Fe^{2+} Binding to Apo and Holo Mammalian Ferritin†

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Received March 24, 1989; Revised Manuscript Received July 3, 1989

ABSTRACT: The binding of Fe^{2+} to both apo and holo mammalian ferritin has been investigated under anaerobic conditions as a function of pH. In the pH range 6.0–7.5, 8.0 ± 0.5 Fe^{2+} ions bind to each apoferritin molecule, but above pH 7.5, a pH-dependent Fe^{2+} binding profile is observed with up to 80 Fe^{2+} ions binding at pH 10.0. This Fe^{2+} binding is reversible and is accompanied by up to two H^+ being released per Fe^{2+} bound at pH 10.0. The Fe^{2+} binding to apoferritin probably occurs in the 3-fold channels. A much larger and more complex pH-dependent Fe^{2+} binding stoichiometry was observed for holoferritin with up to 300 Fe^{2+} ions binding at pH 10.0. This pH-dependent Fe^{2+} binding was interpreted as Fe^{2+} interaction at the FeOOH mineral surface with displacement of H^+ from –OH or phosphate surface groups by the incoming Fe^{2+} ions. Mossbauer spectroscopic measurements using ^{57}Fe-labeled Fe^{2+} under anaerobic conditions showed that $^{57}Fe^{2+}$ binding to holoferritin was accompanied by electron transfer to the core, yielding $^{57}Fe^{3+}$, presumably bound to the mineral surface. Removal of added iron by Fe^{2+}-specific chelating agents yielded $^{57}Fe^{2+}$, demonstrating the reversibility of this electron-transfer process. The Fe^{2+} bound to apo- and holoferritin is readily converted to Fe^{3+} by exposure to O_2 and strongly retained by the respective ferritin species.

Mammalian ferritin accommodates up to 4500 iron atoms within its hollow interior in the form of an $Fe^{III}OOH$ mineral core. The development of this core is generally thought [see Aisen and Listowsky (1980), Thiel (1987), and Crichton and Charloteaux-Wauters (1987) for reviews] to arise from O_2 oxidation of Fe^{2+}, resulting in Fe^{3+} which hydrolyzes within the protein interior. Such a sequence of events implies that Fe^{2+}–ferritin interactions occur during core development. The interaction of Fe^{2+} and other metal ions with apoferritin has been studied recently (Treffry & Harrison, 1984; Chasteen & Theil, 1982; Wardeska et al., 1985; Frankel et al., 1987) in an attempt to discern and evaluate the role of Fe^{2+} in mammalian ferritin core formation. These studies have shown that (1) Fe^{2+} addition to apoferritin produces an initial complex which undergoes O_2 oxidation followed by rearrangement or migration of Fe(III) with polynuclear species ultimately forming within the ferritin core (Treffry & Harrison, 1984; Rosenberg & Chasteen, 1982; Yang et al., 1987; Bauminger et al., 1989), (2) Fe^{2+} initially binds presumably at or near the hydrophilic channels with a stoichiometry of 8–12 Fe^{2+}/apoferritin, and a mixed-valent binuclear iron species forms when Fe^{2+} and the Fe(III) core interact during early core formation (Chasteen et al., 1985; Wardeska et al., 1985, 1986), and (3) $^{57}Fe^{2+}$ binds strongly to both apo- and holoferritin, forming ferritin-bound Fe^{2+}, in the former case, and undergoing electron exchange with the core, apparently forming core surface bound $^{57}Fe^{3+}$ in the latter case (Frankel et al., 1987).

Other studies have examined the properties of Fe^{3+}-bound ferritin resulting from oxidation of Fe^{2+} initially added at low levels to mammalian apoferritin. Mossbauer measurements (Yang et al., 1987; Bauminger et al., 1989) have demonstrated the early formation of both solitary Fe^{3+} and oxygen-bridged Fe(III) species which are replaced by small Fe(III) clusters as more iron accumulates within the ferritin core. EPR measurements (Rosenberg & Chasteen, 1982) demonstrate the presence of about 12 unique Fe^{3+} ions that remain constant in number as the core size increases. NMR relaxometry (Koenig et al., 1986) supports the presence and constancy of six to eight unique Fe(III) sites presumably near the outside of each ferritin molecule that are filled during early core formation and remain constant with additional iron loading. These results are all consistent with Fe^{2+} initially binding at unique apoferritin sites with subsequent conversion to Fe^{3+} upon oxidation. The constant number of unique Fe^{3+} ions suggests a fundamental role for these species in core formation.

We have examined the quantitative binding of Fe^{2+} to both apo and holo mammalian ferritin and report that well-defined Fe^{2+}–ferritin intermediates form in both cases. These results relate to and are discussed in terms of the early events of iron core formation in apoferritin and subsequent core development in holoferritin.

Materials and Methods

Horse spleen ferritin containing 2350 and 2565 iron atoms per ferritin molecule (used for the Fe^{2+} binding and the Mossbauer measurements, respectively) was obtained from Sigma and passed through G-25 columns to remove loosely bound metals. Total iron content was determined by the method of Lovenberg et al. (1963). Apoferritin was prepared by the thioglycolic acid dialysis procedure (Treffry & Harrison, 1978). The concentrated apoferritin at >20 mg/mL was noticeably yellow in color but contained <0.8 Fe/apoferritin molecule.

The equilibrium Fe^{2+} binding experiments, the pH measurements, and all other measurements involving the air-sen-

† This research was supported by the National Science Foundation Biophysics Program (Grants DMB 8512382 and 8902474) and by the Bioelectromagnetics Program of the Office of Naval Research (Contract N0014-88-K-0353).

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0006-2960/89/0428-9216$01.50/0

sitive Fe^{2+} state of ferritin were conducted under anaerobic conditions provided by a Forma (90% argon, 10% H_2, <3 ppm O_2) or a Vacuum Atmospheres (100% argon, <0.5 ppm O_2) glovebox.

Fe^{2+} Binding. Equilibrium binding of Fe^{2+} to apo- or holoferritin (at 2–8 mg/mL) in 0.025 M TES, pH 7.5, and 0.1 M NaCl at a constant pH was measured by three separate methods. The first was the flow dialysis method of Colowick and Womack (1969). Because Fe^{2+} weakly binds to the dialysis membranes, a correction was made by conducting the dialysis reaction in the absence of the ferritin proteins.

The second method consisted of anaerobic ultrafiltration of ferritin containing excess Fe^{2+} with Amicon molecular weight selective XM 100 membranes followed by successive buffer washes until all free Fe^{2+} was removed. The Fe^{2+} bound to the ultrafiltered protein was determined directly by quantitating Fe^{2+} present with bipyridyl (Lovenberg et al., 1963).

The most reliable and convenient method was anaerobic G-25 Sephadex chromatography using 1.0 × 30 cm columns equilibrated with 0.025 M TES/0.1M NaCl, pH 7.5. To the equilibrated anaerobic column was added 0.5 mL of apo- or holoferritin containing excess Fe^{2+} at a given pH which was then eluted anaerobically with Fe^{2+}-free buffer. The emerging protein peak was collected and analyzed for both protein (Lowry) and Fe^{2+} (bipyridyl). At low levels of Fe^{2+} addition, all Fe^{2+} was found in the protein fractions, indicating very strong and complete Fe^{2+} binding, whereas with Fe^{2+} added in excess, Fe^{2+} was found both with the protein fraction and in a band clearly separated from and trailing behind the protein front. The temperature was maintained at 27 ± 1.0 °C during these binding measurements.

H^+ Release as Fe^{2+} Binds. The extent of Fe^{2+} binding to apo- or holoferritin at a selected pH was first determined by one of the procedures described above. The pH change associated with Fe^{2+} binding was then determined by (1) adjusting the pH of apoprotein (1 mL), dissolved in 0.1 M NaCl with no buffer present, to the desired value, (2) adding a known but limiting amount (~20% of the predicted amount) of Fe^{2+}, and (3) adjusting the reaction mixture back to the original pH value with standardized NaOH or HCl. This procedure was repeated until the predicted amount of Fe^{2+} was bound. Control reactions involving the addition of the Fe^{2+} to 0.1 M NaCl (no protein present) corrected for H^+ added from the Fe^{2+} additions.

EPR Spectroscopy. EPR samples of apo- or holoferritin contained in 3-mm i.d. quartz EPR tubes were prepared by (1) adding 250 μL of apoferritin (9–17 mg/mL) to the EPR tube followed by 5–20 μL of a standard Fe^{2+} solution, (2) mixing the reagents, and (3) incubating anaerobically for 30 min. These samples were then opened to the air with gentle shaking for 1 h to oxidize the bound Fe^{2+} to Fe^{3+} and then frozen in liquid nitrogen prior to study at 4–10 K using a Varian 4502 EPR spectrometer at 9.2 GHz. Another identical set of EPR samples was prepared except all operations were carried out aerobically. The EPR signal height at $g = 4.3$ of the resulting Fe^{3+} species was measured as a function of added Fe^{2+} as the Fe^{2+}/apoferritin ratio was varied from 1 to 100.

Mossbauer Spectroscopy. Iron-57 metal enriched to 95% (New England Nuclear–Du Pont) was dissolved in 0.01 M H_2SO_4 (50–70 °C) under anaerobic conditions to produce $^{57}Fe^{2+}$ at concentrations of 0.01–0.1 M at pH 5–6.5. $^{57}Fe^{3+}$ was well below 1% of the total iron present. Excess $^{57}Fe^{2+}$ (50–100-fold excess for apo and 200–500-fold excess for holo) was added to 1.0 mL of apoferritin and holoferritin (20–30 mg/mL) at pH 7.0 or 9.0, incubated anaerobically for 30 min, and subsequently passed through a 1 × 30 cm anaerobic G-25 Sephadex column equilibrated at the corresponding pH to remove excess $^{57}Fe^{2+}$. An aliquot of each ferritin fraction was reacted with bipyridyl to determine the Fe^{2+}/ferritin ratio and to verify that the iron was present as $^{57}Fe^{2+}$.

Table I: Mossbauer Samples Prepared by Anaerobic Binding of Fe^{2+} to Apoferritin and to Holoferritin Containing an Average Number $\langle n \rangle$ = 2565 Fe^{3+} per Molecule

sample	pH	original $^{57}Fe^{3+}$	step 1 added $^{57}Fe^{2+}$ [a]	step 2 added $^{57}Fe^{2+}$ [b]	net $^{57}Fe^{2+}/Fe^{3+}$
holo(1)	7.0	50	107[c]		107/50
holo(2)	7.0	50	107[c]	3[c]	3/157
holo(3)	9.0	50	294[d]		294/50
holo(4)	9.0	50	294[d]		7/344
apo(1)	7.0	0	8		8/0
apo(2)	9.0	0	61		61/0
apo(3)[e]	9.0	0	61		61/0

[a] Fe^{2+} added as 95% iron-57. [b] Fe^{2+} added as 2.2% iron-57 following oxidation of Fe^{2+} added in step 1. [c] Total Fe^{2+} bound = 133. [d] Total Fe^{2+} bound = 309. [e] 1 mM dithionite.

The resulting apoferritin containing bound $^{57}Fe^{2+}$ with no unbound Fe^{2+} present was used to prepare three samples (Table I) for Mossbauer spectroscopy: the first, designated apo(1), was $^{57}Fe^{2+}$ bound to apoferritin at pH 7.0; the second, apo(2), was $^{57}Fe^{2+}$ bound to apoferritin at pH 9.0; and the third, apo(3), was $^{57}Fe^{2+}$ bound to apoferritin at pH 9.0 containing 1.0 mM $S_2O_4^{2-}$ to assure complete reduction.

The holoferritin samples at pH 7.0 and 9.0 each containing bound $^{57}Fe^{2+}$ were divided into two separate portions. One portion was loaded into an air-tight plastic container and frozen. The other portion of each sample was exposed to air for 1 h with gentle stirring to oxidize the added $^{57}Fe^{2+}$ to $^{57}Fe^{3+}$, then made anaerobic, and incubated with excess non-isotopically enriched ferrous sulfate (2.2% iron-57) under argon for 30 min. After passage through an anaerobic Sephadex G-25 column, an aliquot was removed to verify that all iron added in the second step was in the Fe^{2+} state, and the remainder was loaded into an air-tight plastic container and frozen. A total of four samples of ferrous bound holoferritin were thus prepared for Mossbauer spectroscopy (Table I): holo(1), 95% $^{57}Fe^{2+}$-bound holoferritin at pH 7.0; holo(2), natural Fe^{2+} (2.2% ^{57}Fe) bound holoferritin following 95% $^{57}Fe^{2+}$ binding and oxidation at pH 7.0; holo(3), 95% $^{57}Fe^{2+}$-bound holoferritin at pH 9.0; holo(4), natural Fe^{2+}-bound holoferritin following 95% $^{57}Fe^{2+}$ binding and oxidation at pH 9.0.

Mossbauer spectra of the three apoferritin and four holoferritin samples were obtained with a variable-temperature cryostat and a constant-acceleration spectrometer at a number of temperatures between 4.2 and 250 K. Spectra were analyzed with a least-squares fitting program to yield isomer shifts, magnetic hyperfine and electric quadrupole splittings, and relative intensities of spectral components. Isomer shifts were measured with respect to iron metal at room temperature.

Results

Rapid flow dialysis, selective membrane filtration, and G-25 Sephadex chromatography all demonstrate that apo- or holoferritin avidly binds Fe^{2+} under anaerobic conditions. The latter method is direct, convenient, very reproducible, and can be easily adapted to cover wide ranges of pH as well as Fe^{2+} concentrations, ranging from limiting to large excesses. Most results reported here were obtained with the G-25 Sephadex method.

Fe^{2+} Binding to Apoferritin. Figure 1a is a plot of Fe^{2+} bound to apoprotein as a function of pH. These data were

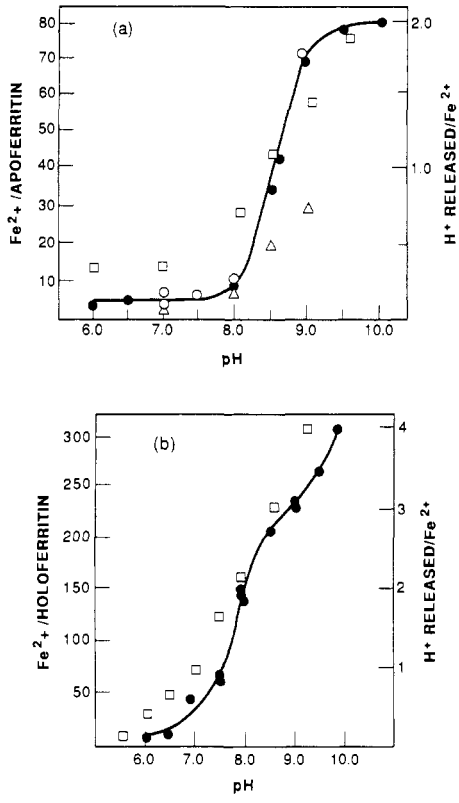

FIGURE 1: Fe^{2+} binding to apo- and holoferritin. (a) Number of Fe^{2+} bound to two separate anaerobic apoferritin samples (○, ●) in 0.025 M TES adjusted to the indicated pH values. Fe^{2+} binding in the presence of 0.5 mM $Na_2S_2O_4$ (△). Proton release (□) as Fe^{2+} binds to apoferritin, right axis. (b) Fe^{2+} binding to holoferritin (2350 Fe^{3+}/ferritin) under the same conditions as (a). Proton release (□) as Fe^{2+} binds to holoferritin, right axis.

obtained after 30-min incubation at the indicated pH with excess Fe^{2+}, passing the sample through the G-25 Sephadex column, and measuring the Fe^{2+} associated with the protein. Longer (>30 min) or shorter (15 min) incubation times did not change the Fe^{2+} binding values. The amount of Fe^{2+} initially added was accounted for either in the protein band alone or in the protein band and in a separate Fe^{2+} band clearly separated from the protein, depending on whether limiting or excess Fe^{2+} was initially added. Control experiments on a calibrated column demonstrated that added Fe^{2+} emerged as a single band well behind the position of the solvent front, indicating that aggregated Fe^{2+} species were absent and that Fe^{2+} found with ferritin was protein associated. Nearly constant values of 8.0 ± 0.5 Fe^{2+}/apo were obtained in the pH range 6–7.5. Above pH 7.5, the Fe^{2+}/apoferritin ratio dramatically increases with increasing pH until at pH 10.0 a value of 80 Fe^{2+}/apoferritin is obtained. Some irreproducibility was observed at pH >9, due perhaps to protein alterations at these higher pH values or $Fe(OH)_2$ formation upon Fe^{2+} addition, but those results presented in Figure 1a were usually observed.

Typically, the thioglycolic acid method for iron removal produces apoferritin with traces of Fe^{3+} (~0.5–3 Fe^{3+}/ferritin molecule) still present. To eliminate the possibility that this remaining iron acts as a nucleation site, we further treated the apoprotein with excess $S_2O_4^{2-}$ containing 0.01 mM methyl viologen in the presence of excess bipyridyl and passed the resulting mixture through an anaerobic G-25 column. The resulting protein had <0.1 Fe^{3+}/ferritin, but still bound Fe^{2+} as shown in Figure 1a, establising that Fe^{2+} binding is a property of the apoferritin molecule. We further observed that changing the apoferritin concentration by a factor of 8, at a carefully controlled pH, produced identical results with those shown in Figure 1a, demonstrating that Fe^{2+} binding is strong and independent of protein concentration over this limited concentration range.

Above pH 7.5, Figure 1a shows a sharp pH-dependent Fe^{2+} binding profile with an apparent pK of 8.65, indicating that proton release is linked to Fe^{2+} binding. This behavior is distinct from $Fe(OH)_2$ precipitation observed by Bryce and Crichton (1973). The nearly symmetrical "S"-shaped curve clearly shows that Fe^{2+} binding is dramatically increased by at least a factor of 10 in the pH range 8–10 but then reaches a plateau above pH 9. The reversibility of Fe^{2+} binding was demonstrated by (1) loading apoferritin with Fe^{2+} (no free Fe^{2+} present) at pH 8.5–10 as shown in Figure 1a, (2) adjusting the pH anaerobically to pH 6 or 7, and (3) passing the protein through a G-25 column equilibrated at the lower pH values. Under these conditions, Fe^{2+} was released as predicted by Figure 1a with six to nine Fe^{2+} remaining bound to the apoferritin at the lower pH and the remaining Fe^{2+}, originally bound at the higher pH, clearly separated from and trailing behind the emerging apoferritin peak. Exposure to O_2 of the samples shown in Figure 1a resulted in the bound Fe^{2+} being converted quantitatively into ferritin-bound Fe^{3+}, demonstrated by both EPR and Mossbauer spectroscopy.

Fe^{2+} Binding in Holoferritin. Figure 1b is a plot of Fe^{2+} bound to holoferritin as a function of pH. As with apoferritin, Fe^{2+} binding to holoferritin is pH dependent, but the degree of Fe^{2+} binding, at a given pH, is much greater with holoferritin than with apoferritin. The binding profile is also more complex with at least two distinct binding regions being clearly visible. Fe^{2+} binding below pH 6 was complicated because of protein precipitation as the isoelectric point (pH ~5.5) of holoferritin was approached. Binding above pH 10 was erratic due to $Fe(OH)_2$ formation and possible alteration in the protein and core structures. The strong pH dependence suggests that proton release accompanies Fe^{2+} binding to holoferritin, a result which has been verified experimentally and will be discussed below.

Reversibility of Fe^{2+} binding to holoferritin was only partially demonstrated. When holoferritin (free of unbound Fe^{2+}) at pH 9–10 was adjusted to pH 6–7, free Fe^{2+} was formed, but only 70–90% of that predicted from Figure 1b. Increasing the incubation period at the lower pH tended to increase the amount of released Fe^{2+}, but still incomplete (5–15% Fe^{2+} remaining) Fe^{2+} release was observed. Exposure of Fe^{2+}-bound holoferritin to O_2 resulted in conversion of the bound Fe^{2+} into Fe^{3+}, retained by the protein.

pH Effects Accompanying Fe^{2+} Binding. Figure 1 demonstrates a strong dependence of Fe^{2+} binding on pH in both apo- and holoferritin, suggesting that protons are released during the Fe^{2+} binding process. Direct pH measurements also shown in Figure 1 quantitate the H^+ release occurring with Fe^{2+} binding to both apo- and holoferritin. Rapid and complete H^+ release occurs within 1–2 min for Fe^{2+} binding to apoferritin, but for holoferritinin, H^+ release is followed by a much slower proton release "drift". This effect was more noticeable at pH >8.5 and occurred even in the absence of added Fe^{2+}, indicating that the core itself is responsible for the "drift". Similar effects were noted by Silk and Breslow (1976) during acid/base titrations of deionized ferritin.

EPR Measurements. Anaerobic incubation of Fe^{2+} with apoferritin followed by air oxidation or addition of Fe^{2+} to aerobic apoferritin equally produced a $g = 4.3$ EPR signal of comparable amplitude measured at 4–10 K. The signal intensity (at constant line width) increased rapidly to 8–12 Fe^{3+}/apoferritin, after which it remained constant with in-

creasing Fe^{3+} accumulation in the ferritin core up to a value of 100. This constant number of EPR-active Fe^{3+} ions is numerically equal to the number of Fe^{2+} binding sites measured above, suggesting the two events are related and may occur at the same site. Our results are consistent with those of Rosenberg and Chasteen (1982) except they observed that the EPR intensity slowly decreased with increasing iron loading, whereas we report a constant EPR signal intensity with increased core development. This difference may be a consequence of the lower temperature (4–10 K) used in our EPR measurements compared to that (77 K) used by Rosenberg and Chasteen.

Mossbauer Measurements. The Mossbauer spectra of apo(1) and apo(2) consisted primarily of a ferrous quadrupole doublet (isomer shift IS = 1.26 mm/s, quadrupole splitting QS = 3.10 mm/s at 80 K) and a residual ferric doublet (IS = 0.47 mm/s, QS = 0.71 mm/s, approximately 6% of ferrous intensity). The residual ferric doublet was present even in samples where extreme measures were taken to assure anaerobic sample preparation. The spectrum of apo(3) was a ferrous doublet with no residual ferric doublet. Thus, the ferric doublet in apo(1) and apo(2) appeared to arise from partial oxidation of the bound Fe^{2+} by the anaerobic apoferritin, in the absence of excess reductant.

Application of an 80-kOe external magnetic field to apo(3) at 4.2 K (data not shown) resulted in a broad, asymmetric spectrum similar to that reported for 10 ferrous ions bound to apoferritin by Yang et al. (1987). The spectrum reflected magnetic hyperfine interactions at the iron nuclei in addition to the applied magnetic field, indicating that the Fe^{2+} ions were bound in the high-spin state and were not magnetically ordered at 4.2 K.

The Mossbauer spectra of holo(1) at 4.2, 20, 40, and 80 K are shown in Figure 2. The spectra of holo(2), holo(3), and holo(4) were similar. The spectra could be decomposed into subspectra corresponding to ferric and ferrous iron. The ferrous subspectrum consisted of a broadened quadrupole doublet at all temperatures. In holo(4), the 80 K ferrous subspectrum could be decomposed into two closely overlapping quadrupole doublets. The ferric subspectrum in all samples exhibited the superparamagnetic transition characteristic of fully oxidized ferritin (Oosterhuis & Spartalian, 1976) with a magnetic hyperfine split sextet at 4.2 K and a quadrupole doublet at 80 K. Between 4.2 and 80 K, the ferric doublet and sextet were superposed with relative intensities that were temperature dependent. By definition, the average superparamagnetic blocking temperature, $\langle T_B \rangle$, is the temperature at which the doublet and sextet have equal intensities. The experimental values of $\langle T_B \rangle$ were 29, 27, 34, and 30 K for holo(1), holo(2), holo(3), and holo(4), respectively. In every holo sample, $\langle T_B \rangle$ was less than the average blocking temperature of fully oxidized ferritin ($\langle T_B \rangle$ = 38–40 K) and of ferritin electrochemically reduced by 40% ($\langle T_B \rangle$ = 51 K) (Frankel et al., 1987).

It should be noted that the total absorption area of the ferric sextet at 4.2 K is somewhat greater than the area of the ferric doublet at 80 K. This appears to be a consistent feature of all the ferritin data we have accumulated. This difference in total absorption areas could be due in part to the temperature dependence of the recoilless fraction. However, data collected at higher temperature (see below) show that the ferric recoilless fraction is practically saturated below 80 K. Another contribution to the difference in total area could arise from differential saturation effects in the two spectra, with the sextet lines less saturated than the doublet lines (Greenwood & Gibb, 1971). Data collected on samples of different thickness (not shown) show this effect does contribute but apparently does not completely account for the difference. We are currently investigating the possibility that there are changes in the recoilless fraction associated with the superparamagnetic transition. In any case, even if the doublet and sextet absorption areas are normalized by the 80 K doublet and 4.2 K sextet absorption areas, respectively, the average blocking temperatures of the Fe^{2+}-bound holo samples are still of the order of 5–10 K less than fully oxidized ferritin.

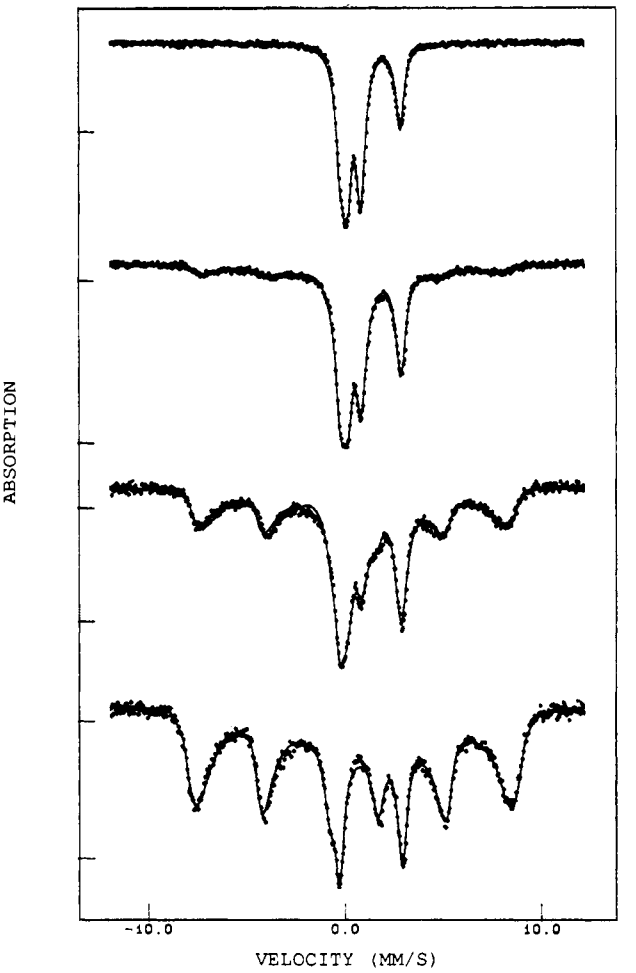

FIGURE 2: Mossbauer spectra of holo(1) at 80, 40, 20, and 4.2 K (top to bottom). The 80 K spectrum was fitted with a ferrous doublet (IS = 1.34 mm/s, QS = 3.04 mm/s) and a ferric spectrum (IS = 0.50 mm/s, QS = 0.71 mm/s). The relative intensities are given in Table II. The ferric sextet spectrum corresponded to a magnetic hyperfine field distribution with mode = 497 kOe.

In spectra obtained between 80 and 200 K, the ferrous absorption area decreased faster with increasing temperature than the ferric absorption area (Figure 3). This is similar to partially reduced ferritin (Frankel et al., 1987). The ferric doublet of all the holo samples had a temperature dependence of the absorption area similar to that of fully oxidized ferritin. The ferrous doublet had a temperature dependence of the absorption area similar to ferrous-bound apoferritin. The ferrous doublet of holo(2) and holo(4) decreased slightly faster with increasing temperature than that of holo(1) and holo(3).

The relative intensities of the ferric and ferrous doublets for the holo samples are shown in Table II. Assuming that no net oxidation of the bound ferrous ions occurred in the initial addition of Fe^{2+} to holoferritin, the ferrous and ferric absorption areas at 80 K would be proportional to the number of the respective ions with iron-57 nuclei in the sample if the recoilless fractions of Fe^{3+} and Fe^{2+} were equal at 80 K.

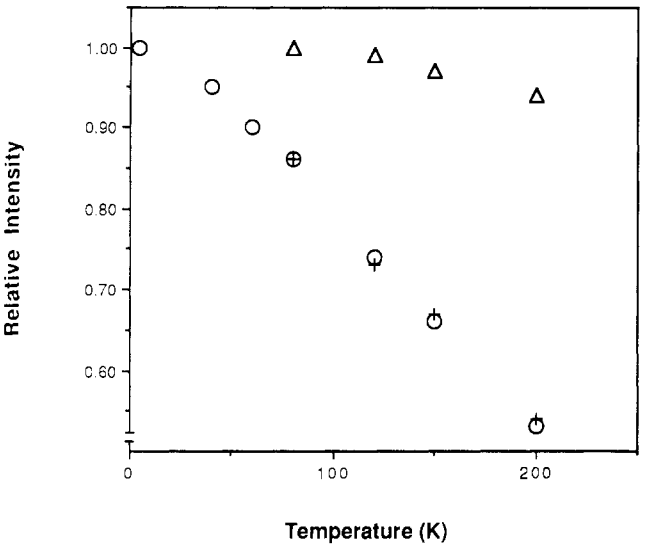

FIGURE 3: Relative Mossbauer absorption areas plotted as a function of temperature for (O) apo(3) ferrous spectrum, (+) holo(1) ferrous spectrum, and (Δ) holo(1) ferric spectrum. The ferrous spectra were normalized at 4.2 K; the ferric spectra were normalized at 80 K.

Table II: Relative Ferric and Ferrous Iron-57 Mossbauer Intensities in Holoferritin Samples

sample	experimental[a]	corrected[b]	theoretical[c]
holo(1) Fe^{2+}	0.34	0.36	0.68 (107)
holo(1) Fe^{3+}	0.66	0.64	0.32 (50)
holo(2) Fe^{2+}	0.17	0.19	0.02 (3)
holo(2) Fe^{3+}	0.83	0.81	0.98 (157)
holo(3) Fe^{2+}	0.56	0.59	0.85 (294)
holo(3) Fe^{3+}	0.44	0.41	0.15 (50)
holo(4) Fe^{2+}	0.23	0.25	0.02 (7)
holo(4) Fe^{3+}	0.77	0.75	0.98 (344)

[a] Experimental relative absorption areas at 80 K. [b] Absorption areas corrected for temperature dependences of the recoilless fractions (see text). [c] Calculated relative concentrations of Fe^{2+} and Fe^{3+} assuming no electron exchange between added ferrous iron and ferric iron in the core particle. Numbers in parentheses are average iron-57 ions of each valence per molecule from Table I.

Considering the temperature dependence of Fe^{2+} in apoferritin, the Fe^{2+} recoilless fraction in holoferritin could be of the order of 10% lower than the Fe^{3+} recoilless fraction at 80 K. The absorption areas corrected for this difference are included in Table II. Even with this correction, the ferric doublet was more intense than expected in holo(1) and holo(3), whereas the ferrous doublet was more intense than expected in holo(2) and holo(4). Let us consider holo(1) and holo(3). If all the original 2565 Fe^{3+} ions per molecule remained Fe^{3+}, and all the added 95% $^{57}Fe^{2+}$ ions remained Fe^{2+}, we would expect, on the average, holo(1) to have 113 $^{57}Fe^{2+}$ and 50 $^{57}Fe^{3+}$, or ferrous and ferric relative absorption areas of 0.69 and 0.21, respectively. The corrected experimental values were 0.36 ferrous and 0.64 ferric for holo(1). Holo(3) would be expected to have 309 $^{57}Fe^{2+}$ and 50 $^{57}Fe^{3+}$, or ferrous and ferric relative absorption areas of 0.86 and 0.14, respectively. The corrected experimental values were 0.59 ferrous and 0.41 ferric for holo(3).

In holo(2) and holo(4), the original 95% iron-57-bound Fe^{2+} ions were oxidized, giving an average of 163 and 359 $^{57}Fe^{3+}$ per molecule, respectively. Subsequent addition of 2.2% iron-57 Fe^{2+} resulted in 2.5 $^{57}Fe^{2+}$ per molecule in holo(2) and 7 $^{57}Fe^{2+}$ per molecule in holo(4). Thus, the expected relative absorption areas were 0.015 ferrous and 0.985 ferric in holo(2); the corrected experimental values were 0.19 ferrous and 0.81 ferric. For holo(4), the expected relative absorption areas were 0.02 ferrous and 0.98 ferric. The corrected experimental values for holo(4) were 0.25 ferrous and 0.75 ferric.

Thus, in holo(1) and holo(3), some of the added $^{57}Fe^{2+}$ was converted to Fe^{3+}, whereas in holo(2) and holo(4), some of the $^{57}Fe^{3+}$ bound in the core was converted to Fe^{2+} after addition of unenriched Fe^{2+}. A consistent interpretation of these results is that the Fe^{2+} ions, when bound to holoferritin under anaerobic conditions, enter the ferritin cavity, bind to the hydrous ferric oxide core, and partially exchange electrons with the ferric ions in the core. In holo(1) and holo(3), this process resulted in partial oxidation of the added $^{57}Fe^{2+}$ by Fe^{3+} ions in the core. In holo(2) and holo(4), this process resulted in partial reduction of $^{57}Fe^{3+}$ in the core by the added Fe^{2+}. The conversion of added $^{57}Fe^{2+}$ to $^{57}Fe^{3+}$ in holo(1) and holo(3) upon binding to the holoferritin core was confirmed to be an "internal oxidation" as opposed to inadvertent oxidation (by O_2, for example) by reacting the Fe^{2+}-bound ferritin with bipyridyl and showing that the original amount of added Fe^{2+} could be recovered. Inadvertent oxidation would have given a smaller Fe^{2+} recovery.

Entrance of Fe^{2+} into the cavity and binding to the core are also consistent with our previous observation that partial reduction by dithionite following Fe^{2+} binding preferentially reduces $^{57}Fe^{3+}$ to $^{57}Fe^{2+}$ (Frankel et al., 1987).

The lower average blocking temperatures of the Fe^{2+}-bound holo samples compared to fully oxidized ferritin presumably reflect binding of the added $^{57}Fe^{2+}$ to the surface of the core particle. The $^{57}Fe^{3+}$ produced by oxygen or ferric ion oxidation would be sited predominantly near or on the surface of the core particle. In the theory of superparamagnetic relaxation as applied to ferritin (Oosterhuis & Spartalian, 1976), the electronic spins of all the ferric ions in the core particle relax at the same rate, which is an exponential function of particle volume/temperature. For a given particle volume, the blocking temperature is the temperature at which the spin relaxation rate equals the Larmor precession frequency of the iron-57 nucleus in the local magnetic hyperfine field. Possible explanations for the experimental decrease in the blocking temperatures of the surface ferric ions compared to the bulk include a decrease in the magnetic hyperfine field in the surface ferric ions. However, measurements of the hyperfine field distributions for the holo samples show they are only marginally different than fully oxidized ferritin. It is also possible that the surface ferric ions form protoclusters that have smaller effective volumes than the bulk core particle with consequently lower blocking temperatures. Finally, it is also conceivable that the surface ferric ions relax at a faster rate than the bulk ions at the same temperature, especially those that are in contact with ferrous ions on the surface. In this regard, it should be noted that in 40% reduced ferritin, the bulk blocking temperature *increases* compared to fully oxidized ferritin (Frankel et al., 1987). This latter effect may arise from the massive number of surface ferrous ions on the magnetic anisotropy of the core particle (Bell et al., 1984). Further investigation is required to elucidate the decrease of the blocking temperatures in the present case.

Discussion

As an iron storage protein, ferritin can accumulate large numbers of iron atoms (up to 4500 Fe/ferritin) in its interior in the form of an iron(III) oxohydroxy mineral core. The stepwise events leading to core formation are beginning to emerge, and prominent in this development is the view that Fe^{2+} interactions occur early and likely produce important intermediates during core development. In fact, recent studies from our laboratory (Watt et al., 1985) demonstrate that even

ferritin cores containing all Fe^{2+} are stable and attest to strong Fe^{2+}–ferritin interactions. In this report, we have clearly established the presence of Fe^{2+}–ferritin intermediates in apoferritin, have measured some aspects of the hydrolytic equilibria associated with Fe^{2+} binding, and have begun to examine the fate of these Fe^{2+}–ferritin intermediates upon O_2 oxidation by both EPR (Rosenberg & Chasteen, 1982) and Mossbauer spectroscopy.

The results in Figure 1 demonstrate, by direct methods, that Fe^{2+} has a strong affinity for and binds reversibly to apoferritin with a stoichiometry of 8.0 ± 0.5 Fe^{2+}/apoferritin in the pH range 6.0–7.5. Fe^{2+} and not Fe^{3+} is clearly the species bound because of the anaerobic conditions maintained during the experiments and because the analysis for bound iron measured both total iron as well as Fe^{2+} (as the bipyridyl complex) present in apoferritin. The value of 8 Fe^{2+}/apoferritin (0.33 Fe^{2+}/subunit) confirms the results of Wardesks et al. (1986), and the small proton release value upon Fe^{2+} binding (Figure 1a) suggests that Fe^{2+} is bound as solitary, unhydrolyzed ions (probably as carboxylate complexes) in each of the 8 hydrophilic channels located on 3-fold axes leading to the protein interior. Exosure of the 8 Fe^{2+}–apoferritin intermediate to O_2 produces the corresponding Fe^{3+}–ferritin with apparently isolated Fe^{3+} ions as judged by the development of an Fe^{3+} EPR signal at $g = 4.3$ and a quadrapole doublet in the Mossbauer spectrum. Similar EPR results and conclusions have been reported by Rosenberg and Chasteen (1982) and recently supported by NMR relaxometry (Konig et al., 1986). The O_2 oxidation of well-defined Fe^{2+} intermediates in the absence of excess Fe^{2+} may be useful in delineating in further detail the stepwise Fe^{3+}–ferritin reactions (Bauminger et al., 1989) occurring in early core development.

Above pH 7.5, the dramatic increase in Fe^{2+} binding accompanied by the release of up to two H^+ probably results from the formation of ferritin-bound $Fe(OH)_2$. The leveling off in Figure 1a may be due to the saturation or filling of the region of ferritin near the initial Fe^{2+} binding sites because otherwise Fe^{2+} hydrolysis would not be expected to reach this limiting value. Fe^{2+} binding at these high pH values is reversible, a result consistent with the neutralization of $Fe(OH)_2$ aggregates forming free Fe^{2+}. Thus, at physiological pH values, a well-defined Fe^{2+}–ferritin intermediate is clearly established. Above this pH, $Fe(OH)_2$–ferritin complexes readily form.

Fe^{2+} binding to holoferritin is dramatically different compared to apoferritin, a result which is directly attributable to the presence of the FeOOH core. The large numbers of Fe^{2+} ions that bind at pH >6.5 indicate that Fe^{2+} binding is complex and is probably occurring at multiple, pH-dependent binding sites (most likely deprotonated –OH or phosphate groups) present on the mineral surface. This complexity is further demonstrated by the incomplete reversibility of Fe^{2+} binding to holoferritin as well as the proton release results (Figure 1b). Some insights into this Fe^{2+} binding process are revealed by the Mossbauer results in Table II which show that Fe^{2+} binding is accompanied by an electron-transfer reaction resulting in the formation of Fe^{3+}. Hydrolysis of the surface-bound Fe^{3+} would give rise to the high proton release values and is probably responsible for the partial Fe^{2+} binding irreversibility. Thus, the nature of the Fe^{2+} interactions with the surface groups on the mineral surface and how these interactions promote electron-transfer and hydrolysis reactions of the bound metal ions are important questions to pursue in gaining an understanding of ferritin core development.

Registry No. Fe, 7439-89-6.

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