Iron Core Formation in Horse Spleen Ferritin: Magnetic Susceptibility, pH, and Compositional Studies

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ABSTRACT

Horse spleen ferritin (HoSF) reconstituted with small iron cores ranging in size from 8 to 500 iron atoms was studied by magnetic susceptibility and pH measurements to determine when the added Fe^{3+} begins to aggregate and form antiferromagnetically coupled clusters and also to determine the hydrolytic state of the iron at low iron loading. The Evans NMR magnetic susceptibility measurements showed that at iron loadings as low as $8 \text{ Fe}^{3+}/\text{HoSF}$, at least half of the added iron atoms were involved in antiferromagnetic exchange interactions and the other half were present as isolated iron atoms with $S = 5/2$. As the core size increased to about 24 iron atoms, the antiferromagnetic exchange interactions among the iron atoms increased until reaching the limiting value of 3.8 Bohr magnetons per iron atom, the value present in holo HoSF. HoSF containing eight or more Fe^{3+} to which eight Fe^{2+} were added showed that the Fe^{2+} ions were at sites remote from the Fe^{3+} and that the resulting HoSF consisted of individual, noninteracting Fe^{2+} and the partially aggregated Fe^{3+} . pH measurements for core reduction showed that $\text{Fe}(\text{OH})_3$ was initially present at all iron loadings but that in the absence of iron chelators the reduced iron core is partially hydrolyzed. Proton induced x-ray emission spectroscopy showed that Cl^- is transported into the iron core during reduction, forming a stable chlorohydroxy $\text{Fe}(\text{II})$ mineral phase.

Ferritin from animal sources is a multisubunit protein with an internal cavity that sequesters a core of iron atoms as the ferric oxy-hydroxide mineral ferrihydrite, nominally $5\text{Fe}_2\text{O}_3 \cdot 9\text{H}_2\text{O}$, associated with variable amounts of

inorganic phosphate [1–4]. Formation of the iron mineral core is presumably a multistep, protein-mediated process which involves binding of Fe^{2+} to protein sites, oxidation of bound Fe^{2+} to Fe^{3+} by molecular oxygen or other oxidants, hydrolysis of the resulting Fe^{3+} , and finally, nucleation of ferrihydrite within the ferritin cavity. Apoferritin is therefore a ferroxidase, catalyzing the oxidation of ferrous ions [5, 6], and a putative ferroxidase center situated within the four-helix subunit bundle has been identified on H-chain subunits, but L-chain subunits lack this center [7]. It has been proposed that O_2 -oxidation of Fe^{2+} by apoferritin initially occurs at the ferroxidase centers, followed by migration of Fe^{3+} to mineral nucleation sites in the cavity [8]. Oxidation of the ferrous ions in the ferroxidase center is thought to involve formation of isolated Fe^{3+} [9, 10] as well as oxo dimers [10, 11]. Because bacterial ferritins have only one subunit type, the concept of H subunit containing the ferroxidase center needs to be further examined for the bacterial ferritin system. However, once the core has been nucleated, it presumably provides additional surface sites [6, 12] for the binding and oxidation of Fe^{2+} . Details of the stoichiometry of Fe^{2+} oxidation by horse spleen ferritin (HoSF) and evidence for oxidation at mineral surface sites has recently been reported [13].

Under anaerobic conditions, apo HoSF binds only eight Fe^{2+} at pH 6.0–8.0, even if incubated with excess Fe^{2+} [14, 15]. The binding of these eight Fe^{2+} ions presumably represents the initial binding step of the multistep process leading to core formation. After oxidation of bound Fe^{2+} , forming HoSF with eight Fe^{3+} present [$\text{HoSF}(8 \text{ Fe}^{3+})$], eight more Fe^{2+} bind when the $\text{HoSF}(8 \text{ Fe}^{3+})$ is again incubated with excess Fe^{2+} under anaerobic conditions. Oxidation of the Fe^{2+} ions must involve their translocation to other sites in the protein because, as demonstrated [18], the eight hydrophilic Fe^{2+} sites are then available for binding additional incoming Fe^{2+} ions. Cycles of oxidation and binding of eight Fe^{2+} have been repeated up to ten times [14]. This result is consistent with one Fe^{2+} binding site in each of the eight hydrophilic, threefold symmetric channels [14, 15]. Minimal proton release accompanies Fe^{2+} binding to apoferritin at pH 6.0–8.0 [18], suggesting that Fe^{2+} is bound as solitary, unhydrolyzed ions, probably as carboxylate or histidine [16] complexes.

The location of the Fe^{3+} binding sites which are filled subsequent to Fe^{2+} oxidation are not known, although it has been proposed [8] that Fe^{3+} ions pass through the H-chain ferroxidase center on their way to nucleation sites in the cavity. However, an Fe^{3+} binding site involving tyrosine has been suggested in amphibian ferritin [17]. The final state of hydrolysis of the Fe^{3+} during initial iron deposition was inferred [13] to be $\text{Fe}(\text{OH})_3$, as summarized by reaction (1), but the point in the overall process where hydrolysis first occurs remains undetermined.

Additional information regarding the nature of Fe^{3+} during early core formation can be obtained from the complementary process of proton uptake during iron reduction from ferritin containing native or reconstituted iron cores. Holo HoSF containing 2300 iron atoms was reported [19] and recently confirmed [20] to undergo reduction in the absence of Fe^{2+} chelators with retention of Fe^{2+} in the core. Electron transfer to the core was accompanied by two H^+/e at pH 7.0,

as represented by reaction (2), suggesting the presence of a stable, and partially hydrolyzed Fe^{2+} mineral phase within the ferritin cavity.

In the presence of bipyridine, Fe^{2+} was removed completely from ferritin as $\text{Fe}(\text{bipyd})_3^{2+}$, a result which implies reaction (3).

Evaluating the proton stoichiometry for core reduction and iron release should provide information on the nature of the redox properties and hydrolytic state of iron at low Fe/HoSF ratios. For example, if at low $\text{Fe}^{3+}/\text{HoSF}$ ratios unhydrolyzed Fe^{3+} ions are bound at protein sites consisting of amino acid residues exclusively, then no proton uptake at pH 7–8 would be expected upon reduction. However, if Fe^{3+} is completely hydrolyzed at low iron loading, then reduction of iron to Fe^{2+} state should release 3H^+ , unless Fe^{2+} formed from reduction of $\text{Fe}(\text{OH})_3$ remains partially hydrolyzed as shown in reaction (2). Thus, measurement of proton uptake accompanying iron core reduction could provide a measure of the degree of ferric iron hydrolysis in the protein as a function of iron loading.

Related to the hydrolytic state of iron in early core formation is the degree of aggregation of Fe^{3+} ions in the ferritin cavity. By determining the magnetic state of the iron for lightly loaded HoSF, it should be possible to determine whether Fe^{3+} is present as isolated ions, or whether Fe^{3+} ions have aggregated to form magnetically-coupled clusters. In the latter case, the effective magnetic moment per iron will be decreased from the free ion value. Antiferromagnetic exchange coupling in holo HoSF ferrihydrite cores decreases the effective magnetic moment per iron, μ_{eff} , from the free ion value of $5.92\mu_B$ to $3.85\mu_B$ [21].

In this paper, we report measurements of proton uptake accompanying reduction of small, reconstituted iron cores in HoSF ($8\text{--}100 \text{ Fe}^{3+}$) at physiological pH. The results indicate that iron is fully hydrolyzed even for HoSF containing 8 Fe^{3+} , $\text{HoSF}(8 \text{ Fe}^{3+})$. In addition, we report the use of nuclear magnetic resonance to measure the effective moment per iron of HoSF with small iron cores in order to detect antiferromagnetic exchange interactions which indicate formation of clusters in the core. Finally, we report analytical data on fully reduced HoSF cores prepared anaerobically by reaction (2), and show that chloride ion is taken up during reduction of the cores forming stable chlorohydroxy $\text{Fe}(\text{II})$ complexes within the HoSF interior.

MATERIALS AND METHODS

Sample Preparation

Holo HoSF was purchased from Sigma or Boehringer and the iron cores removed to form apo HoSF by the thioglycolic acid-methyl viologen procedure [19]. Reconstituted HoSF containing $8\text{--}500 \text{ Fe}^{3+}/\text{HoSF}$ at $10\text{--}30 \text{ mg/mL}$ was prepared by incrementally adding an anaerobic Fe^{2+} solution to a rapidly

stirred, air saturated, apo HoSF solution maintained at pH 7.0. Following oxidation, the reconstituted HoSF solutions were filtered through a 0.45 μ millipore filter to remove small particles. Iron was determined by inductively coupled plasma (ICP) spectroscopy or by reducing the reconstituted HoSF anaerobically with dithionite, adding excess α , α -bipyridyl and determining iron concentrations as the ferrous chelate, using 8400 $\text{cm}^{-1} \text{ M}^{-1}$ at 520 nm.

The HoSF samples were then dialyzed against 0.15 M NaCl for the pH measurements or 0.05 M TES, pH 7.5, for the NMR magnetic susceptibility measurements described below. The NMR and pH measurements could only be made within 1–2 hr following reconstitution, due to the time required for sample preparation. Reduced methyl viologen (MV) was prepared by the method of Watt and Corbin [22]. Vacuum Atmospheres glove boxes containing nitrogen ($\text{O}_2 < 1 \text{ ppm}$) were used for reactions involving air sensitive reagents.

Proton Uptake

Reconstituted HoSF in 0.15 M NaCl containing variable amounts of iron (no buffer present) was diluted to 5–10 mg/mL inside the glove box and the pH adjusted to 5.0 or 7.0 with 1.0 mM NaOH. Excess reduced MV previously adjusted to the desired pH was then added and the pH change occurring upon reduction of the iron was recorded. After reduction of the iron, the pH was readjusted to its original value with 1.00 mM NaOH and the volume required was measured. From the measured amount of NaOH and the amount of iron reduced by MV in the HoSF samples, the H^+/Fe ratio was established for iron reduction in HoSF with different core sizes. HoSF used in these studies had been prepared a minimum of one hour and typically one to two days prior to the pH measurements.

NMR Magnetic Susceptibility

The magnetic susceptibility of: 1) $\text{HoSF}(8 \text{ Fe}^{2+})$ prepared anaerobically; 2) reconstituted HoSF containing variable amounts of Fe^{3+} prepared by O_2 oxidation of added Fe^{2+} ; and 3) reconstituted HoSF containing known numbers of Fe^{3+} , but to which 8 Fe^{2+} had been added anaerobically, was determined by the Evans NMR method [23], using a 300 MHz Bruker spectrometer. The resonance shifts of *t*-butyl alcohol methyl protons in aqueous HoSF solutions were measured relative to *t*-butyl alcohol in buffer in the absence of HoSF, or in some cases in the presence of apo HoSF at the same concentration as the reconstituted HoSF, using a coaxial Wilmad NMR tube assembly. The *t*-butyl alcohol shifts were determined as a function of the concentration of HoSF with a fixed Fe/HoSF ratio to provide a linear plot of NMR shift against HoSF concentration from which the slope was determined. The process was then repeated for other HoSF samples with different numbers of iron atoms per HoSF.

For a sample geometry in which the axis of the coaxial NMR tube assembly is parallel to the applied magnetic field, the gram susceptibility of the Fe bound to HoSF is given by equation (4):

$$\chi_g = (3/4\pi)(\Delta f/f)(1/c) + \chi_o \quad (4)$$

where f is the spectrometer frequency (300 MHz), Δf is the proton resonance shift in hertz, c is the iron concentration in g/cm^3 , and χ_o is the diamagnetic gram susceptibility of water (-0.72×10^{-6}). Since Δf is proportional to iron concentration, measurement of Δf as a function of iron concentration can be fitted with a straight line to yield $\delta(\Delta f)/\delta c$, and this quantity then used to determine χ_g . With iron concentrations expressed in moles per liter, M , χ_g is given by

$$\chi_g = 1.42 \times 10^{-8} (\delta(\Delta f)/\delta M) + \chi_o \quad (5)$$

where the diamagnetism of apoferritin has been combined with that of water. The effective moment per iron atom bound to HoSF is then given by equation (6):

$$\mu_{\text{eff}} = 2.84 (\chi_m T)^{1/2}, \quad (6)$$

where χ_m is the molar paramagnetic susceptibility of iron, after correction for diamagnetism (-1×10^{-5} emu/mole).

The reliability of the method was tested with Mn^{2+} ($S = 5/2$) and Cu^{2+} ($S = 1/2$) in aqueous solutions. The measured effective moments were $5.7 \mu_B$ for Mn^{2+} and $1.8 \mu_B$ for Cu^{2+} , which compare well with the theoretical values of 5.92 and $1.73 \mu_B$, respectively. A sample of HoSF(8 Mn^{2+}) had a similar effective moment per Mn^{2+} to that obtained for aqueous Mn^{2+} , indicating that the 8 Mn^{2+} were bound as isolated ions in HoSF.

Chloride Analysis of HoSF

Holo HoSF (0.5 mL, 12.5 mg/mL) containing 2186 iron atoms was added to a water equilibrated 1×30 cm column of Sephadex G-25 and eluted with water to remove free chloride ion. 5 μL of the emerging holo HoSF with all iron in the Fe^{3+} state was then dried in plastic cell holders and analyzed for iron, phosphorous, and chlorine by proton induced x-ray emission spectroscopy (PIXE) [24]. The same HoSF used above but in which the core was in the Fe^{2+} state was prepared by first reducing holo HoSF with excess MV, in the presence of 0.15 M NaCl, in a Vacuum Atmospheres glove box and then passing 0.5 mL of this sample through a 1×30 cm anaerobic Sephadex G-25 column, also contained within the glove box, equilibrated with water. 5- μ samples were dried and analyzed for iron, phosphorous, and chlorine by PIXE.

RESULTS

Proton Uptake

Table 1 summarizes the H^+/Fe values obtained for the reduction by MV of HoSF samples reconstituted with various numbers of iron atoms. In the absence of Fe^{2+} chelators, the reduced iron remains associated with HoSF, presumably within the ferritin cavity [19,20]. Under these conditions the H^+/Fe remains constant at a value near 2.0, independent of the initial iron loading. The reaction is best represented by reaction (2), which describes reduction of holo HoSF with retention of the core in the reduced state [23,25]. When the iron

TABLE 1. Proton Uptake During Reduction of Reconstituted HoSF

$\text{Fe}^{3+}/\text{HoSF}^a$	$\text{OH}^-/\text{Fe}^{3+}{}^b$	$\text{OH}/\text{Fe}^{3+}(\text{bipyd})^c$
8.0	2.12 (2.14)	3.12
14.0	2.03 (2.04)	3.05
23.8	2.04 (2.04)	2.85 (2.90)
46.1	2.10	2.71 (2.66)
80.0	2.02 (2.05)	2.75 (2.68)
88.3	2.25	2.96
101	2.08	2.87

^aThe number of iron atoms determined by ICP for reconstituted HoSF. The reduction of iron was carried out with MV at pH 7.0 in 0.15 M NaCl.

^bThe values in parentheses were obtained under the conditions described in footnote a except the pH was 5.0.

^cThe values were obtained as in footnote a except 1.0 mM bipyridine was present to complex and remove the reduced iron core.

core in HoSF is reduced in the presence of excess bipyridine, Table 1 shows that 3 H^+/Fe are observed, consistent with the presence of $\text{Fe}(\text{OH})_3$ as outlined by reaction (3).

The measurements are consistent with reactions (2) and (3) for all iron loadings down to 8 Fe/HoSF and indicate that Fe^{2+} in the reduced iron core in HoSF is partially hydrolyzed, i.e., FeOH^+ . Similar results had been reported from variation in the HoSF midpoint reduction potential with pH and by direct pH measurements for the reduction of holo HoSF containing over 2000 iron atoms to form HoSF with very large reduced iron cores [19]. The formation and binding of FeOH^+ by HoSF formed from reduction of $\text{Fe}(\text{OH})_3$ must be fundamentally different from the binding of externally added Fe^{2+} , because the latter is not partially hydrolyzed at pH 6–8 [14], whereas $\text{Fe}(\text{II})$ formed by reduction of an $\text{Fe}(\text{III})$ core is partially hydrolyzed. Insights into this stable, partially hydrolyzed $\text{Fe}(\text{II})$ core will be discussed below.

From Table 1, the reduction stoichiometry of 2 H^+/Fe for core reduction of iron in HoSF at pH 5.0 is no different than that at pH 7.0. This surprising result may have several interpretations. It is possible that the FeOH^+ phase remains stable at pH 5.0 and that even lower pH values will be required to protonate the OH^- matrix and release Fe^{2+} as the aqueous ions. This possibility suggests that the $\text{Fe}(\text{II})$ core is more stable than just $\text{Fe}(\text{OH})_2$ which should dissolve at pH 5.0. Support for a chlorohydroxy $\text{Fe}(\text{II})$ phase is discussed below which may be more stable at pH 5 than $\text{Fe}(\text{OH})_2$.

Since the electrophoretic mobility of holo HoSF with a reduced core containing $\text{Fe}(\text{OH})^+$ was not measurably different from holo HoSF with an $\text{Fe}(\text{OH})_3$ core, we previously concluded [19] that a charge compensation process had to be operating in order to neutralize the ca. 2000 positive charges that would have been produced by the FeOH^+ in the reduced core. This same charge compensation process must be operating in HoSF with small reduced cores because the reduction stoichiometry presented in Table 1, even at very low iron loadings, is consistent with reaction (2). As we will discuss below, PIXE measurements for large reduced iron cores show the presence of large amounts of chloride transported into the core during the reduction process. Thus, for these large cores, the charge compensation process required for reaction (2) appears to be

satisfied by chloride ion. We have not been able to measure the chloride content for the small cores with as much precision, but it appears this same process is operative upon reduction of both small and large iron cores.

Magnetic Susceptibility

Figure 1 shows the resonance of methyl protons in *t*-butyl alcohol in aqueous HoSF(8 Fe³⁺) solution, relative to *t*-butyl alcohol in aqueous solution without HoSF. Figure 1 also shows that protons in TES and HDO are also shifted to about the same extent in the presence of paramagnetic HoSF. Figure 2 shows the increase in the shift of the *t*-butyl alcohol methyl proton resonance as the concentration of the paramagnetic HoSF(8 Fe³⁺) is increased. The shift increases linearly with increasing HoSF concentration, and extrapolates to zero at zero HoSF concentration, indicating that no anomolous magnetic effects are present over a three- to fourfold variation in protein concentration. Also shown in Figure 2 are proton shifts for Mn²⁺ and Cu²⁺ in aqueous solutions. The slopes and effective magnetic moments per iron for all HoSF samples evaluated are listed in Table 2.

The effective moment per iron of 4.8 μ_B for HoSF(8 Fe²⁺) is close to the free ion theoretical value of 4.90 μ_B for $S = 2$ (four unpaired electrons), indicating that the Fe²⁺ ions are bound at isolated sites. The effective moment per iron for HoSF(8 Fe³⁺), produced by air oxidation of HoSF(8 Fe²⁺), is decreased to 4.7

FIGURE 1. Nuclear magnetic resonance shifts of 0.2 M *t*-butyl alcohol (*t*-BuOH) and 0.025 M TES and HDO in aqueous solution in the 1.0 mm i.d. NMR tube insert (right peak), compared with the same resonances at the same concentrations in the 4.0 mm i.d. NMR tube containing 1.0×10^{-4} M HoSF which has 8 Fe³⁺/HoSF present (left peak). For these conditions, the shift of the methyl protons in *t*-butyl alcohol in the presence of 1×10^{-4} M HoSF containing 8 Fe³⁺ relative to the insert which has no HoSF present is 9.20 Hz.

FIGURE 2. Resonance shift of the methyl protons in *t*-butyl alcohol as a function of transition metal ion concentration for (●) Mn^{2+} ions ($S = 5/2$); (○) $\text{HoSF}(8 \text{ Fe}^{3+})$, and (□) Cu^{2+} ions ($S=1/2$).

μ_B from the free ion $S = 5/2$ value of $5.92 \mu_B$, expected for isolated Fe^{3+} . The effective moments per iron of $\text{HoSF}(16 \text{ Fe}^{3+})$ and $\text{HoSF}(24 \text{ Fe}^{3+})$ are even more attenuated from the free ion value, and approach the effective moment per iron of $3.8 \mu_B$ observed in holo HoSF .

For HoSF containing both Fe^{2+} and Fe^{3+} , $\text{HoSF}(8 \text{ Fe}^{2+} + 8 \text{ Fe}^{3+})$ and $\text{HoSF}(8 \text{ Fe}^{2+} + 16 \text{ Fe}^{3+})$, the experimental values for μ_{eff} in Table 2 can be compared with equation (7):

$$(\mu_{\text{eff}})^2 = b[\mu_{\text{eff}}(\text{Fe}^{2+})]^2 + (1-b)[\mu_{\text{eff}}(\text{Fe}^{3+})]^2 \quad (7)$$

TABLE 2. Effective Magnetic Moments from the Evans NMR Magnetic Susceptibility Data

Sample	Shift/Conc. (Hz/mols·L ⁻¹) $\times 10^{-2}$	Effective Moment ^a (Bohr magnetons)
Mn^{2+}	169	5.7
Cu^{2+}	17	1.8
$\text{HoSF}(8 \text{ Fe}^{2+})$	1.22	4.9
$\text{HoSF}(8 \text{ Fe}^{3+})$	109	4.8
$\text{HoSF}(8 \text{ Fe}^{2+} + 8 \text{ Fe}^{3+})$	112	4.8
$\text{HoSF}(16 \text{ Fe}^{3+})$	76	3.8
$\text{HoSF}(8 \text{ Fe}^{2+} + 16 \text{ Fe}^{3+})$	94	4.3
$\text{HoSF}(24 \text{ Fe}^{3+})$	78	3.9
holo HoSF	75	3.8

^aPer metal ion. Theoretical effective moments in Bohr magnetons: $S = 5/2$, $\mu_{\text{eff}} = 5.92$; $S = 2$, $\mu_{\text{eff}} = 4.90$; $S = 3/2$, $\mu_{\text{eff}} = 3.87$; $S = 1$, $\mu_{\text{eff}} = 2.83$; $S = 1/2$, $\mu_{\text{eff}} = 1.73$.

where b is the fraction of iron in the $2+$ state, $\mu_{\text{eff}}(\text{Fe}^{2+})$ is the effective moment for Fe^{2+} , and $\mu_{\text{eff}}(\text{Fe}^{3+})$ is the effective moment for Fe^{3+} . Taking the measured μ_{eff} for $\text{HoSF}(8 \text{ Fe}^{2+})$ and $\text{HoSF}(16 \text{ Fe}^{3+})$ for $\mu_{\text{eff}}(\text{Fe}^{2+})$ and $\mu_{\text{eff}}(\text{Fe}^{3+})$, respectively, in equation (7), the measured effective moments of $\text{HoSF}(8 \text{ Fe}^{2+} + 8 \text{ Fe}^{3+})$ and $\text{HoSF}(8 \text{ Fe}^{2+} + 16 \text{ Fe}^{3+})$ both correspond to $\mu_{\text{eff}}(\text{Fe}^{2+}) = 4.8 \mu_{\text{B}}$, which is close to the $S = 2$ free ion value of $4.9 \mu_{\text{B}}$. This means that the eight ferrous ions which bind to HoSF under anaerobic conditions bind at isolated sites in the protein and do not interact magnetically with each other or with the Fe^{3+} present. This supports the view that oxidation of Fe^{2+} involves transport of the iron out of the original Fe^{2+} binding sites.

Chloride Content of Oxidized and Reduced HoSF

Passing native holo HoSF containing 0.15 M NaCl through Sephadex G-25 columns equilibrated with water gave the PIXE spectrum shown in Figure 3 from which Fe/P and Fe/Cl ratios of 7–8.5 and 13–15 were obtained, respectively. Iron was determined from the Fe K_{α} peak at 6.4 Kev. Additional passage through water equilibrated Sephadex G-25 columns did not appreciably change these values. The Fe/P value is typical of native holo HoSF but the presence of low levels of chloride in the HoSF iron core has not been previously reported. Carrying out the same experiments with HoSF reduced anaerobically in the

FIGURE 3. PIXE spectrum of native HoSF. The Cl, P, and Fe x-ray emission lines along with other trace elements are indicated above the dotted x-ray baseline. The relative abundances of these elements are obtained by integration of the respective emission lines.

presence of 0.15 M NaCl, followed by anaerobic chromatography as described above, produced a PIXE spectrum (not shown) for HoSF which yielded a Fe/P value of 15, indicating some phosphate loss occurs with reduction, as previously reported [26]. However, upon reduction, Cl levels dramatically increased over native HoSF to Fe/Cl values 0.80–1.05. This result indicates that one Cl^- is transferred to the reduced HoSF core for each electron transferred to the iron atoms of the core. Additional passage through the water equilibrated anaerobic columns did not alter the values, indicating that the chloride was an integral part of the reduced mineral core. The optical spectrum of the reduced, Cl-containing core showed decreased absorption in the 400–550 nm region and an increase in absorption in the 550–800 nm region, as previously reported [14].

The results clearly show that low levels of chloride, just smaller than the phosphate levels, are present in the oxidized HoSF core but that in the reduced HoSF core, levels of chloride are equivalent to the iron present. Equation (2) and previous analysis [14, 28] predicts that one anion must be transported into the mineral core upon reduction of each iron atom in order to maintain electrical neutrality of the mineral core, as was measured previously by electrophoresis [14, 28]. Our analysis indicates that Cl^- is the anion that is associated with the reduced core. This resulting chlorohydroxy Fe(II) mineral phase present in HoSF, thus, may be the reason for the reduced mineral phase being stable to low pH values as discussed above. Our experiments have only examined the Cl content of large, reduced cores (1500–2300 Fe or Cl/HoSF) where Cl is abundant and the measurements by PIXE produce high intensity Cl x-ray lines for analysis. As we refine the analytical procedures, measurements of Fe/Cl ratio upon reduction will be extended to HoSF with smaller iron cores. However, the results in Table 1 clearly show that a 2 H^+/e value is measured for reduction of HoSF with 8 Fe^{3+} within its core, a value consistent with reaction (2) and consistent with 1.0 Cl^- taken up for each iron reduced.

Figure 3 also shows the presence of several other chemical elements in addition to Fe, P, and Cl in native holo HoSF. The silicon is from the sample holders and the sulfur is from the sulfur-containing amino acids present in the HoSF protein shell. K, Ca, V, Cu, and Cr are all present in relatively low amounts. However, Zn is found in relatively high amounts, presumably within the mineral core. Upon reduction, all the elements stay relatively constant except for Cl^- , which dramatically increases, indicating that it is an integral reactant in core reduction.

DISCUSSION

The data in Table 1 show that two protons per iron atom are taken up during iron reduction in the absence of Fe^{2+} chelators and that three protons are taken up during reduction in the presence of bipyridine for all iron loadings studied, down to 8 Fe^{3+} /HoSF. These results correspond to reactions (2) and (3), respectively, and demonstrate that the ferric ions in reconstituted ferritin are fully hydrolyzed as $\text{Fe}(\text{OH})_3$. Our results are consistent with those of Xu and Chasteen [13], who observed a proton release stoichiometry during Fe^{2+} oxidation by O_2 , consistent with the formation of $\text{Fe}(\text{OH})_3$ and H_2O_2 as shown in reaction (1) during the deposition of 24 iron atoms into HoSF. Because the $\text{H}^+/\text{Fe}^{3+}$ value shown in Table 1 is constant over the entire range of iron core

sizes studied, we interpret this result in terms of reaction (2), i.e., as the formation of an FeOH^+ moiety associated with HoSF. The PIXE results showing Fe/Cl ratios near 1.0 for reduced HoSF indicates further that reaction (2) should be reformulated as reaction (8):

The nature of the FeClOH core remains poorly defined at this point but its enhanced stability over $\text{Fe}(\text{OH})_2$ may be the reason why the reduced HoSF iron core is stable. Reaction (3) and the results shown in Table 1 show that this mineral phase is unstable to bipyridine chelation and provides a way to release the iron as the bipyridine chelate. Our results predict that Cl^- will also be released upon addition of bipyridine to reduced holo HoSF when reaction (3) is carried out.

The minimum time for the pH and magnetic susceptibility measurements after preparation of the reconstituted ferritins was at least one hour and typically 24 hours. Previous Mossbauer measurements [11] of reconstituted HoSF have shown a succession of transient, monomeric, dimeric, and iron cluster species, with concentrations changing as a function of time as measured in minutes. The results reported here probably represent relatively "static" iron cores in HoSF but nevertheless suggest the presence of isolated Fe^{3+} comparable in concentration to those reported for short term measurements [11].

For HoSF(16 Fe^{3+}) and HoSF(24 Fe^{3+}), $\mu_{\text{eff}} = 3.8$ and $3.9\mu_{\text{B}}$, respectively, the effective moments are similar to the effective moment of $\mu_{\text{eff}} = 3.8\mu_{\text{B}}$ per iron in holoferritin. These results suggest that for these iron loadings, the Fe^{3+} ions are clustered in sites with strong, antiferromagnetic exchange interactions. In HoSF(8 Fe^{3+}), the decrease from the free ion value to $\mu_{\text{eff}} = 4.7\mu_{\text{B}}$ is likely due to partial clustering of Fe^{3+} . Assuming that the Fe^{3+} ions are either in isolated or in sites with strong exchange interactions the degree of clustering can be estimated from equation (7), with b the fraction of isolated Fe^{3+} ions ($\mu_{\text{eff}} = 5.9\mu_{\text{B}}$), and $1-b$ the fraction of the clustered Fe^{3+} ($\mu_{\text{eff}} = 3.8\mu_{\text{B}}$). Using these values in equation (7) gives $b = 0.5$. This result suggests that about one-half of Fe^{3+} has aggregated within HoSF even for iron loadings as low as eight Fe^{3+} per molecule. This is consistent with Mossbauer results [11,25] which show some cluster formation occurs even for low iron loading. We are unable to determine from the magnetic measurements alone if the iron atoms are all present in the same molecule or if redistribution of the iron has occurred among HoSF molecules, forming some HoSF with larger cores and some empty. However, capillary electrophoresis measurements which are quite sensitive to HoSF containing small numbers of iron atoms suggest a homogeneous loading of iron atoms [28]. When taken together, our measurements suggest that iron is fully hydrolyzed and that clustering begins with 8 or fewer Fe^{3+} ions per HoSF.

The final step in ferritin core formation must involve formation of the mineral ferrihydrite at the nucleation site. While the magnetic measurements could detect iron aggregation, they could not distinguish iron in clusters from iron in ferrihydrite (as in holo ferritin). Thus, the point in iron loading at which ferrihydrite formation occurs was not determined in this study.

From this study we conclude that core cluster formation is initiated for as few as 8 Fe^{3+} /HoSF. Core Fe^{3+} is best represented as $\text{Fe}(\text{OH})_3$, and reduction of

the core in the presence of Cl^- results in the formation of a species best represented FeClOH , which is strongly associated with HoSF . The sites that bind partially hydrolysed Fe^{2+} formed by reduction remain unknown but must be different from those that bind unhydrolysed Fe^{2+} from the external medium under anaerobic conditions. The former are probably bound at sites in the cavity, whereas the latter are likely bound at sites in or near the threefold channels.

We are now determining if other anions (Br^- , I^- , phosphate, etc.) are incorporated into the reduced HoSF interior or if Cl^- is unique in this reaction. We are also examining if the Cl^- present in the reduced mineral core is released totally upon oxidation or if the low Cl^- values observed in the oxidized HoSF core are remnants of previous redox history.

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