Mössbauer Spectroscopic Study of the Initial Stages of Iron-Core Formation in Horse Spleen Apoferritin: Evidence for both Isolated Fe(III) Atoms and Oxo-Bridged Fe(III) Dimers as Early Intermediates†

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ABSTRACT: Ferritin stores iron within a hollow protein shell as a polynuclear Fe(III) hydrous oxide core. Although iron uptake into ferritin has been studied previously, the early stages in the creation of the core need to be clarified. These are dealt with in this paper by using Mössbauer spectroscopy, a technique that enables several types of Fe(II) and Fe(III) to be distinguished. Systematic Mössbauer studies were performed on samples prepared by adding $^{57}Fe(II)$ atoms to apoferritin as a function of pH (5.6–7.0), n [the number of Fe/molecule (4–480)], and t_f (the time the samples were held at room temperature before freezing). The measurements made at 4.1 and 90 K showed that for samples with $n \leq 40$ at pH ≥ 6.25 all iron was trivalent at $t_f = 3$ min. Four different Fe(III) species were identified: solitary Fe(III) atoms giving relaxation spectra, which can be identified with the species observed before by EPR and UV difference spectroscopy; oxo-bridged dimers giving doublet spectra with large splitting, observed for the first time in ferritin; small Fe(III) clusters giving doublets of smaller splitting and larger antiferromagnetically coupled Fe(III) clusters, similar to those found previously in larger ferritin iron cores, which, for samples with $n \geq 40$, gave magnetically split spectra at 4.1 K. Both solitary Fe(III) and dimers diminished with time, suggesting that they are intermediates in the formation of the iron core. Two kinds of divalent iron were distinguished for $n = 480$, which may correspond to bound and free Fe(II).

Ferritin stores iron as an inorganic complex or "core", encompassed by a protein composed of 24 polypeptide chains (Ford et al., 1984; Theil, 1987). This method of storage is relatively efficient, since up to 4500 Fe(III) atoms can be deposited in a protein of molecular weight 480 000, and an otherwise insoluble ferric oxy–hydroxide complex known as ferrihydrite is rendered soluble. The means by which the iron-free protein, apoferritin, is converted to ferritin is the subject of this paper, and we are concerned especially with the crucial early stages of this process. Our focus is on iron–apoferritin complexes [mainly Fe(III)] that form when small numbers of Fe(II) atoms are added aerobically and not on the Fe(II)–apoferritin that may form under anaerobic conditions (Watt et al., 1985, 1988).

The three-dimensional structure of horse spleen apoferritin is known (Ford et al., 1984), and previous studies on iron uptake and deposition have also mainly been done on this ferritin (Macara et al., 1972, 1973; Treffry & Harrison, 1984; Yang et al., 1987). These have shown that Fe(II) oxidation by dioxygen is accelerated in the presence of apoferritin (Niederer, 1970; Bryce & Crichton, 1973; Macara et al., 1973; Bakker & Boyer, 1986). The oxidized iron is hydrolyzed and deposited as ferrihydrite within the apoferritin cavity. In the presence of transferrin, however, as much as 80% of the Fe(III) can be donated to this protein (Bakker & Boyer, 1986). This has been interpreted as indicating the presence of a ferroxidase center on or near the outside of the molecule, although this remains unconfirmed. In contrast, iron-core nucleation, or initiation, must occur on the inside surface of the protein shell, nucleation being followed by growth of the core into the cavity space (Macara et al., 1972). Potential ligands that may participate in nucleation have been identified by X-ray crystallography (Ford et al., 1984; Harrison et al., 1986). The route by which iron ions may traverse the apoferritin shell are also suggested, since the three-dimensional structure has revealed the presence of narrow intersubunit channels connecting the cavity to the external surface. The eight hydrophilic channels lying along the molecular 3-fold axes are known to bind metals and thus could be the route selected by iron (Rice et al., 1983; Ford et al., 1984; Harrison et al., 1986). The site of initial Fe(II) oxidation has not yet been established.

The formation of the ferritin iron core has been studied by a number of spectroscopic methods. The first few Fe(III) atoms produced after apoferritin is titrated with Fe(II) in air can be detected by EPR spectroscopy since they give a $g' = 4.3$ signal (Rosenberg & Chasteen, 1982). As soon as these atoms have been incorporated into the core, however, they become EPR silent through antiferromagnetic coupling. An initial Fe(III)–apoferritin complex is also seen by UV difference spectroscopy, when 4–8 atoms/apoferritin molecule are added at pH 6.5 (Treffrey & Harrison, 1984). The spectrum corresponding to this species is gradually replaced by a different spectrum corresponding to the ferrihydrite core complex. A 10 Fe(III)–apoferritin complex has also been analyzed by EXAFS measurements made 24 h after the addition of iron as Fe(II) (Yang et al., 1987). The results suggest the presence of Fe(III) as small clusters. Mössbauer spectroscopy has frequently been used to study preformed native or reconstituted ferritin iron cores, which have been shown by this method to exhibit small particle superparamagnetism (Blaise et al., 1965; Bell et al., 1984; St. Pierre et al., 1986; Mann et al., 1987). Such cores exhibit a six-line magnetic spectrum at 4.1 K, but as the temperature is raised, this is changed to a quadrupole doublet at a temperature, dependent

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Table I: Mössbauer Parameters of Different Iron Species[a]

species	T (K)	LW (mm/s)	QS (mm/s)	IS (mm/s)	H_{eff} (kOe)	T_r (ns)
(a) isolated Fe(III)	90	0.5	–	0.51 (1)	550 (10)	7 (2)
(b) small Fe(III) clusters	90	0.44 (2)	0.66 (2)	0.50 (1)	–	–
(c) dimeric Fe(III)	90	0.30 (2)	1.50 (4)	0.50 (2)	–	–
(d) Fe(II)	90	0.45 (2)	3.15 (2)	1.36 (2)	–	–
(d_1) Fe(II)	90	0.30 (2)	3.36 (1)	1.38 (1)	–	–
(d_2) Fe(II)	90	0.44 (2)	3.03 (1)	1.37 (1)	–	–
(e) larger Fe(III) clusters	90	0.40 (2)	0.73 (2)	0.48 (2)	–	–
n = 40	4.1	0.50	–	0.50 (1)	370 (15)	–
n = 150 + 4[b]	4.1	0.50 (2)	–	0.50 (1)	460 (10)	–
n = 480	4.1	0.50 (2)	–	0.50 (1)	460 (2)	–

[a] LW = full line width at half-maximum; QS = quadrupole splitting; IS = isomer shift; H_{eff} = effective magnetic field; T_r = relaxation time. The numbers in parentheses give the error on the last digits. [b] 150 ^{56}Fe + 4 ^{57}Fe.

on the number of Fe atoms per molecule present, between 4.1 and 50 K. It has also been used to study the dynamic behavior of iron in ferritins of different iron contents, and this has been contrasted with that of iron in other proteins (Bauminger et al., 1987). Hitherto, apart from one study in which 10 Fe(II) atoms/molecule were added and the product examined immediately and after 24 h in air, this technique has not been applied to the processes of iron oxidation and core initiation. In many ways it provides an ideal tool, since, with a powerful γ-ray source and use of ^{57}Fe-enriched iron, small numbers of iron atoms can be detected, reactions can be stopped by freezing in liquid nitrogen, and chemically different species of Fe(II) and Fe(III) may be identified. Here we examine successive stages in the oxidation of Fe(II) at a variety of pH values, when as few as 4 Fe atoms/horse spleen apoferritin molecule have been added. We provide evidence for the binding of Fe(II) to apoferritin and show the presence first of isolated Fe(III) atoms and at later times of Fe(III) clusters. A μ-oxo-bridged Fe(III) dimer intermediate has been found for the first time, and we suggest that this dimer probably represents the initial step in core nucleation. A preliminary account of some of the work described here is given in Bauminger et al. (1988). We also provide some information on the rate at which Fe(II) is oxidized in the presence of apoferritin and show that although Fe(II) is oxidized rapidly at pH 6.4 or above at low iron concentration and low Fe atoms per molecule, Fe(II) can persist for longer periods at high Fe atoms per molecule, as reported previously (Rohrer et al., 1987), or at low pH.

Materials and Methods

Horse spleen ferritin was purchased from Boehringer Corp. (London) and reduced with thioglycolic acid. Elemental ^{57}Fe and ^{56}Fe were obtained from the Atomic Energy Research Establishement, Harwell, U.K., and dissolved in a small molar excess of H_2SO_4 (1:1.1) to give a final H_2SO_4 concentration of 10% (v/v). Before use, the samples were further diluted to give H_2SO_4 not higher than 2%. All other chemicals were purchased from Sigma (London).

Iron additions to apoferritin and ferritin were made at pH values between 5.6 and 7.0 in 0.1 M Mes (pH 5.6–6.5) or Mops (pH 6.4–7.0) buffers containing 7.0 mM NaCl. For Fe(II) additions to apoferritin, 5 μL of ^{57}Fe or ^{56}Fe (containing 10 μg of Fe) was added to 0.5 mL of the protein solution in buffer, and the protein concentration was varied in the range 42.6–4.26 mg/mL (89–8.9 μM) to give the required Fe/protein ratio (4–40 Fe atoms/molecule) while the Fe(II) concentration (0.35 mM) was kept constant. For the addition of 480 Fe atoms/molecule, 20 μL of ^{57}Fe (40 μg) was added to 0.5 mL containing 0.7 mg of apoferritin (to give 1.37 mM).

A small core of average size of 150 Fe atoms was built up in ferritin molecules by making 10 successive additions of ^{56}Fe to apoferritin (2.84 mg/mL) in 0.1 M Mops, pH 7.0, and allowing oxidation to go to completion before the next iron addition. The final concentration was 1 mM Fe. The solution was then concentrated by ultrafiltration and dialyzed against 10 mM NaCl. Four ^{57}Fe atoms/molecule was added to this sample as above, 1 week after the ^{56}Fe. This sample is referred to as $150^{56}+4^{57}$. In one pair of samples, 4 ^{56}Fe atoms/molecule were added first to the apoferritin solution, followed 3 min later by 4 ^{57}Fe atoms/molecule, and the reverse procedure was applied to the second sample. These samples are referred to as $4^{56}+4^{57}$ and $4^{57}+4^{56}$, respectively.

The samples were put into Lucite holders and frozen at different times, t_f (1 min or longer), after iron loading by throwing them into liquid nitrogen. The samples were stored in liquid nitrogen until the measurements were performed.

Conventional Mössbauer spectroscopy with a 100 mCi ^{57}Co(Rh) source at room temperature and a Harwell proportional counter was used. Velocity calibration was performed with a metallic iron foil at room temperature. Isomer shifts are quoted with respect to this absorber. Measurements were performed in cryostats at temperatures between 4.1 and 200 K. Computer fits were performed to all measured spectra. In spectra in which relaxation subspectra were clearly seen, computer fits using different relaxation models (Hoy, 1984) were tried. Various models gave equally good fits and similar relaxation times. The results quoted were obtained by assuming the simplest relaxation model with one hyperfine field and one relaxation time (Wickman et al., 1966). From these fits the Mössbauer parameters and relative intensities of all subspectra were obtained. The errors on the relative intensities of the relaxation spectra are quite large (about 5%), but the general trend can easily be followed.

Results

Spectra and Their Interpretation. (1) pH Dependence of Mössbauer Spectra: 4 Fe Atoms/Molecule, t_f = 3 min. Some typical Mössbauer spectra at 90 K are shown in Figures 1 and 2 for samples at various pH values between 5.6 and 7.0. All samples contained 4 ^{57}Fe atoms/molecule, except that of Figure 2e, for which n = 20. Similar spectra were obtained at 4.1 K. The spectra obtained at both 4.1 and 90 K are composed of four subspectra: a magnetically split relaxation spectrum, a, and three different types of quadrupole doublets, b, c, and d. The Mössbauer parameters of the different subspectra are summarized in Table I. The four subspectra are outlined in the following paragraphs.

(a) Fe(III) Relaxation Spectrum. A magnetically split relaxation spectrum, a, is present in all samples. These relaxation spectra are very similar at 90 and 4.1 K as is to be expected for spin–spin relaxation. In spectra in which relaxation subspectra were clearly seen, the hyperfine field, the relaxation time, and the relative intensities were free param-

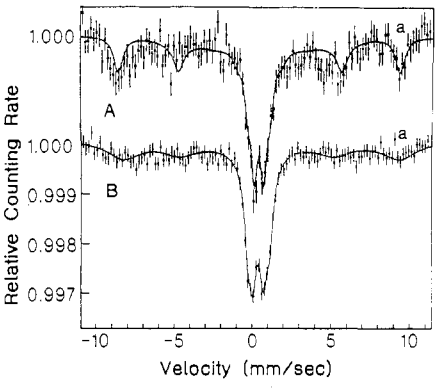

FIGURE 1: Mössbauer spectra obtained at 90 K in ferritin samples loaded with four ^{57}Fe atoms/ferritin molecule, frozen 3 min after loading, prepared at (A) pH 6.4 and (B) pH 7.0. The figure demonstrates the lower intensity of the relaxation spectrum (a), in samples prepared at higher pH. The solid lines are computer fits to the spectra.

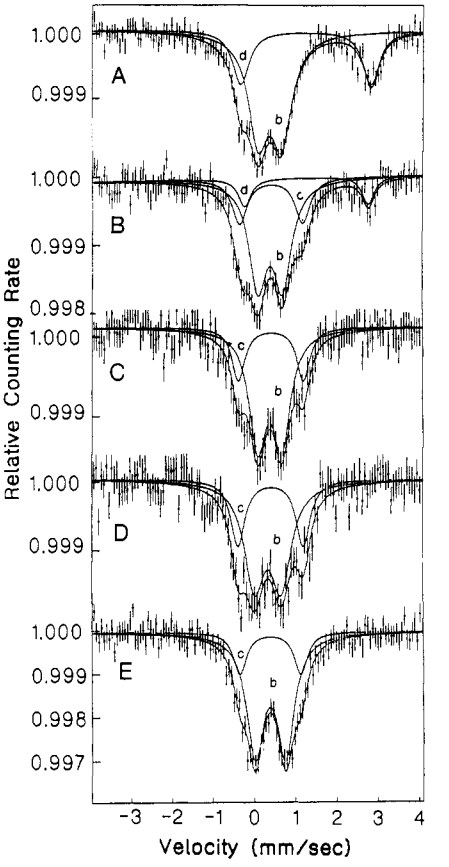

FIGURE 2: Mössbauer spectra obtained at 90 K on an extended velocity scale in ferritin samples loaded in (A)–(D) with $n = 4$ Fe atoms/ferritin molecule, frozen at times t_f after iron loading, prepared at (A) pH 5.6, $t_f = 17$ min; (B) pH 6.25, $t_f = 3$ min; (C) pH 6.4, $t_f = 3$ min; (D) pH 7.0, $t_f = 3$ min; and (E) with $n = 20$, pH 6.4, $t_f = 3$ min. Doublet b corresponds to iron in small clusters, doublet c to the dimer, and doublet d to divalent iron. The figures shows the higher intensity of doublet d at lower pH and the higher intensity of doublet c at higher pH, as well as the lower relative intensity of doublet c at higher n. The solid lines are computer fits to the spectra.

eters in the computer fits. Examples of such fits are shown in Figure 1 for samples prepared at pH 6.4 and 7.0 that were frozen 3 min after iron loading. The observed hyperfine field and isomer shift fit high-spin Fe(III) in octahedral coordination (Mørup & Knudsen, 1986). In other spectra, where the relative intensity of the relaxation spectrum was small, the hyperfine field and relaxation time were fixed at the values given in Table I and only the relative intensities were left as free parameters.

The paramagnetic relaxation spectra are probably due to solitary high-spin Fe(III) ions, such as give rise to an EPR signal at $g' = 4.3$ (Chasteen et al., 1985). The farther solitary Fe ions are from each other, the longer are the relaxation times. The relation between Fe–Fe distances and relaxation times was estimated from measurements performed on frozen solutions containing iron salts. Such a calibration indicates that the iron atoms which yield these relaxation spectra are about 17 Å apart from each other, since the average relaxation time, 7 ns, is similar to that observed in frozen solutions of 0.3 M Fe(III) salts, (Mørup & Knudsen, 1986). This implies that they are bound to protein, since the concentration of Fe was only 0.36 mM. The calibration must not be taken too literally. It may well be that the observed relaxation spectrum is itself a superposition of relaxation spectra with faster and slower relaxation times, due to pairs of atoms that are separated by distances that are greater or less than 17 Å, which on the average yield the above-mentioned relaxation time. As seen in Figure 1 the relative intensity of the relaxation spectrum is considerably higher in the pH 6.4 than in the pH 7.0 sample.

(*b*) *Fe(III) Cluster Doublet.* Figure 2 shows the central part of some typical spectra on an extended velocity scale. In all samples a quadrupole doublet, b, whose parameters are given in Table I, is seen. This doublet is probably due to iron in small clusters in which high-spin Fe(III) atoms are close enough so that the relaxation is fast. These clusters are not large enough to yield magnetic spectra at 4.1 K, but again iron in these clusters must be attached to the protein and they probably represent the beginning of the core.

(*c*) *Fe(III) Dimer Doublet.* In the samples with pH $\geq$ 6.25, an additional doublet, c (Table I), is seen. The Mössbauer parameters of this doublet are the same at 90 and 4.1 K. The isomer shift is typical of trivalent iron. The quadrupole splitting (QS) is large compared to QS found usually in trivalent iron compounds and reflects the low symmetry of the iron environment. Similar parameters were observed heretofore only in μ-oxo-bridged Fe(III) dimers (Bancroft et al., 1968; Lippard; 1988), and we therefore attribute this subspectrum to such dimers, which are probably an intermediate stage between the solitary iron ions and the Fe(III) in larger groups. The relative intensity of this subspectrum is larger in samples that were prepared at higher pH.

(*d*) *Fe(II) Doublet.* In the samples prepared at pH $\leq$6.25, subspectra corresponding to Fe(II) were observed (doublet d, Table I). The relative intensity of doublet d is smaller in samples prepared at higher pH, and in samples prepared at pH 6.4 and higher, no divalent iron is seen. This divalent iron may be due to Fe(II) at initial binding sites on the protein shell or to Fe(II) that is in the solution and has not yet reacted with ferritin or which may have reached the protein cavity without oxidation.

The areas under the various subspectra obtained at low temperatures are proportional to the amount of iron present in the different species that yield the respective spectra (Goldanskii & Makarov, 1968). The relative amounts of the different species obtained in samples prepared at different pH values are summarized in Figure 3A. The results show that in all samples which were held for 3 min at room temperature after loading with iron a relatively large amount of solitary ions is present. At lower pH values some divalent iron is present, whereas at higher pH values a larger proportion is present as dimers and in larger trivalent iron clusters.

(*2*) *Effect of Varying the Number of Fe Atoms/Molecule on Mössbauer Spectra: pH 6.4, t_f = 3 min.* The Mössbauer spectra obtained at 90 K in samples with different amounts

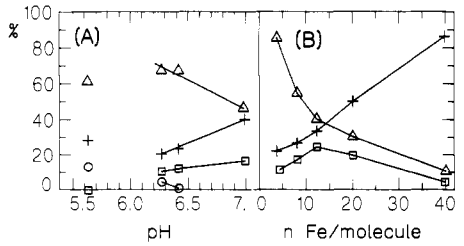

FIGURE 3: Percentage of different iron species: (△) solitary Fe(III); (+) Fe(III) clusters; (□) Fe(III) dimers; (○) Fe(II). (A) Percentage as a function of pH in samples prepared with 4 Fe atoms/molecule. All samples were frozen 3 min (t_f) after iron loading, except the pH 5.6 sample, for which t_f = 17 min. (B) Percentage as a function of the number n of Fe atoms per molecule, in samples prepared at pH 6.4 and t_f = 3 min. The solid lines in (A) and (B) serve only as guides for the eye.

Table II: Relative Amounts of Different Iron Species in Samples Held at Room Temperature for Different Times t_f[a]

pH	n (Fe atoms/molecule)	t_f	% solitary Fe(III)	% Fe(III) clusters	% Fe(III) dimers	% Fe(II)
5.6	4	17 min	60	28	–	12
5.6	4	3 h	40	53	–	7
6.25	4	3 min	65	20	10	5
6.25	4	24 h	30	67	2	1
6.4	4	3 min	65	23	12	–
6.4	4	20 min	63	31	6	–
6.4	4	2 h	50	44	6	–
6.4	$150^{56}+4^{57}$	3 min	10	78	12	–
6.4	480	3 min	–	19	–	81
6.4	480	2 h	–	58	–	42
7.0	4	1 min	50	22	28	–
7.0	4	3 min	45	40	17	–
7.0	8	1 min	50	25	25	–
7.0	8	3 min	35	35	30	–

[a] The concentration of added ^{57}Fe(II) was 0.35 mM in all samples except for those with n = 480, for which it was 1.37 mM. In sample $150^{56}+4^{57}$ an iron core containing 150 ^{56}Fe(III) was built prior to the addition of 4 ^{57}Fe(II).

of Fe per ferritin molecule (n = 4, 8, 12, 20, 40, and 480 and also $150^{56}+4^{57}$), which were held at room temperature 3 min after iron loading, are shown in Figure 4. These show clearly that the relative intensity of the relaxation spectrum diminishes with increasing iron concentration, but even in samples with n = 40, there are still traces of solitary iron. The relative amounts of the different iron species for $n \leq 40$ are summarized in Figure 3B. Sample $4^{56}+4^{57}$ gave 35% as solitary ions, 38% in clusters, and 27% in dimers, and sample $4^{57}+4^{56}$ gave 30% as solitary ions, 52% in clusters, and 18% in dimers, thus showing similar trends with respect to iron. Although not clearly resolved in the velocity scale shown in Figure 4, doublet c, corresponding to iron dimers, is present in the spectra of all samples with $n \leq 20$. The amount of these dimers is insignificant (4%) in samples with $n \geq 40$, yet they can be distinguished clearly (12%) in sample $150^{56}+4^{57}$ (Table II). Traces of the relaxation spectrum (about 10%) are seen in this sample too. The sample with 480 Fe atoms/molecule shows 80% of the iron in the divalent state and the rest in doublet b. In this sample two different subspectra, d_1 and d_2, corresponding to two different Fe(II) sites, can be distinguished. The Mössbauer parameters of these sites are given in Table I. We will deal with these two sites later.

Some of the spectra obtained at 4.1 K are shown in Figure 5. There is no appreciable difference between the spectra obtained at 90 and 4.1 K in the samples with $n \leq 20$. In the samples with $n \leq 20$, one observes the three subspectra, corresponding to the isolated Fe ions, to the larger clusters, and

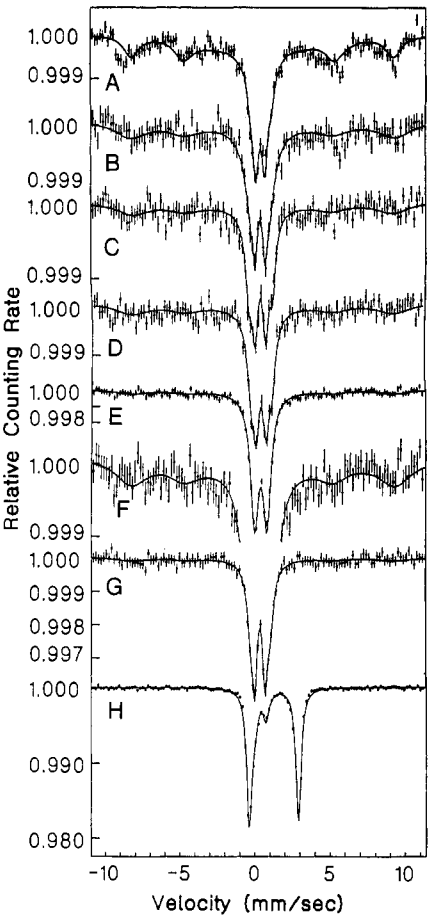

FIGURE 4: Mössbauer spectra obtained at 90 K in ferritin samples loaded with (A) n = 4, (B) n = 8, (C) n = 12, (D) n = 20, (E,F) n = 40, (G) $n = 150\ ^{56}\text{Fe} + 4^{57}\text{Fe}$, and (H) n = 480, prepared at pH 6.4 and frozen 3 min after iron loading. The spectra demonstrate the lower intensity of the relaxation spectrum at higher n. (F) is the same as (E), drawn on an extended scale to show that in this sample indeed traces of the relaxation spectrum are still seen. (G) was obtained in sample $150^{56}+4^{57}$, which had a core of 150 ^{56}Fe atoms/molecule to which 4 ^{57}Fe atoms/molecule was added, and the sample was frozen 3 min afterward; traces of the relaxation spectrum are seen. With n = 480, most of the iron is divalent. The solid lines are computer fits to the spectra.

to the dimers. No magnetic subspectra, other than the relaxation spectra, are seen. In the sample with 20 Fe atoms/molecule the traces of the relaxation spectrum are hardly distinguished. Only in the sample with n = 40, there is, besides doublet b, a magnetic subspectrum, e, with H_{eff} = 370 ± 15 kOe. The relative area of the magnetic subspectrum, e, is about 50%, showing that in this sample about 50% of the iron is in larger aggregates in which the iron atoms interact magnetically and 50% are in smaller groups, which, due to a lower blocking temperature (which is proportional to the aggregate volume), do not show magnetic spectra at 4.1 K (Mørup et al., 1980). The spectra obtained for sample $150^{56}+4^{57}$ at 90 and 4.1 K show that the added 4 ^{57}Fe distribute within 3 min as about 60% of the magnetic species with H_{eff} = 460 ± 10 kOe, 18% as doublet b, 12% as dimer, and 10% as isolated Fe(III). The size of the magnetic field is an indication of the larger aggregates in this sample (Mørup et al., 1980). This field is still smaller than the field found in iron-rich ferritin (500 kOe) (Bell et al., 1984). As the Mössbauer effect is sensitive only to ^{57}Fe, these results show that most of the last four iron atoms join the larger iron aggregates within the first 3 min and only a minority is present as solitary ions, as dimers, and in smaller groups. In the spectra obtained in these samples at 4.1 K, the relaxation

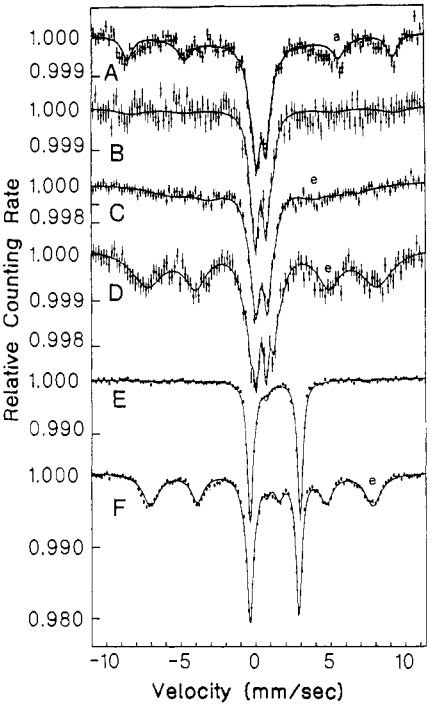

FIGURE 5: Mössbauer spectra obtained at 4.1 K in ferritin samples prepared at pH 6.4 and loaded with (A) $n = 4$, (B) $n = 20$, (C) $n = 40$, (D) $n = 150$ $^{56}Fe + 4$ ^{57}Fe, and (E,F) $n = 480$, frozen 3 min after iron loading, except for (F) for which $t_f = 2$ h. The spectra demonstrate the disappearance of the relaxation spectrum and appearance of the magnetic spectrum at higher n. The solid lines are computer fits to the spectra.

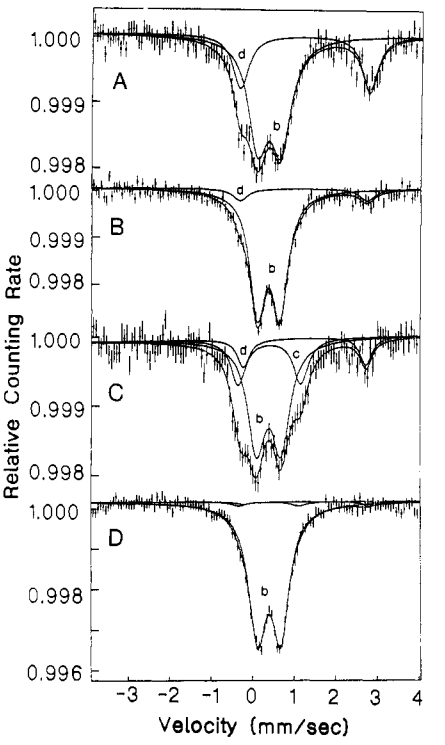

FIGURE 6: Mössbauer spectra obtained at 90 K in samples loaded with 4 Fe atoms/molecule and held at room temperature for different times: (A) pH 5.6, $t_f = 17$ min; (B) pH 5.6, $t_f = 3$ h; (C) pH 6.25, $t_f = 3$ min; (D) pH 6.25, $t_f = 24$ h. The figure demonstrates the decrease of the relative intensity of doublet d, corresponding to Fe(II) [(A) and (B)], and doublet c, corresponding to dimer [(C) and (D)], with time. The solid lines are computer fits to the spectra.

spectra seen at 90 K cannot be distinguished. Due to their weak intensity, they are disguised here under the much stronger magnetic spectrum. The sample with 480 Fe atoms/molecule shows 80% as the Fe(II) doublet as at 90 K [384 Fe(II) atoms/molecule] and 96 Fe atoms as Fe(III). About 50% of the Fe(III) spectrum is now split magnetically with $H_{eff} = 460 \pm 2$ kOe and the rest gives doublet b. (Compare Figure 4H with Figure 5E.) In this experiment, a relatively large volume of acidic $^{57}Fe(II)$ was added, which may have caused some reduction in pH (this was not checked). However, since at least 50% of the Fe was in magnetic clusters at 2 h, the final pH was probably not much diminished.

(*3*) *Dependence on t_f, the Time the Sample Is Held at Room Temperature.* There is an appreciable change of the relative intensities of the various subspectra with t_f, the length of time for which the sample is held at room temperature. The relative amounts of the different iron species in samples held at room temperature for different times t_f are summarized in Table II. In all spectra with $n = 4$ the relative intensities of the relaxation subspectrum, of doublet c, and of doublet d, corresponding to solitary Fe(III), dimer, and divalent iron, respectively, decrease with t_f, and the relative amount of Fe(III) clusters increases with t_f. The decrease of the relative intensity of doublet c and doublet d is demonstrated in Figure 6 for pH 5.6 and 6.25. In samples prepared at pH 7.0 the relative intensity of the dimer decreases to about half of its initial value between the first and third minutes. This change is about the same as that observed in a sample prepared at pH 6.4 within 2 h. The results show therefore a flow of iron from the solitary ions, dimers, and Fe(II) to the larger Fe(III) aggregates. This flow is faster the higher the pH.

The spectra obtained in samples prepared at pH 6.4 with $n = 480$ at 4.1 K are shown in Figure 5E,F. Whereas at $t_f = 3$ min most of the iron is divalent and only 10% are magnetic in the core, after 2 h, about 42% are still divalent, 53% give a magnetic spectrum with $H_{eff} = 460 \pm 2$ kOe, and the other 5% are in doublet b. Thus, we observe here in the first 2 h the creation of the magnetic iron core. The iron ions responsible for doublet b are in smaller clusters, which due to superparamagnetism are not large enough to show a magnetic spectrum at 4.1 K. If one assumes the same anisotropy constant as in iron-full ferritin, the aggregates responsible for the magnetic spectrum must be larger than 18 Å in diameter. Although over the first 3 min only about 90 of the 480 Fe(II) atoms had been oxidized, the rate of oxidation was 23 times that observed when only 4 Fe atoms/molecule was added at pH 6.25 (or up to 22.5 times that at pH 6.4). If the rates were linear in protein and iron concentration, a marked decrease in oxidation rate would have been expected, since the decrease in protein concentration (30-fold) greatly exceeded the increase in iron concentration (4-fold). Thus, the iron:protein ratio may be an important determinant of the rate of oxidation, as well as of the fraction present as clusters (see also Figure 3B). The drop in the rate of oxidation of the remaining 390 Fe(II) atoms may have been due to the exhaustion of the dissolved oxygen in the first 3 min during which 0.27 mM Fe(II) was oxidized, with only slow replenishment through the air–liquid interface of the Eppendorf tube.

(*4*) *Bonding Strength of Different Species.* The f factor, or the probability for recoilless resonance absorption (the Mössbauer effect), obtained from the Mössbauer spectra is a measure of the bonding strength of the iron atoms in the lattice (Goldanskii & Makarov, 1968). This probability is given by $f = \exp(-\langle x^2 \rangle / \lambda\!\!\!^{-})$, where $\langle x^2 \rangle$ is the mean square displacement of the iron atoms in the direction of the γ ray and $\lambda\!\!\!^{-}$ is the reduced wavelength of the γ ray. The area under a Mössbauer spectrum is proportional to the f factor. At low temperatures the f factor for all iron compounds is similar, and therefore the area under the different subspectra is pro-

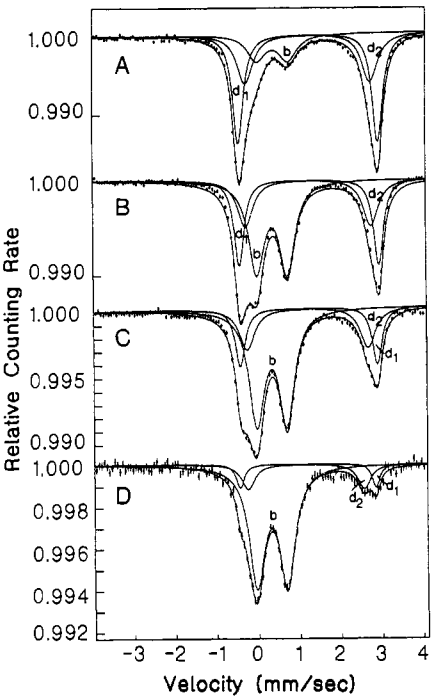

FIGURE 7: Mössbauer spectra obtained at different temperatures in ferritin samples prepared at pH 6.4 and loaded with 480 Fe atoms/molecule: (A) 90 K, t_f = 3 min; (B) 90 K, t_f = 40 min; (C) 150 K, t_f = 40 min; (D) 200 K, t_f = 40 min. (A) and (B) demonstrate the resolution of doublet d into doublets d_1 and d_2 and the increase of the intensity of doublet b with time. (B)–(D) show the different temperature dependences of the intensities of the subspectra. The solid lines are computer fits to the spectra.

portional to the abundance of the different iron-containing species in the sample. Yet at higher temperatures, the f factor differs considerably in different iron compounds and is a measure of the bonding strength of iron in the compound. The more the f factor (or area under the Mössbauer spectra) changes with temperature, the weaker is the bonding of iron in that compound (Goldanskii & Makarov, 1968). Thus, Mössbauer spectra were obtained at temperatures between 90 and 200 K for some of the samples. Spectra obtained in the sample with 480 Fe atoms/molecule at t_f = 40 min at some temperatures are shown in Figure 7. One observes immediately the change in the relative areas of the different subspectra with temperature. Thus, in Figure 7 (480 Fe atoms/molecule) the relative intensities of the doublets corresponding to divalent iron decrease appreciably with temperature, and whereas at 90 K the area under doublet d_1 (30%) was about twice as large as the area under doublet d_2 (12%), at 200 K, doublet d_2 is even of higher intensity than doublet d_1. In spectra of samples with 4 Fe atoms/molecule, the relaxation spectrum is hardly observed at 150 K, though it is of appreciable intensity at 90 K. Figure 8 shows the change of the areas under the different subspectra with temperature. The steeper the slope of the lines, the weaker is the bonding of the Fe atoms. Thus, not only is Fe(II) relatively weakly bound, but the temperature dependences of the spectral area of the two divalent iron species also show different slopes. It is reasonable to assume that one of the divalent species (d_1) corresponds to iron in the solution, which has not yet reacted with the ferritin or free Fe(II) inside the molecule; the other divalent species (d_2), amounting to 58 Fe(II) atoms/molecule, corresponds probably to Fe(II) that is bound by protein side chains or to core particle surfaces.

Among the trivalent iron species, the bonding of the solitary iron ions, which yield the relaxation spectra, is the weakest

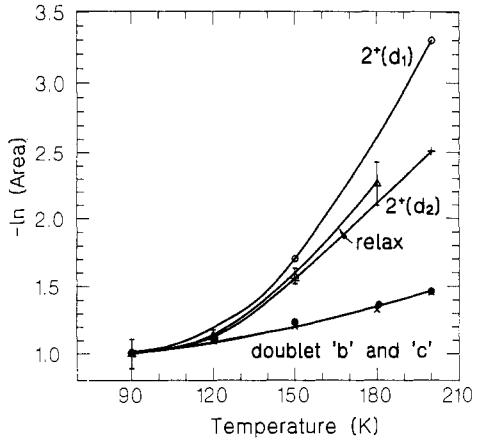

FIGURE 8: Temperature dependence of the areas under the various subspectra, corresponding to solitary iron ions (relax), to two kinds of divalent iron [(d_1) and (d_2)], to dimers (c), and to trivalent iron in larger clusters (b).

and is similar to that of one of the divalent species (d_2). Both these iron species could be bound at the same protein site. The intensities of doublets b and c show a similar small change with temperature, indicating that the iron in dimers and in large aggregates is the most firmly bound. The similarity of the bonding of iron in the dimers and in larger groups may indicate that the dimers are the initial species of the iron core, around which the larger aggregates are then built.

Discussion

The results described here nicely complement and extend those of our earlier studies by UV difference spectroscopy (Treffrey & Harrison, 1984), EPR studies of Rosenberg and Chasteen (1982) and Chasteen et al. (1985), and EXAFS studies by Yang et al. (1987). We equate the species responsible for the EPR g' = 4.3 signal with that giving the relaxation spectra seen by Mössbauer spectroscopy. Our data indicate that the isolated Fe(III) atoms must be bound to the protein since their separation is about 17 Å, or about $^1/_{10}$ of the average separation of Fe atoms in free solution at the concentration used. We are also able to observe species that are EPR silent, namely, divalent iron and iron(III) in binuclear and larger clusters, and have followed their development and decline as a function of the number of Fe atoms per molecule, of pH, and of time. The behavior of these various species allows us to identify the isolated Fe(III) also with the initial Fe(III)–apoferritin species absorbing in the region of 290 nm seen by UV difference spectroscopy (Treffry & Harrison, 1984) and the cluster and magnetic spectra with UV spectra seen at later times or with more Fe per molecule. Yang et al. (1987) measured Mössbauer spectra of 10 Fe–apoferritin after incubation in air overnight at pH 7.0. From EXAFS spectra of the 10 Fe(III)–apoferritin complex they deduced that it contained polynuclear clusters attached to the protein, but they did not examine the formation of these clusters as a function of pH or time. Although we did not measure Mössbauer spectra of our samples at such long intervals after iron addition, we have done so previously (Bauminger et al., 1987) with samples that were dialyzed and crystallized before examination. The spectra we obtained for 10 Fe–apoferritin and those of Yang et al. (1987) were similar. They showed the presence of magnetic spectra as well as those of smaller clusters. Now, at only 3 min after Fe(III) addition, we see no trace of magnetic spectra with samples containing less than 40 Fe atoms/molecule. They seem to develop slowly after the initial oxidation. Although an Fe(III) oxo-bridged dimer is

seen here in ferritin for the first time, an EPR signal, attributed to a Fe(II)–Fe(III) mixed valence dimer, was found before when Fe(II) was added anaerobically to a 12 Fe(III)–apoferritin complex (Chasteen et al., 1985). The relationship between these dimers remains to be investigated.

Our data, building on those of earlier studies, suggest the following picture of events after small numbers of Fe(II) atoms (4–40/molecule) are added to horse spleen apoferritin. The finding of Fe(III) relaxation spectra as the major component at early times with small numbers of Fe per molecule indicates that Fe(II) is first bound and oxidized at isolated sites on the protein. This and the absence of binuclear Fe(III) at lower pH values suggest that oxidation is through a single-electron transfer to dioxygen and not through two-electron transfer to Fe(II) pairs. Some of the Fe(III) atoms must migrate toward each other from these sites, since the relative areas of the relaxation spectra decrease with time and the numbers of Fe(III) atoms in clusters increases. Vacation of iron-binding sites after oxidation has also been suggested by competition experiments with Fe(II) or Fe(III) and VO(IV) (Chasteen & Theil, 1982). The decrease in percent dimer with time, in parallel with the decrease in solitary Fe(III) and the increase in Fe(III) clusters, may mean that clusters form around the binuclear centers. The similar binding strength of iron in dimers and larger groups supports this hypothesis. Nevertheless, dimers do not seem to be essential for cluster formation, which occurs even at low pH (5.6), when dimers are not seen. Such clusters may contain Fe(III) atoms that are close, but not covalently linked. However, such linkage is likely to stabilize Fe(III) in this form, and this is indicated by the larger percent of Fe(III) clusters at higher pH values, at which hydrolysis is favored. Not surprisingly, the rate of clustering is increased as the number of Fe atoms per molecule is increased. The number and percent of Fe(III) in dimers fall markedly when Fe is increased from 20 to 40 Fe atoms/molecule, as the dimers become incorporated in large clusters. When 4 ^{57}Fe is added to molecules with preformed cores (150 ^{56}Fe atoms/molecule), over 3 times as much of the added Fe is found in Fe(III) clusters at 3 min as compared with a simple addition of 4 ^{57}Fe to apoferritin and the percent of solitary Fe(III) falls from 65 to 10. This may result from some of the Fe(II) bypassing oxidation sites on the protein and being oxidized in newly created Fe(II)-binding sites on the iron core as has been suggested before (Macara et al., 1972). Such positive cooperativity is likely to lead to uneven distributions of Fe atoms per molecule in the product of Fe(II) oxidation with apoferritin, and indeed, this has been found previously when much larger numbers of Fe atoms per molecule were added (Macara et al., 1972). In this case analytical centrifugation showed a tendency toward "all-or-none" distribution in the resulting ferritin. Such nonrandom distributions are clearly shown again here even with only 4 Fe atoms/molecule.

In a sample with $n = 40$, about 60% of the Fe(III) atoms were in magnetic clusters 2 h after iron addition. If the magnetic anisotropy constant, k, of such clusters is assumed to be the same as in the iron-rich ferritins, a cluster of about 18-Å diameter containing about 120 Fe atoms should be present. This indicates either that k is larger for smaller aggregates, as has been suggested previously (Yang et al., 1987), or that clusters with $n \gg 40$ are present in some molecules and others contain littler or no iron. Recent data indicate k to be independent of particle size (Dickson et al., 1988). Since magnetic spectra are observed in preparations containing 10 Fe(III) atoms/molecule if they have been left for several hours before measurement (Yang et al., 1987; Bauminger et al., 1988), a redistribution of Fe atoms between molecules may be indicated. This could be tested by measuring spectra of 4 ^{57}Fe–apoferritin mixed at various times with molecules already nucleated with ^{56}Fe. Such a redistribution, if it does happen, is likely to occur only from isolated Fe(III), which are more weakly bound, and perhaps from "immature" clusters. Once iron-core nuclei have grown above a certain size and have developed a stable crystalline ferrihydrite structure, the probability of these nuclei losing iron is greatly decreased. On the contrary, a thermodynamic driving force to increase their size is evident.

The data in Figure 8 show that Fe(II) is more weakly bound than Fe(III) atoms present in clusters. A similar result has been obtained by Frankel et al. (1987) in ferritin that had been partially reduced. However, the two types of Fe(II), which may represent bound and unbound atoms, have not been clearly distinguished previously. Rohrer et al. (1987) reported that when 480 Fe atoms/molecule was added, at a much higher concentration (20 mM) and higher pH (7.0) than those used here (1.37 mM and 6.4), much of the iron becomes unavailable to react with *o*-phenanthroline. At 2 h, they found 68% unavailable, a figure that agrees well with one calculated from our results, namely, 58% in Fe(III) clusters plus 12% bound Fe(II), although such close agreement may be fortuitous in view of the different conditions used. However, these authors also measured the near-edge structure of the X-ray absorption spectrum and could detect no Fe(III) within the first 2 h after addition of Fe(II) and concluded that the sequestered iron must be Fe(II). On the other hand, Rohrer et al. (1987) also observed an increase in visible absorbance at 420 nm; if this were due to the presence of a polynuclear iron core, as may be expected (Macara et al., 1972), then nearly 10% of the iron was converted to Fe(III) in 10 min and nearly 20% after 2 h in their samples. The reason for these discrepancies is unclear. Although Fe(II) can evidently persist under some aerobic conditions, as well as under anaerobic (Watt et al., 1985, 1988), especially if a partial Fe(III) core is present to bind Fe(II), the physiological importance of ferritin as an Fe(II) store [suggested by Rohrer et al. (1987)] remains an open question.

The data we have presented here are relevant to the more general field of biomineralization, where mineral is laid down on an organic matrix. Similar processes may occur in other systems, even in the absence of an oxidation step: the binding at initial sites, the migration of some atoms from these sites to form clusters, and the dissolution of some of these clusters in parallel with the formation and growth of stable nuclei around others. The next stage in our understanding of these processes in ferritin will be the determination of the ligands and ligand geometries of the various types of iron observed. Although some proposals have been made (for example, the possible location of nucleation centers) on the basis of the binding sites of other metals in horse spleen apoferritin crystals (Harrison et al., 1986), solution of this complex problem requires the application of site-directed mutagenesis techniques. Such studies are in progress in our and other laboratories.

Registry No. Fe, 7439-89-6.

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