

# BINDING OF $\text{Fe}^{2+}$ BY MAMMALIAN FERRITIN

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## Abstract

An average of 140  $\text{Fe}^{2+}$  ions bind to mammalian ferritin that has an average core content of 1876  $\text{Fe}^{3+}$  ions per molecule. The  $\text{Fe}^{2+}$  ions enter the protein interior and exchange electrons with the core  $\text{Fe}^{3+}$  ions. A non-homogeneous model for the iron-containing core of ferritin is proposed.

## 1. Introduction

Strategies for the biological storage of iron in such a way that it is available for metabolic needs, yet nontoxic, center on a widely distributed class of proteins, the ferritins. Ferritins are found in organisms as diverse as bacteria [1,2] and mammals [3]. Mammalian ferritin is a roughly spheroidal, 120 Å diameter protein with a core of up to 4500 iron atoms in the 70 Å diameter protein interior cavity [4]. The protein shell is composed of 24 nearly identical sub-units that are arranged to isolate the iron-containing core from the cellular environment. Six hydrophilic and eight hydrophobic channels provide access to the protein interior, presumably for electrons, protons and iron ions, and other small ions and molecules.

The ferritin iron core is a hydrous ferric oxide phosphate with nominal formula  $(\text{FeOOH})_8 (\text{FeO} \cdot \text{H}_2\text{PO}_4)$  and a structure similar to the protocrystalline mineral ferrihydrite, in which  $\text{Fe}^{3+}$  ions have six-fold oxygen coordination and oxygens are hexagonally close-packed [5]. Iron is removed from the protein slowly by  $\text{Fe}^{3+}$  chelators and more rapidly by reductants and  $\text{Fe}^{2+}$  chelators [6]. In the absence of chelators, the core can be reduced by up to one electron per iron atom, with all the reduced iron retained in the protein [7]. Reduction is accompanied by the uptake of two protons from the external medium for every electron transferred to  $\text{Fe}^{3+}$ .

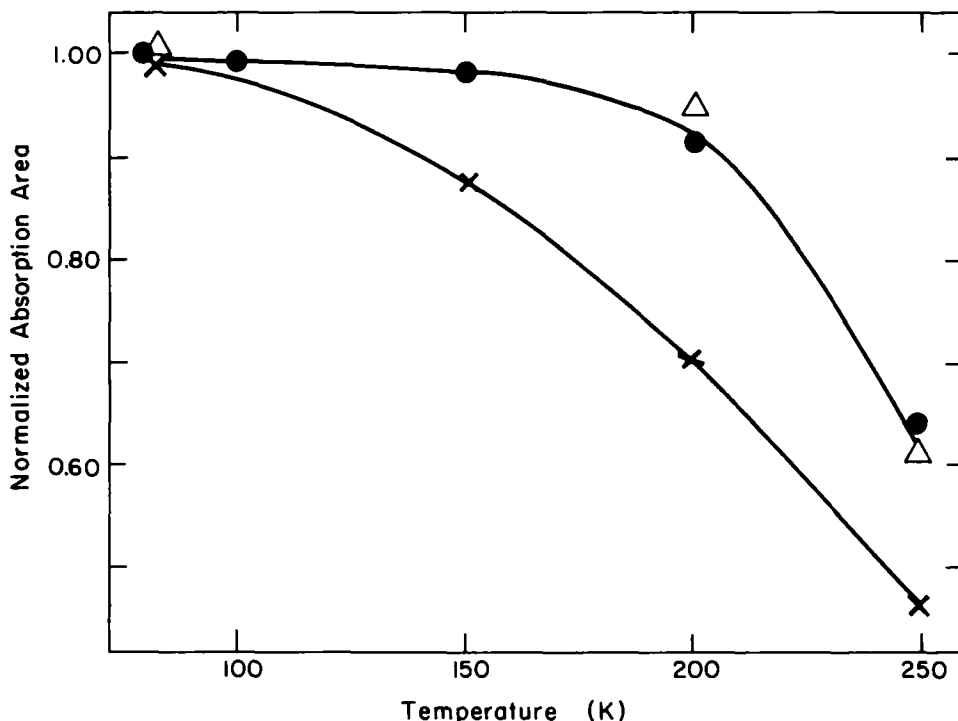


Fig. 1. Temperature dependence of the absorption intensity for (●) Fe<sup>3+</sup> in ferric ferritin, (Δ) Fe<sup>3+</sup> in 50% reduced ferritin, and (X) Fe<sup>2+</sup> in 50% reduced ferritin, normalized to 80 K.

Mössbauer spectroscopy has been used to distinguish Fe<sup>2+</sup> and Fe<sup>3+</sup> in partially reduced mammalian ferritin [7]. The spectra consist of partially resolved ferrous and ferric quadrupole doublets with relative intensities at 80 K that correspond to the degree of reduction of the protein. For  $80 \leq T \leq 300$  K, the ferrous and ferric quadrupole doublets have different temperature dependencies of their recoil-free fractions (fig. 1). For  $4.2 < T < 80$  K, the ferric subspectrum undergoes a superparamagnetic transition, with an average blocking temperature that is higher than in fully oxidized ferritin with the same average iron content (fig. 2). The ferrous subspectrum does not participate in the superparamagnetic transition, but is broadened below 20 K. These results suggest that Fe<sup>3+</sup> and Fe<sup>2+</sup> ions constitute separate phases within the protein cavity. Alternatively, the Fe<sup>2+</sup> ions might be bound to the outer surface of the protein shell.

In this paper we present evidence that Fe<sup>2+</sup> ions are bound from the external medium by mammalian ferritin. Further, the Fe<sup>2+</sup> ions enter the protein interior cavity and exchange electrons with Fe<sup>3+</sup> in the core. Thus, Fe<sup>2+</sup> ions could function as mediators of iron reduction during iron release from ferritin.

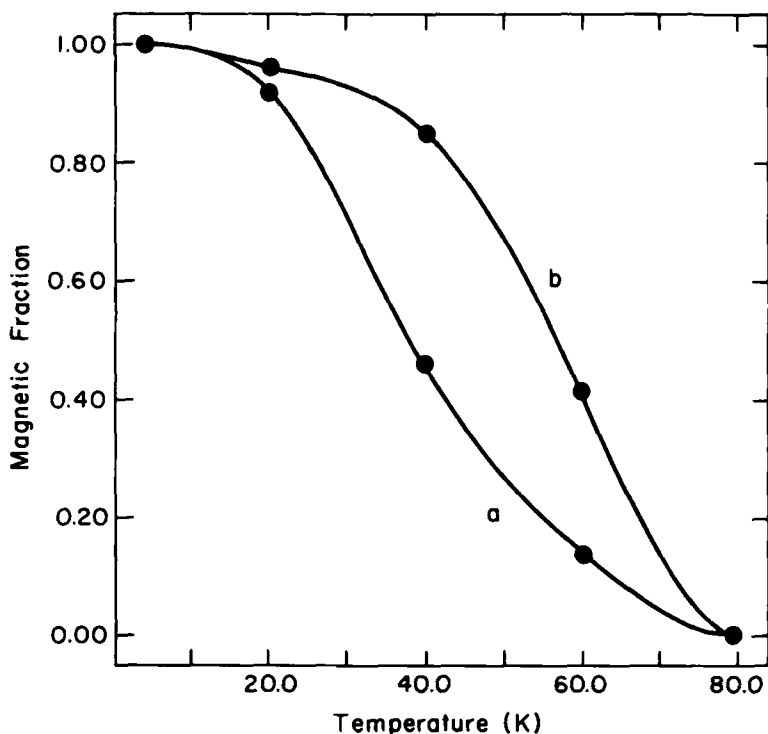


Fig. 2. Magnetic fraction of the Fe<sup>3+</sup> spectrum in (a) ferric ferritin and (b) 50% reduced ferritin. The average blocking temperature is defined as the temperature at which the magnetic fraction is 0.5.

## 2. Methods

Horse spleen ferritin with normal <sup>57</sup>Fe isotopic abundance (2.2%) was obtained from the Sigma Chemical Company. It was passed through a 1 × 13 cm Sephadex G-25 column equilibrated in a 0.25 M Tris buffer at pH = 7.0 prior to use. Total iron and total protein were determined by the bathophenanthroline and the Lowry methods, respectively. The results gave an average number of 1876 Fe<sup>3+</sup> ions per ferritin molecule. Isotopically enriched ferrous sulfate solutions with 95% <sup>57</sup>Fe were prepared from iron metal (New England Nuclear). Ferrous sulfate and ferritin were incubated together under argon for thirty minutes at room temperature, then passed through an anaerobic Sephadex G-25 column to separate ferritin from unbound Fe<sup>2+</sup>. An aliquot of the ferritin fraction was extracted with orthophenanthroline to yield the average number of Fe<sup>2+</sup> ions bound per ferritin molecule. With an initial excess of Fe<sup>2+</sup> (500 Fe<sup>2+</sup>/ferritin), it was found that an average of 140 Fe<sup>2+</sup> ions were bound per molecule. This was confirmed by titration of ferritin with increasing amounts of Fe<sup>2+</sup> per ferritin

molecule. These results showed that all the  $\text{Fe}^{2+}$  ions were bound up to 140  $\text{Fe}^{2+}$  ions per molecule and that beyond 140  $\text{Fe}^{2+}$  ions per molecule, a band of unbound  $\text{Fe}^{2+}$  could be detected trailing the ferritin band after passage through the Sephadex column. Subsequent orthophenanthroline extraction of the ferritin band gave 140  $\text{Fe}^{2+}$  per molecule. This showed that no net oxidation of the bound iron took place under the anaerobic conditions of the experiment.

Samples of ferritin with bound  $\text{Fe}^{2+}$  were anaerobically loaded into sealed plastic containers and frozen in liquid nitrogen. The samples were kept frozen during transfer to the Mössbauer spectrometer. Following Mössbauer spectroscopy, the samples were thawed and incubated anaerobically with 0.5 reducing equivalents of dithionite (1  $\bar{e}$  per 2  $\text{Fe}^{3+}$ ), then resealed and refrozen for Mössbauer spectroscopy.

### 3. Results

The 80 K Mössbauer spectrum of ferritin with bound  $^{57}\text{Fe}^{2+}$  is shown in fig. 3(a). The spectrum was fit with two overlapping quadrupole doublets corresponding to ferric and ferrous iron with relative intensities of 0.69 and 0.31, respectively (table 1). For  $4.2 < T < 80$  K, the  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  subspectra behave as those in partially reduced ferritin obtained by electrochemical reduction [7]. For  $80 < T < 300$  K, the temperature dependencies of the recoil-free fractions of the  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  subspectra are similar to those in partially reduced ferritin (fig. 1). Subsequent partial reduction by addition of 0.5 reducing equivalents of dithionite per  $\text{Fe}^{3+}$  ion yielded the 80 K spectrum shown in fig. 3(b), with ferric and ferrous relative intensities of 0.25 and 0.75, respectively. On a per ferritin molecule basis, if all the 1876 original  $\text{Fe}^{3+}$  ions remained  $\text{Fe}^{3+}$  and all the 140 bound  $\text{Fe}^{2+}$  ions remained  $\text{Fe}^{2+}$ , we would expect 41 atoms of  $^{57}\text{Fe}^{3+}$  and 133 atoms of  $^{57}\text{Fe}^{2+}$ , or ferric and ferrous relative intensities of 0.24 and 0.76, respectively. The experimental ferric and ferrous relative intensities are 0.69 and 0.31, respectively, or 120 atoms of  $^{57}\text{Fe}^{3+}$  and 54 atoms of  $^{57}\text{Fe}^{2+}$ . Since no net oxidation of the added  $\text{Fe}^{2+}$  occurred, we interpret the experimental result as an exchange of electrons between the added  $\text{Fe}^{2+}$  ions and the  $\text{Fe}^{3+}$  ions in the ferritin core.

Addition of 0.5 reducing equivalents of dithionite per  $\text{Fe}^{3+}$  ion should have resulted in reduction of one half of the  $^{57}\text{Fe}^{3+}$  ions, if all the  $\text{Fe}^{3+}$  ions in the molecule have equal probability of being reduced. This would give 60  $^{57}\text{Fe}^{3+}$  ions and 114  $^{57}\text{Fe}^{2+}$  ions, or ferric and ferrous relative intensities of 0.34 and 0.65, respectively. The respective experimental values are 0.25 and 0.75. We interpret this to mean that the added  $^{57}\text{Fe}^{2+}$  which underwent electron exchange to form  $^{57}\text{Fe}^{3+}$  have a higher probability of being reduced than the original  $\text{Fe}^{3+}$  ions of the core. This is consistent with a "last in - first out" process for release of ferritin iron [8].

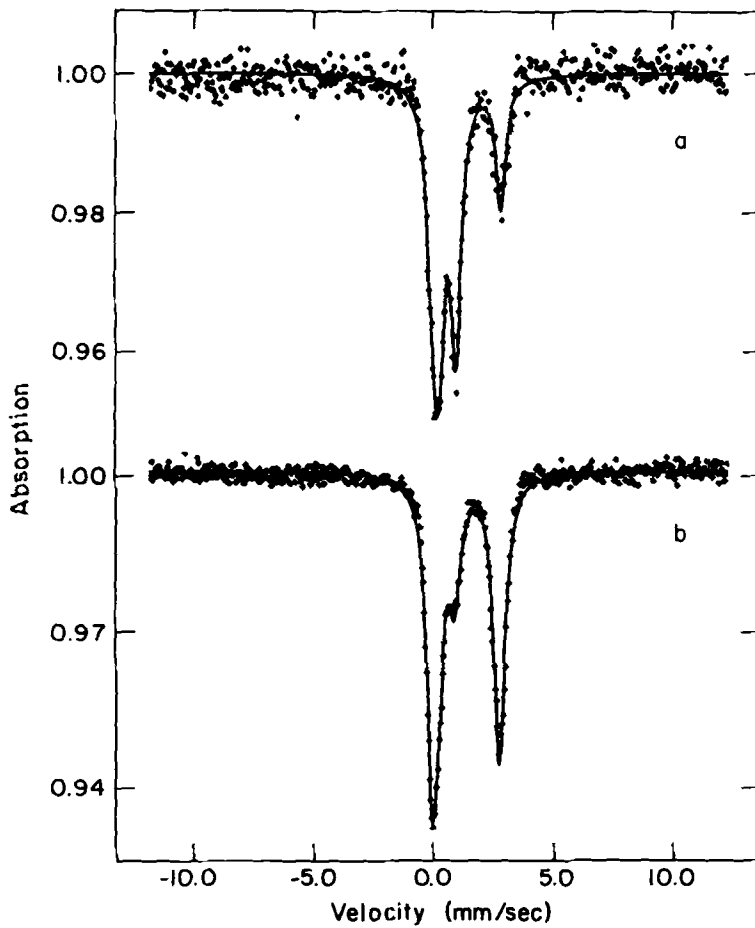


Fig. 3. Mössbauer spectra at 80 K; (a) 140 atoms of 95%  $^{57}\text{Fe}$  enriched  $\text{Fe}^{2+}$  bound to ferritin with an average iron content of 1876  $\text{Fe}^{3+}$  ions per molecule (2.2%  $^{57}\text{Fe}$ ); (b) following reduction by 0.5 reducing equivalents of dithionite per  $\text{Fe}^{3+}$ . The solid line is a theoretical least-squares fit of two quadrupole doublets to the data.

Table 1  
Mössbauer parameters at 80 K

Sample	Isomer shift <sup>a</sup> (mm/s)	Quadrupole splitting (mm/s)	Relative area	Theoretical area <sup>b</sup>
Ferritin + <sup>57</sup> Fe <sup>2+</sup>	0.50 ± 0.03 1.30	0.72 ± 0.05 2.87	0.69 (Fe <sup>3+</sup> ) 0.31 (Fe <sup>2+</sup> )	0.24 0.76
Ferritin + <sup>57</sup> Fe <sup>2+</sup> + 0.5 e <sup>-</sup> /Fe <sup>3+</sup>	0.50 1.29	0.66 2.78	0.25 (Fe <sup>3+</sup> ) 0.75 (Fe <sup>2+</sup> )	0.34 0.66

<sup>a</sup>Relative to Fe metal at 300 K.

<sup>b</sup>See "results" section (sect. 3).

#### 4. Discussion

The experimental results can be summarized as follows: (a) Fe<sup>2+</sup> binds to ferritin under anaerobic conditions; (b) the bound Fe<sup>2+</sup> ions exchange electrons with the Fe<sup>3+</sup> ions of the core; (c) the last added Fe<sup>3+</sup> ions, those produced by oxidation of the originally added Fe<sup>2+</sup> ions, are preferentially reduced when ferritin is incubated with a reductant such as dithionite.

The results imply that Fe<sup>2+</sup> ions enter and are bound within the ferritin cavity. Since apoferritin binds ~ 12 Fe<sup>2+</sup> ions per molecule [8], the binding of > 100 Fe<sup>2+</sup> ions by the holoprotein implies many more binding sites, perhaps on the surface of the core. Binding on the surface of the core would also facilitate the exchange of electrons with the Fe<sup>3+</sup> ions of the core. Since addition of Fe<sup>2+</sup> ions produces a core that is spectroscopically indistinguishable from a core which is partially reduced electrochemically [7], redox states formed by partial reduction are thermodynamically as well as kinetically stable. Higher states of reduction, in contrast, may be only kinetically stable. The number of bound Fe<sup>2+</sup> ions could depend on the average iron concentration per molecule and, because the reduction potential is pH dependent [7], on the pH of the medium.

The ferritin iron cores are usually pictured as homogeneous three-dimensional solids with well-defined two-dimensional surfaces [10]. However, the rapid oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup>, followed by hydrolysis, might produce more open fractal structures such as those produced by diffusion limited aggregation (DLA) [11]. DLA structures are characterized by highly invaginated surfaces and fluctuations in local density. The ferritin core might thus be a heterogeneous solid with a dense nucleus at the point of attachment to the interior of the protein, and domains of variable size or density that spread out into the cavity like the root structure of a tree or a pile of rocks. The

opening in and between the domains would provide access for the binding of  $\text{Fe}^{2+}$  ions and exchange of electrons with the ferric ions. They would also provide access for protons which are taken up in the course of core reduction [7].

We originally interpreted the increase in the average superparamagnetic blocking temperatures of partially reduced ferritin compared to fully oxidized ferritin in terms of preferential reduction of ferritin molecules with fewer iron atoms than average per molecule [7]. This interpretation was based on the model in which the distribution of blocking temperatures reflects the distribution of core volumes in the population of ferritin molecules [12]. With DLA core structures, however, the distribution of blocking temperatures could be ascribed to the domains of differing size or density within a single ferritin molecule. Preferential reduction of the last formed, smaller domains within a given molecule would shift the average blocking temperature to higher values and sharpen the superparamagnetic transition. The higher average blocking temperatures would correspond to the denser or more crystalline nuclei of the core structures.

Bell et al. [13] have noted that hemosiderin has a higher average blocking temperature than ferritin, although transmission electron microscopy (TEM) suggests a smaller average core diameter for the former. Hemosiderin cores could be considered ferritin cores with higher average density perhaps due to aging and collapse of the DLA structure. This would decrease the core volume as determined by TEM, yet result in an increase of the average blocking temperature. It is also consistent with the fact that iron in hemosiderin is less labile than in ferritin [9].

Ferritin from *E. coli* (EC) [14] and *P. aeruginosa* (PA) [15] have blocking temperatures below 4 K. Yet TEM gives core volumes comparable with mammalian ferritin [16]. This could be due to a lower average density of the EC and PA ferritin. *A. vinelandii* (AV) ferritin has an average blocking temperature which is intermediate between EC and PA ferritin and mammalian ferritin [17]. Partial reduction of AV ferritin results in an increase of the average blocking temperature as in mammalian ferritin. This suggests that the bacterial ferritins might also have DLA core structures. The bacterial ferritins generally have higher phosphate/iron ratios than in mammalian ferritin [16,17]. This could affect the average core density as well as the domain size distribution within the cores.

Reduction of  $\text{Fe}^{3+}$  in the ferritin core could occur by diffusion of reductants into the protein cavity. However, it has been shown that diffusion of even small molecules such as MeOH into ferritin is very slow compared to the rate of reduction [18]. If even a small number of the core ferric ions are labile, or are in equilibrium with sites in the hydrophilic channels of the protein shell [4], reductants need not enter the protein cavity. Rather, electrons might be carried in by the  $\text{Fe}^{2+}$  ions produced by reduction of the ferric ions in the channels.

## Acknowledgements

R.B.F. and G.C.P. were partially supported by the Office of Naval Research. The Francis Bitter National Magnet Laboratory is supported by the National Science Foundation. G.D.W. was supported by the Metabolic Biology Program of the NSF.

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