Mössbauer Spectroscopy of Reduced Ferritin

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1. Introduction

Iron is an important element in the physiology of living organisms. Nature has elaborated an elegant system for modulating and controlling the balance between iron insufficiency and iron toxicity by developing the protein molecule ferritin. This protein is similar in form and function in species ranging from bacteria to mammals. Its basic function seems to be that of storing iron during growth cycles when this element is abundant and distributing it controllably during metabolic demand.[1–4]

Mammalian ferritin is a roughly spherical, 120 Å diameter protein with a 70 Å interior cavity. The three-dimensional structure of horse spleen ferritin has been refined to 2.8 Å[5,6] and shows a highly symmetrical arrangement of 24 nearly identical subunits in the protein shell (Figure 1). Six hydrophilic and eight hydrophobic channels provide access to the protein's interior. The mineral core of ferritin is a polymeric hydrous ferric oxide associated with phosphate. The nominal composition is $(FeOOH)_8(FeO{\cdot}H_2PO_4)$ and the x-ray and electron diffraction patterns indicate a structure similar to that of the mineral ferrihydrite $(5Fe_2O_3{\cdot}9H_2O)$[7] in which Fe^{3+} ions have sixfold oxygen coordination. Particles very similar in size and structure to the mineral core of ferritin can also be synthesized by hydrolysis of ferric nitrate solutions.[8]

Typically, ferritin is heterogeneous as isolated with an average iron content of 2000–3000 iron atoms per molecule although the maximum

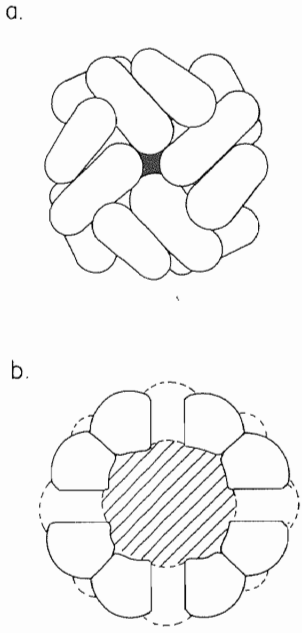

FIGURE 1. Schematic representation of the ferritin molecule (after references 6 and 13). (a) View looking down on a fourfold axis of symmetry showing pairs of protein subunits and a hydrophilic channel (center). (b) Cross-section showing the 70 Å diameter interior cavity (hatched area) which can accommodate up to 4500 iron atoms as a hydrous iron oxide associated with phosphate.

loading is thought to approach 4500 Fe atoms/mole.[9] Apoferritin (ferritin with the iron core missing) can be obtained by fractionating heterogeneous ferritin samples by ultracentrifugation, or more commonly by reduction of the core iron, followed by exhaustive dialysis against iron complexing reagents. Ferritin can be reconstituted from apoferritin by reaction with Fe^{2+} and oxidizing agents (O_2, IO_3^-, $S_2O_3^{2-}$).[10,11] The mechanism of iron release from ferritin and the reconstitution of ferritin from apoferritin have been studied extensively. However, the detailed mechanism of iron uptake and deposition in ferritin is still uncertain.

Several kinetic models have been proposed[12,13] and a reasonably successful one is the crystal growth model.[12] This model assumes that the rate of deposition or release of iron from the ferritin core is a function of the surface of the hydrous iron oxide crystallite. The six channels[6] seen in the three-dimensional structure of ferritin are thought to be the ingress and egress pathways for Fe^{2+}, Fe^{3+}, reductant, chelators, and so on during the functioning of this protein.

2. *Mössbauer Spectroscopy of Oxidized Ferritin*

Iron in ferritin from several sources has been studied by Mössbauer spectroscopy.[14–18] The high-temperature spectrum ($T > 60$ K) typically

consists of a broadened quadrupole doublet with isomer shift and quadrupole splitting characteristic of Fe^{3+}. At helium temperatures ($T \simeq 4.2$ K), the spectrum is magnetically split with a ~490 kOe field at the nucleus. The lines are broad, indicating a distribution of hyperfine fields. As the temperature is increased from 4.2 K, the magnetically split spectrum gradually decreases in intensity and the quadrupole doublet increases in intensity. The temperature at which the doublet and sextet have equal intensities is called the average blocking temperature. The temperature range over which the magnetically split spectrum and quadrupole doublet coexist depends on the source of the ferritin (mammalian, plant, or bacterial in origin) and the degree of iron loading of the ferritin molecules.

The temperature dependence of the Mössbauer spectrum is due to superparamagnetic behavior in the small particles of the ferritin core.[14] In this phenomenon, the iron atoms are antiferromagnetically coupled at low temperature. The sublattice magnetizations lie along particular crystallographic orientations in the crystal, the easy magnetic axes. At finite temperature, there is a certain probability that the sublattice magnetizations will undergo a transition to an energetically equivalent easy axis. The sublattice relaxation time τ is an exponential function of magnetic anisotropy K, the volume V of the particle, and the temperature:

$$\tau = \tau_0 \exp(KV/kT) \tag{1}$$

where τ_0 is a constant and k is Boltzmann's constant. When the relaxation time in a particle is of the order of or faster than the Larmor precession time of the 14.4 keV excited state, the hyperfine field will be wiped out and the spectrum will consist of the quadrupole doublet. For particles of a given volume, this condition will occur at a definite temperature called the blocking temperature. If there is a distribution of particle volumes in the sample, there will be a distribution of blocking temperatures, and the magnetic spectrum and the quadrupole doublet corresponding to the larger particles and the smaller particles, respectively, can coexist. Determination of the relative intensities of the two subspectra as a function of temperature is a means of measuring the distribution of particle volumes.

Cohen and coworkers[19,20] have discovered an interesting dynamic effect in the Mössbauer spectra of crystals of horse spleen ferritin molecules when the crystals are warmed through the freezing point of water (~265 K). Below 265 K the spectrum consists of the quadrupole doublet referred to above. Above 265 K, the spectrum consists of a narrow line quadrupole doublet superposed on a broad spectrum of width ~4 cm/s. This effect has been interpreted in terms of bounded or

localized diffusive motions of the ferritin molecules after interstitial water in the crystal has melted. Analysis has been made in terms of discrete transitions between a number of fixed points, or in terms of continuous harmonic motion driven by Brownian forces.[20] For small particles in water, frictional forces are large compared to interstitial forces and the situation corresponds to strong overdamping. It has been shown that the theoretical spectrum based on these models does indeed consist of a narrow and a broad component, with parameters determined by a diffusion constant D and the ratio of harmonic-to-frictional forces. The intensity of the narrow line and the width of the broad line can be used to calculate the mean squared displacement and the diffusion constant, respectively, of the iron atoms participating in the diffusive motions.

3. *Reduction of Ferritin*

Coulometric reduction[21] of horse spleen ferritin at pH 8, containing an average of 2300 iron atoms per molecule at -500 mV versus the normal hydrogen electrode (NHE), using methyl viologen as a mediator, demonstrates that one electron per iron atom (2300 electrons per molecule of ferritin) is transferred during the electrochemical reaction. More detailed coulometric reduction experiments of ferritin as a function of applied potential indicates that reduction occurs over a narrow, well-defined potential range as shown in Figure 2. Analysis of these curves for reduction of the core iron atoms in ferritin suggests an apparently reversible electrochemical reduction reaction with an $E_{1/2}$ value of -310 mV at pH 8 and an n value of one. Reduction measurements to extreme negative potentials, where water reduction imposes a limit, showed that no further reduction occurs. Both the n value and the measured stoichiometry are consistent with a single-electron reduction of each core iron atom.

Figure 2 also shows that the reduction of the core iron atoms is pH dependent, becoming more difficult with increasing pH. This result is consistent with proton uptake, presumably by proton transfer into the core upon reduction. From the magnitude of the potential change with pH (115 mV between pH 7 and 8, 106 mV between pH 8 and 9), values of 1.92 and 1.78 protons transferred per electron transferred were obtained. This result was confirmed by direct pH measurements during $Na_2S_2O_4$ reduction of ferritin, which demonstrated that 2.06 H^+ were transferred to ferritin for each Fe^{3+} reduced to Fe^{2+} in the ferritin core.

The apparent stability of the Fe^{2+} formed by reduction and its retention within the ferritin core suggested by the coulometric results in Figure 2 were verified by direct, longer-term chromatography experi-

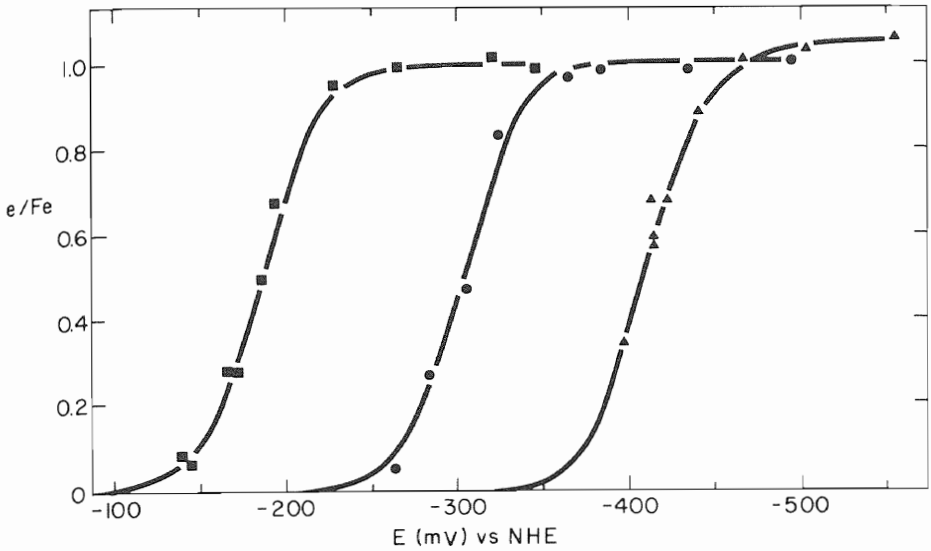

FIGURE 2. Coulometric reduction of ferritin at 25°C. Ferritin samples added to $1 \times 10^{-5}M$ mediator solutions, $0.05M$ Tris, $0.25M$ NaCl controlled at the indicated potentials. ■, pH 7.0; ●, pH 8.0; and ▲, pH 9.0. All potentials are relative to the normal hydrogen electrode (NHE). (After reference 21.)

ments. Five identical 1 ml samples of ferritin of known iron content were each placed in separate containers in a vacuum atmosphere anaerobic enclosure under argon (<0.5 ppm O_2) and 0, 0.25, 0.5, 0.75, and 1.0 electron equivalent of $S_2O_4^{2-}$ per iron atom were added and allowed to react 20 min. Following this reaction interval, each ferritin sample was passed through an anaerobic G-25 Sephadex column to remove $S_2O_4^{2-}$ oxidation products and any other small ions/molecules (i.e., Fe^{2+}, Fe^{3+}) and the iron and protein concentrations were redetermined on these gel-filtered ferritin samples. Except for the one electron/Fe^{3+} sample, much of the original iron is retained and the expected amount of unreduced Fe^{3+} present is commensurate with the amount of added reductant. The one electron/Fe^{3+} result has been repeated several times and variable results have been obtained ranging from little iron loss to as much as 25% iron loss. Such factors as length of reduction time, amount and quality of $S_2O_4^{2-}$ used as reductant (SO_3^{2-}, S^{2-}, and other products of $S_2O_4^{2-}$ reaction or decomposition may serve as Fe^{2+} chelators), and the origin and previous history of ferritin samples (aging, hydration, damaged ferritin molecules) tend to influence the rate and amount of Fe^{2+} loss after reduction. Where an iron decrease is noted in the protein emerging from the column, the missing iron is found trailing behind the protein band in the free Fe^{2+} form. It thus appears that, as reduction of

ferritin approaches one electron per iron atom, some iron is unstable with respect to retention by the core but that, at values less than 1:1, nearly complete retention of Fe^{2+} by the core occurs.

4. *Mössbauer Spectroscopy of Reduced Ferritin*

Evidence for the reduction of Fe^{3+} and retention as Fe^{2+} in ferritin was obtained by Mössbauer spectroscopy.[21] Samples were prepared by freezing the ferritin molecules in air-tight, plastic containers immediately following reduction and passage through a Sephadex column in an inert atmosphere to remove nonferritin-bound Fe^{3+} and Fe^{2+} ions. Mössbauer spectra of fully oxidized ferritin, 50% reduced ferritin (0.5 electron/iron atom), and 75% reduced ferritin (0.75 electron/iron atom) at 100 K are shown in Figure 3. Whereas the spectrum of oxidized ferritin is a broadened quadrupole doublet with parameters corresponding to ferric iron, the spectra of the reduced samples consist of the ferric quadrupole doublet with a superposed ferrous quadrupole doublet. Both ferric and ferrous doublets have broadened lines. The relative intensities of the ferric and ferrous quadrupole doublets in each case are consistent with the electrochemically determined degree of reduction, considering the uncertainties in the relative recoilless fractions. Exposure of the reduced samples to air at room temperature resulted in the Mössbauer spectra of oxidized ferritin.

For $80 \leq T \leq 300$ K, the ferrous and ferric quadrupole doublets have different temperature dependencies of their recoil-free fractions (Figure 4). 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 (Figure 5). The 4.2 K magnetic hyperfine fields (Figure 6) are the same as in fully oxidized ferritin, which indicates that the ferric material in the partially reduced samples is the same hydrous ferric oxide as in the fully oxidized ferritin. Moreover, the ferric sextet flanking the ferric doublet is observable in the 60 K spectrum of all three samples. Also, the ferric doublet persists at lower temperatures in the oxidized ferritin than in either reduced sample. Since at any temperature the ferric doublet and sextet correspond to smaller and larger particles, respectively, this observation suggests that those molecules with fewer iron atoms are preferentially reduced, perhaps on an "all-or-nothing" basis. All-or-nothing accumulation of iron in ferritin has been noted under conditions of rapid oxidation.[11,12]

The ferrous spectra of the partially reduced samples have different temperature dependencies than the ferric spectra for $4.2 \leq T \leq 80$ K.

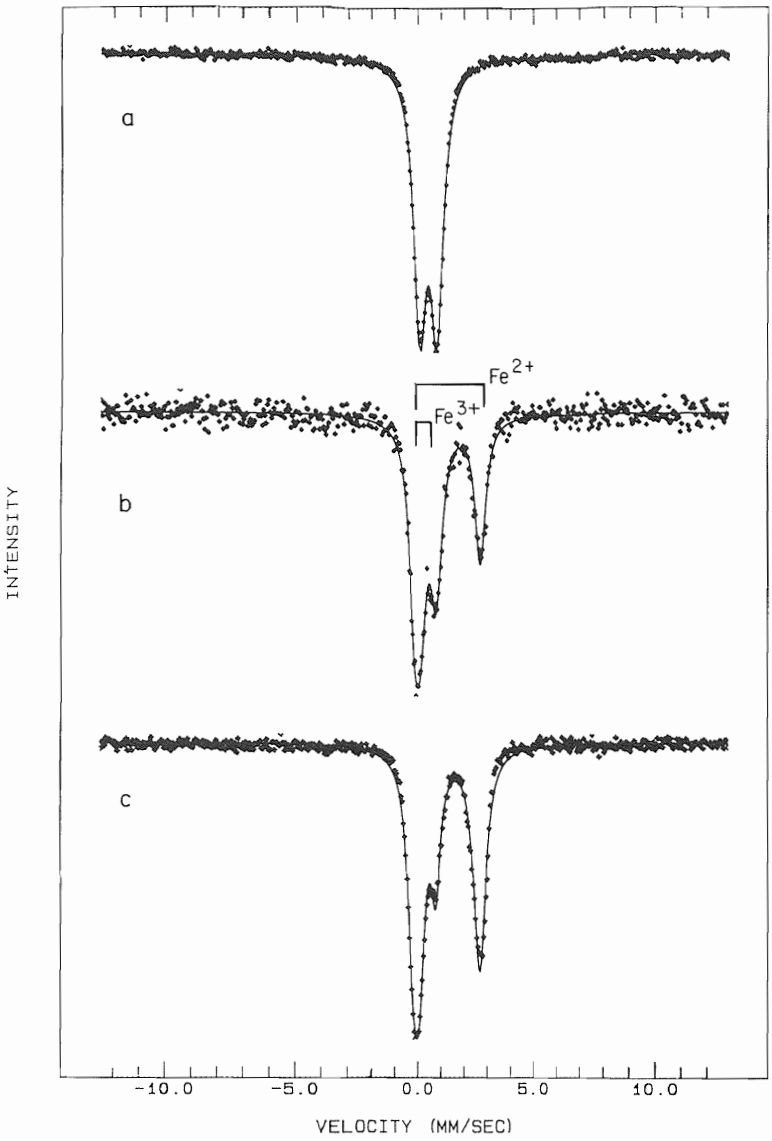

FIGURE 3. Mössbauer spectra at 100 K: (a) fully oxidized ferritin; (b) 50% reduced ferritin, and (c) 75% reduced ferritin. The solid lines are theoretical fits assuming a ferric doublet ($\Delta E_Q = 0.72$ mm/s, $\delta = 0.45$ mm/s) in (a), and ferric and ferrous doublets ($\Delta E_Q = 2.85$ mm/s, $\delta = 1.27$ mm/s) in (b) and (c)

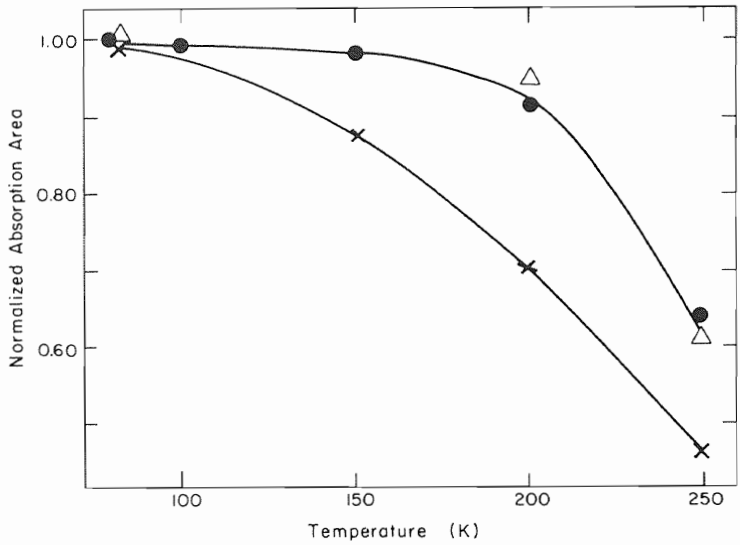

FIGURE 4. Temperature dependence of the absorption intensity for (●) Fe^{3+} in ferric ferritin, (△) Fe^{3+} in 50% reduced ferritin, and (×) Fe^{2+} in 50% reduced ferritin, normalized to 80 K.

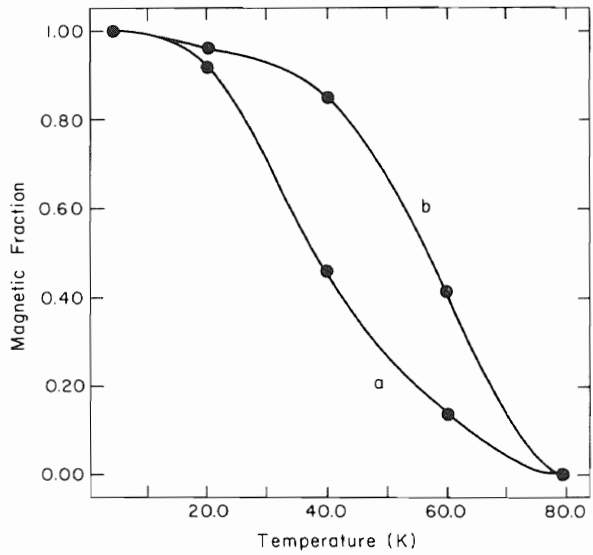

FIGURE 5. Magnetic fraction of the Fe^{3+} spectrum in (a) ferric ferritin and (b) 50% reduced ferritin. The average blocking temperature is defined as the temperature at which the magnetic fraction is 0.5.

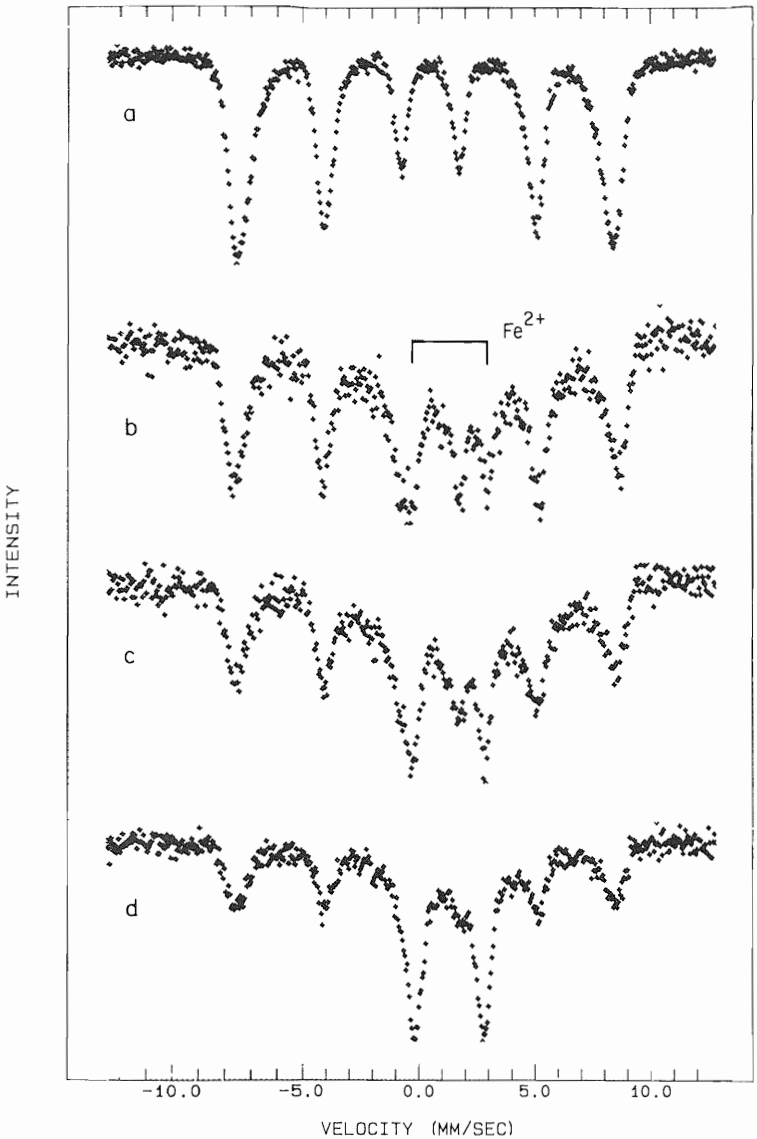

FIGURE 6. Mössbauer spectra at 4.3 K: (a) fully oxidized ferritin; (b) 50% reduced ferritin; and (c) 75% reduced ferritin. Spectrum (d) is 75% reduced ferritin at 10 K. The brackets indicate the position of the ferrous quadrupole doublet.

The ferrous subspectrum does not show the effects of superparamagnetism as the ferric spectrum does, but is broadened below ~20 K. The 4.2 K ferrous spectra (Figure 6) are somewhat obscured by the ferric lines, but in each case they appear to consist of a low-intensity quadrupole doublet superposed on a broad, magnetically split background which appears as an asymmetry in the overall spectrum. The intensity of the quadrupole doublet increases with increasing temperature, reaching full intensity between 10 and 20 K. These results suggest that the ferrous material might be noncrystalline with a range of magnetic exchage interactions between Fe^{2+} ions resulting in a nonunique magnetic hyperfine splitting at 4.2 K. Certainly the ferric and ferrous materials in the partially reduced samples behave independently of each other, suggesting that certain ferritin molecules are preferentially reduced or that reduction occurs in a localized region in or on the hydrous ferric oxide cores.

5. *Binding of Fe^{2+} by Ferritin*

It has also been shown that Fe^{2+} ions are bound from the external medium by ferritin.[22] The Fe^{2+} ions enter the protein interior cavity and exchange electrons with Fe^{3+} in the core. Thus, Fe^{2+} ions could function as mediators of iron reduction during iron release from ferritin.

In these experiments, horse spleen ferritin with normal iron-57 isotopic abundance (2.2%) and an average number of 1876 Fe^{3+} ions per ferritin molecule were incubated with isotopically enriched ferrous sulfate solutions with 95% iron-57 prepared from iron metal. After incubation for 30 min under argon at room temperature, the mixture was passed through an anaerobic Sephadex G-25 column to separate ferritin from unbound Fe^{2+}. An aliquot of the ferritin fraction was extracted with orthophenanthroline to yield the average number of Fe^{2+} ions bound per ferritin molecule. With an initial excess of Fe^{2+} (500 Fe^{2+}/ferritin), it was found that an average of 140 Fe^{2+} ions were bound per molecule. This was confirmed by titration of ferritin with increasing amounts of Fe^{2+} per ferritin molecule. These results showed that all the Fe^{2+} ions were bound up to 140 Fe^{2+} ions per molecule and that beyond 140 Fe^{2+} ions per molecule, a band of unbound Fe^{2+} could be detected trailing the ferritin band after passage through the Sephadex column. Subsequent orthophenanthroline extraction of the ferritin band gave 140 Fe^{2+} per molecule. This showed that no net oxidation of the bound iron took place under the anaerobic conditions of the experiment.

The 80 K Mössbauer spectrum of ferritin with bound $^{57}Fe^{2+}$ is shown in Figure 7a. The spectrum was fit with two overlapping quadrupole

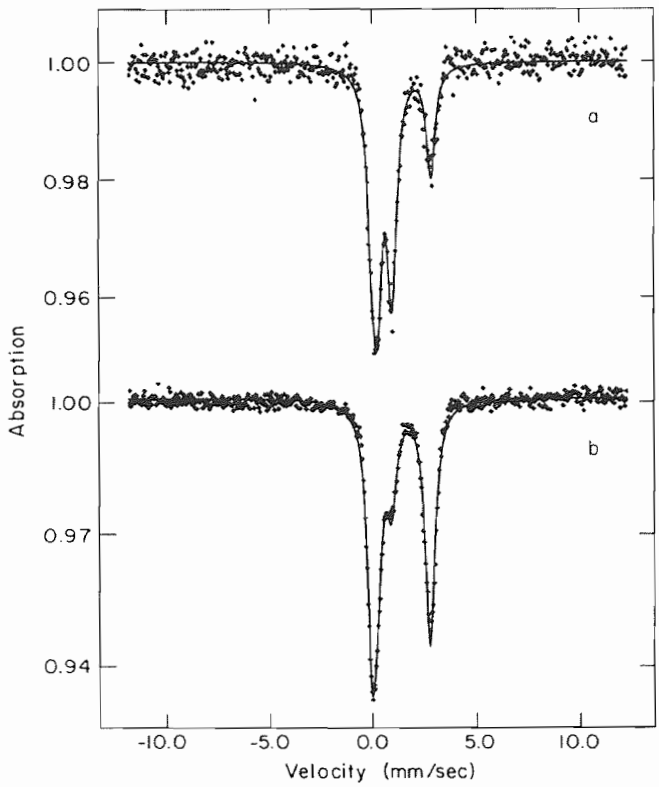

FIGURE 7. Mössbauer spectra at 80 K: (a) 140 atoms of 95% iron-57 enriched Fe^{2+} bound to ferritin with an average iron content of 1876 Fe^{3+} ions per molecule (2.2% iron-57); and (b) following reduction by 0.5 reducing equivalents of dithionite per Fe^{3+}. The solid line is a theoretical least-squares fit of two quadrupole doublets to the data.

doublets corresponding to ferric and ferrous iron with relative intensities of 0.69 and 0.31, respectively. For $4.2 < T < 80$ K, the Fe^{3+} and Fe^{2+} subspectra behave as those in partially reduced ferritin obtained by electrochemical reduction. For $80 < T < 300$ K, the temperature dependencies of the recoil-free fractions of the Fe^{2+} and Fe^{3+} subspectra are similar to those in partially reduced ferritin. Subsequent partial reduction by addition of 0.5 reducing equivalents of dithionite per Fe^{3+} ion yielded the 80 K spectrum shown in Figure 7b, with ferric and ferrous relative intensities of 0.25 and 0.75, respectively. On a per ferritin molecule basis, if all the 1876 original Fe^{3+} ions remained Fe^{3+} and all the 140 bound Fe^{2+} ions remained Fe^{2+}, we would expect 41 ions of $^{57}Fe^{3+}$ and 133 ions of $^{57}Fe^{2+}$, 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 ions of $^{57}Fe^{3+}$ and 54

ions of $^{57}Fe^{2+}$. Since no net oxidation of the added Fe^{2+} occurred, this must result from exchange of electrons between the added Fe^{2+} ions and the Fe^{3+} ions in the ferritin core.

Addition of 0.5 reducing equivalents of dithionite per Fe^{3+} ion should have resulted in reduction of one-half of the $^{57}Fe^{3+}$ ions, if all the Fe^{3+} ions in the molecule had equal probability of being reduced. This would give 60 $^{57}Fe^{3+}$ ions and 114 $^{57}Fe^{2+}$ ions, or ferric and ferrous relative intensities of 0.34 and 0.65, respectively. The respective experimental values are 0.25 and 0.75. This means that the added $^{57}Fe^{2+}$ which underwent electron exchange to form $^{57}Fe^{3+}$ had a higher probability of being reduced than the original Fe^{3+} ions of the core. This is consistent with a "last in–first out" process for release of ferritin iron.[23]

6. *Conclusions*

To summarize, the hydrous ferric oxide cores of horse spleen ferritin can be reduced by one electron per iron atom with an $E_{1/2}$ value of −310 mV at pH 8.0. Reduction is accompanied by an uptake of two protons per electron from the surrounding medium and the Fe^{2+} produced by reduction is retained in the cores. The reduction potential for mammalian ferritin is ~100 mV more positive than that reported[24] for the corresponding bacterial ferritin. Since the latter appears to retain Fe^{2+} much more strongly,[25] this result implies that Fe^{2+} retention is related to reduction potential. These characteristics raise the following questions:

1. Are the cores of these two proteins somewhat different?
2. Are the protein shells in these two proteins influencing their redox potentials?
3. Is some other undisclosed feature of these proteins causing variation of the reduction properties?

Reduction measurements using ferritins from other sources would be valuable in providing additional information leading to a better understanding of the reduction step of ferritins that precedes Fe^{2+} release.

The uptake of two protons, which occurs upon reduction, implies that the oxidation of Fe^{2+} to Fe^{3+} in ferritin and deposition of the hydrous ferric oxide core involves export of protons. Perhaps phosphate, which is also a constituent of the ferritin cores, is involved in buffering the pH changes within the cores resulting from iron redox reactions. It is intriguing to consider that ferritin in cells could function as a system in which redox reactions involving iron are coupled with the translocation of protons in the cell.

The results of the binding experiments can be summarized as follows:

1. Fe^{2+} binds to ferritin under anaerobic conditions.
2. The bound Fe^{2+} ions exchange electrons with the Fe^{3+} ions of the core.
3. The last added Fe^{3+} ions, those produced by oxidation of the originally added Fe^{2+} ions, are preferentially reduced when ferritin is incubated with a reductant such as dithionite.

The results imply that Fe^{2+} ions enter and are bound within the ferritin cavity. Since apoferritin binds ~12 Fe^{2+} ions per molecule,[26] the binding of more than 100 Fe^{2+} 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^{3+} ions of the core. Since addition of Fe^{2+} ions produces a core that is spectroscopically indistinguishable from a core that is partially reduced electrochemically,[21] 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^{2+} ions could depend on the average iron concentration per molecule and, because the reduction potential is pH dependent, on the pH of the medium.

The ferritin iron cores are usually pictured as homogeneous three-dimensional solids with well-defined two-dimensional surfaces.[26,27] However, the rapid oxidation of Fe^{2+} to Fe^{3+} followed by hydrolysis might produce more open fractal structures such as those produced by diffusion limited aggregation (DLA) or cluster aggregation.[28] 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 openings in and between the domains would provide access for the binding of 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.[21]

The increase in the average superparamagnetic blocking temperatures of partially reduced ferritin compared to fully oxidized ferritin was originally interpreted in terms of preferential reduction of ferritin molecules with less iron atoms than average per molecule.[21] 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. With DLA core structures, however, the

distribution of blocking temperatures would 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.*[18] 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.[27]

Ferritin from *E. coli* (EC)[29] and *P. aeruginosa* (PA)[30] have blocking temperatures below 4 K. Yet TEM gives core volumes comparable with mammalian ferritin.[31] This could be due to lower average density of the EC and PA ferritins. *A. vinelandii* (AV) ferritin has an average blocking temperature that is intermediate between EC and PA ferritin and mammalian ferritin.[24] 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.[24,31] This could effect the average core density as well as the domain size distribution within the cores.

Reduction of 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.[32] If even a small number of the core ferric ions are labile or are in equilibrium with sites in the hydrophilic channels of the ptorein shell,[6] reductants need not enter the protein cavity. Rather, electrons might be carried in by the Fe^{2+} ions produced by reduction of the ferric ions in the channels.

Acknowledgments

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