm. Dps_{tery} (70 nM Dps_{tery}, 20 μM Fe) was incubated with 100 μM ferrozine and 1 mM ascorbate acid. All experiments were repeated in duplicate sets (except for HoSF in 500 μM DFB). The results are expressed as the mean ± SD. For the determination of the rate constants, the molar absorption of $\varepsilon_{562} = 27.9\ mM^{-1}\ cm^{-1}$ for the Fe–ferrozine complex and for desferrioxamine B $\varepsilon_{493} = 2280\ M^{-1}\ cm^{-1}$ were used. Iron release from ferritin in the presence of sulfoxine was followed at the 572 nm. Ferritin (18 nM ferritin, 35 μM Fe) was incubated with the appropriate sulfoxine concentration (100 μM, 200 μM, 500 μM, and 1 mM) in 50 mM Tris–HCl buffer (pH 7.0). The molar absorption

of the Fe(III)–sulfoxine complex, $\varepsilon_{572} = 5230\ M^{-1}\ cm^{-1}$ was used to determine the rate constants.

2.2. Dialysis of ferritin against apotransferrin

A solution of ferritin (1.24 μM ferritin, 2.48 mM Fe) was placed in Slide-A-Lyzer 10 K cassette (PIERCE), 10,000 MWCO (molecular weight cut-off) and placed in trace-metal clean polycarbonate bottle that contained a solution of 0.27 mM of apotransferrin in 1 M HEPES, 50 mM $NaHCO_3$, pH 7.4. The dialysis cassette was soaked in a solution of 1 M EDTA for 3 days prior to the experiment to remove metal contamination and rinsed and soaked in MilliQ water for three days. Dialysis was performed at 4 °C with constant stirring in the dark. One ml aliquots of the transferrin solution were measured at a wavelength of 465 nm with a Cary 100 Bio UV–visible spectrophotometer. The same experiment was repeated with a 1 M Tris–HCl, 50 mM $NaHCO_3$, pH 7.4 and 0.27 mM of apotransferrin solution and ferritin (1.22 μM ferritin, 2.44 mM Fe). Apotransferrin in TRIS or HEPES but in the absence of ferritin was used to normalize the absorbance measured for dialysis of apotransferrin and ferritin.

3. Results

3.1. Dissolution of the Fe core by chelation and reduction

We observed increasing rates of Fe release from Dps_{tery} and horse spleen ferritin (HoSF) with increasing concentration of DFB (Fig. 1a and b). The release rate remained constant for at least 10 h for DFB concentrations up to 200 μM. But at the concentration of 1 mM DFB we observed for both proteins a biphasic reaction which slowed down around 2–3 h. A similar biphasic dissolution occurred in the presence of 1 mM ascorbic acid (plus 100 μM ferrozine to trap Fe(II)) (Fig. 1a). But the initial rate in the presence of ascorbic acid was much faster than in the presence of DFB resulting in nearly 23% of the core dissolved within 120 min. Both the acceleration of the dissolution in the presence of reductant and its biphasic kinetics are consistent with previous observations [15,18]. The removal of ca. 250 Fe/ HoSF in 5 h in the presence of 1 mM DFB is about twice as much as observed in a previous study [17], probably reflecting a higher Fe content per protein in our study.

The initial dissolution rates of both Dps_{tery} and ferritin cores (estimated from the initial slopes of the graphs in Fig. 1) increased linearly with DFB concentrations. The second order rate constants obtained by normalization the slope of the regression lines to the Fe concentration in the proteins are essentially identical for Dps_{tery} and HoSF: $8.75 \times 10^{-3}\ M^{-1}\ s^{-1}$ $(=1.05 \times 10^{-5}\ min^{-1}/(2 \times 10^{-5}\ M \times 60\ s\ min^{-1}))$ and $8.84 \times 10^{-3}\ M^{-1}\ s^{-1}$ $(=1.75 \times 10^{-5}\ min^{-1}/(3.3 \times 10^{-5}\ M \times 60\ s\ min^{-1}))$, respectively.

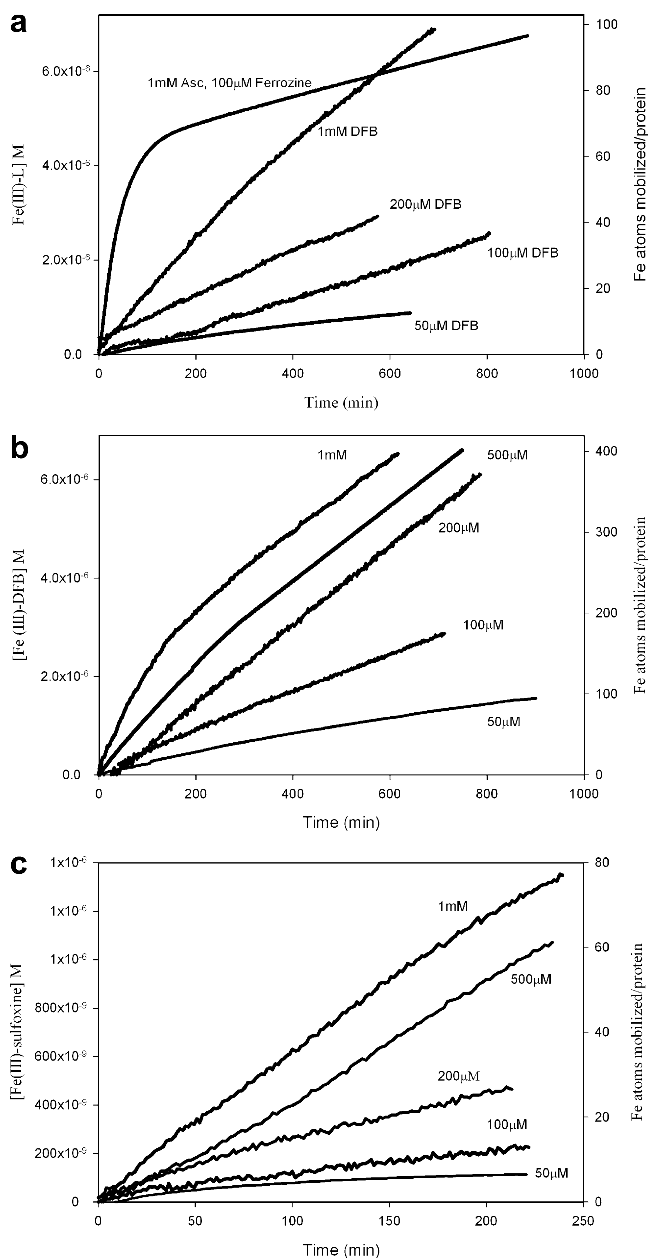

Fig. 1. Iron core dissolution of ferritin and Dps_{tery}. The dissolution of 20 μM Fe–Dps_{tery} (a) and of 33 μM Fe–HoSF (b) in the presence of DFB was followed spectrophotometrically at 493 nm. Dissolution of the Fe core of Dps_{tery} by ascorbic acid was followed spectrophotometrically at 562 nm (a). Dissolution in the presence of sulfoxine was followed spectrophotometrically at 572 nm for 35 μM Fe–HoSF (c).

Extrapolation of the data of Fig. 2 to zero DFB concentration shows a significant intercept ($k_0 = 3.5\ nM\ min^{-1}$ for Dps, and $k_0 = 3.33\ nM\ min^{-1}$ for HoSF) implying a small but significant rate of Fe release may be occurring even in the absence of chelating agents. To verify that this phenomenon is not resulting from some different reaction mechanism at very low DFB concentrations, we repeated the experiment, using sulfoxine as the chelating agent. The dissolution kinetics of HoSF in the presence of sulfoxine are much lower than with DFB but they extrapolate to a reasonably similar value, $k_0 = 2.74\ nM\ min^{-1}$, for a

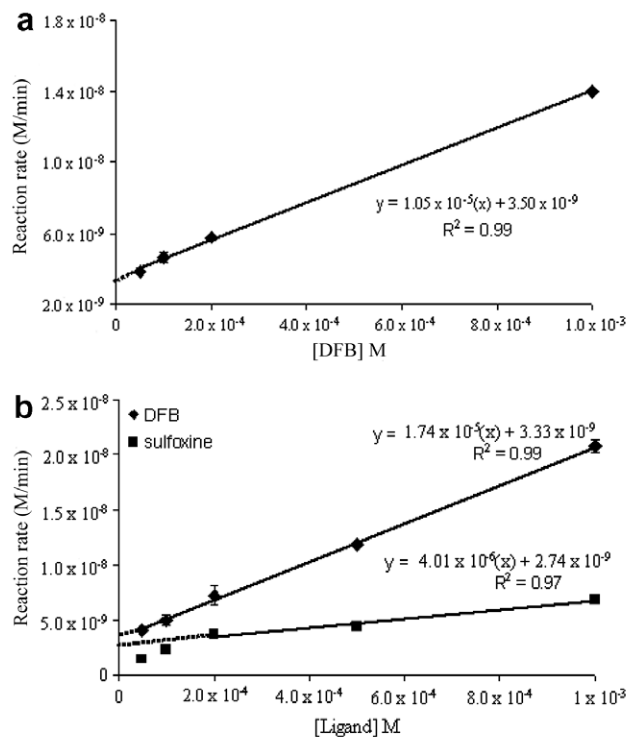

Fig. 2. First-phase dissolution reaction rates for Dps_{tery} (a) and HoSF (b) in the presence of Fe(III) chelators. Initial dissolution rates in the presence of ligands appear to be first order with respect to the ligand concentration. Extrapolation of the reaction rates to zero ligand concentrations show a ligand independent dissolution process with reaction rates of 3.50 nM min^{-1} for 20 μM Fe–Dps_{tery} in the presence of DFB, 3.33 nM min^{-1} for 33 μM Fe–HoSF (b) in the presence of DFB, and 2.75 nM min^{-1} for 35 μM M Fe–HoSF in the presence of sulfoxine.

similar protein concentration. The second order rate constant of 1.91 $mM^{-1} s^{-1}$ for dissolution by sulfoxine, obtained by dividing the slope of the line on Fig. 2b by the Fe concentration in the protein ($=4.01 \times 10^{-6}$ min^{-1}/(3.5 10^{-5} M × 60 s min^{-1})) is markedly lower than the value obtained for DFB. As discussed below, this slower release rate with sulfoxine as well as the progressive deviation from the linear trend at low ligand concentrations (seen for the sulfoxine but not the DFB data) is consistent with the inherently slow rate of reaction of sulfoxine with Fe(III).

3.2. Spontaneous dissolution of the Fe core

In an attempt to verify that Fe release from the core of the protein indeed occurs in the absence of a chelator, as implied from the extrapolation of the above data to zero ligand concentrations, we performed an experiment where holoferritin was placed in a dialysis bag and dialyzed against apotransferrin. The dialysis membrane prevented direct interaction of the two proteins. The experiment was carried out in the dark to prevent any possible photochemical reduction of the Fe core and at 4 °C to minimize protein degradation. To remove all Fe associated with the outer surface of the protein, the ferritin was carefully washed with an EDTA–oxalate solution prior the experiment (see Section 2). As seen in Fig. 3, we observed a steady formation of the Fe(III)–transferrin complex, indicating release of Fe from HoSF in the absence of chelating or reducing agent. Repeating the experiment in the presence of two different buffers (TRIS and HEPES) yielded similar kinetics, making it unlikely that the metal exchange is caused by the buffer itself. After 4 days, the Fe concentration in the transferrin matched approximately the measured decrease in the concentration of Fe in the ferritin (0.17 mM vs. 0.37 mM in TRIS and 0.20 mM vs. 0.21 mM in HEPES).

4. Discussion

Relatively little is known of the release of Fe from Dps. Our results show that the presence of the Fe(III) chelator, DFB, leads to similar rates of release of ferric iron from Dps_{tery} and HoSF. Reduction of Fe(III) by ascorbic acid results in a faster initial mobilization of Fe from the Dps core. The two kinetic phases observed at high concentrations of DFB and ascorbate have been reported in *E. coli* Dps, pig spleen ferritin, and *Azotobacter vinelandii* bacterioferritin [15,25,26]. Different Fe populations within the protein cage, or heterogeneity in the iron core are thought to be responsible for the two kinetic phases. The first phase likely corresponds to the dissolution of Fe(III) at the surface of the core and the second phase to bulk mineral.

Analysis of the kinetics for the first phase of the Fe release yields remarkably similar second order rate constants for Dps_{tery} and HoSF in the presence of DFB. This is somewhat unexpected since the phosphate content, which has been shown to accelerate the release of Fe from

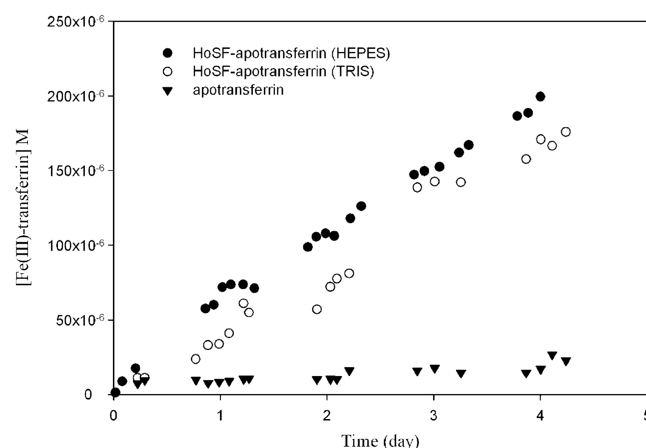

Fig. 3. Time-course of the formation of the Fe(III)–transferrin complex. Self-dissolution of the HoSF Fe core releases Fe(III) that is then complexed by transferrin. An apotransferrin solution lacking ferritin shows minimal formation of Fe(III)–transferrin complex indicating that background Fe contamination is not responsible for the observed Fe(III)–transferrin complex in the presence of ferritin. TRIS and HEPES did not promote Fe core dissolution as the reaction rate is the approximately the same for both experiments (3.29×10^{-8} M min^{-1} for TRIS and 3.07×10^{-8} M min^{-1} for HEPES).

HoSF [27], is twice as high in our sample of HoSF compared to Dps_{tery} (P:Fe = 1:13 vs. 1:26) [16]. Perhaps phosphate effects are greater for the bulk mineral than for the surface, or the effect of the lower P content of Dps_{tery} is fortuitously compensated by the smaller core. The relatively large surface/volume of smaller Fe cores results in a greater area for Fe release that leads to a faster rate of dissolution [28]. The smaller cavity size of Dps might be expected to result in smaller Fe cores and inherently faster dissolution rates for a similar P content. A property of the protein nanocages that has not been compared is the differential stability of Dps and mammalian ferritin protein subdomains. Overall, the similar kinetics and pattern of release of Fe from Dps_{tery} and HoSF in the presence of desferroxamine B and ascorbic acid are consistent with similar core structures in the two proteins.

The fact that the kinetics of release of Fe from the storage proteins are roughly first order with respect to the ligand concentration suggests that the part of the mineral that is dissolving is in direct contact with the ligand. If this is case the dissolution of the core must occur via formation of a surface complex [29] as has been invoked in the case of dissolution by reducing agents [19,30]. The difference that is seen in the kinetics of the ligand-assisted dissolution of the protein core between the DFB and sulfoxine experiments is consistent with the much slower rate of reaction of sulfoxine with Fe compared to DFB [31,32].

In all our experiments in the presence of ligands, the extrapolation of the Fe release data to zero ligand concentrations yields finite Fe release rates, indicating that a small fraction of the Fe may be released from the proteins in the absence of ligands. Such spontaneous Fe release likely represents simply the dissolution of the Fe(III) core in an undersaturated solution, or the dynamics of pore unfolding [10,11]. Like the rate of ligand-assisted dissolution, the rate of spontaneous dissolution should not be exactly first order with respect to the Fe concentration since it must depend on the size of the individual cores. Nonetheless a normalization of the extrapolated release rates to the Fe concentration in the proteins yields first order rate constants for self release that are convenient for comparison purposes. The calculated rate constant for the ligand-independent release from Dps_{tery} is roughly twice that from HoSF: $2.92 \times 10^{-6}\ s^{-1}$ vs. $1.71 \times 10^{-6}\ s^{-1}$ (calculated from the extrapolation of the DFB data: $3.5 \times 10^{-9}\ M\ min^{-1}/(2 \times 10^{-5}\ M \times 60\ s\ min^{-1})$ for Dps, and $3.33 \times 10^{-9}\ M\ min^{-1}/(3.3 \times 10^{-5}\ M \times 60\ s\ min^{-1})$ for HoSF). The values obtained for HoSF in the presence of DFB and sulfoxine, $1.71 \times 10^{-6}\ s^{-1}$ and $1.31 \times 10^{-6}\ s^{-1}$ ($2.75 \times 10^{-9}\ M\ min^{-1}/(3.5 \times 10^{-5}\ M \times 60\ s\ min^{-1})$) are reasonably similar, particularly in view of the factor of five difference in the second order rate constants obtained from the data at high ligand concentrations. There is a wide range of values reported for the dissolution rate of ferrihydrite, the structure of which is thought to be similar to that of the protein core. The first order dissolution rate constant reported for 4–7 day old ferrihydrite, whose nuclearity is presumably higher than that of Dps or ferritin cores, ranges from $1.5 \times 10^{-7}\ s^{-1}$ to $4.8 \times 10^{-6}\ s^{-1}$ [33,34].

Slow complexation kinetics explain the deviation from linearity observed at low sulfoxine concentrations (only the data for three highest sulfoxine concentrations were used in the extrapolation; see Fig. 2b). Using lower and upper limits on the complexation rate constants of $<5 \times 10^3\ M^{-1}\ s^{-1}$ for sulfoxine and $>10^6\ M^{-1}\ s^{-1}$ for DFB (obtained from ionic strength corrections of the data in [31,32]), consider, for example the data at 5×10^{-5} M ligand concentrations. For a release rate of ca. 3 nM min^{-1}, a dissolved Fe(III) concentration at steady state with sulfoxine binding should be larger than 200 pM while it should be at least 200 times smaller (<1 pM) at steady state with DFB. The former is above the solubility limit for Fe(III) at neutral pH while the latter is not [33]. Net self dissolution requires efficient scavenging of the dissolved Fe(III). This is not the case at low sulfoxine concentrations, resulting either in re-precipitation of Fe(III) outside the protein or a slower net dissolution of the core.

The problems of precipitation of Fe(III) or its back reaction with the protein preclude a direct study and quantification of the spontaneous release of Fe(III) from ferritin in the absence of chelating or reducing agents. But a qualitative confirmation of that process is given by the transfer of Fe(III) from ferritin to transferrin across a dialysis membrane. Unfortunately the slow transfer of Fe(III) through the dialysis membrane does not permit the calculation of a rate constant from this experiment. We note that great care was taken to control Fe contamination during this experiment and that about 10% of the initial Fe concentration was transferred from the ferritin to the transferrin, much more than could be accounted for from the trace concentrations that could have remained bound to the outside of the protein after the oxalate–EDTA wash.

Many studies of Fe release from storage proteins have focused on the role of chelating and reducing agents [17,18,27]. A few previous studies also suggest that small amount of Fe are released in the absence of added chelator. For example, Bauminger et al. showed that monomeric Fe(III) can be exchanged among ferritin minerals in different ferritin molecules [35]. It has also been reported that transfer of Fe(III) to heterologous ferritins occurs suggesting that this process does not requires specific inter-molecular contact [36]. In addition, Bakker and Boyer demonstrated that in the presence of buffer ions that can facilitate Fe transfer Fe(II) oxidized by the ferroxidase center of ferritin could be transferred to transferrin in excess rather than incorporated in the ferritin core [37]. Finally in a recent study, continuous release of Fe was observed from the Dps protein of *Deinococcus radiodurnas*, a radiation resistant chemohetero-organotroph, and, based on the crystal structure, attributed to the unusual properties of the protein Fe-exit channels [38]. (Ferritin protein channels are reviewed in Ref. [39].)

It is thought that Fe(III) concentrations in cells are below saturation of Fe oxyhydroxide precipitates. If this

is the case, spontaneous release of Fe from Fe-storage proteins might occur *in vivo*, as it does in our experiments. This Fe release would then have to be balanced by re-incorporation into the core. Such re-incorporation should be efficient in view of the generally reducing conditions inside cells. On the basis of our results, we thus propose the hypothesis that a small amount of Fe might be continuously released from the core and re-incorporated into Fe storage proteins *in vivo*, unless the Fe- exit channels were closed by a cytoplasmic regulator absent in the *in vitro* studies. Such continuous dissolution-precipitation process might insure that the Fe core is always "freshly precipitated" and thus highly bioavailable [33]. As demonstrated in many studies, including ours, the presence of reducing or chelating agents can greatly accelerate the rate of release of small amounts of Fe from ferritin proteins when needed.

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