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Iron(III) Clusters Bound to Horse Spleen Apoferritin: An X-ray Absorption and Mössbauer Spectroscopy Study That Shows That Iron Nuclei Can Form on the Protein†

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Received June 9, 1986; Revised Manuscript Received August 27, 1986

ABSTRACT: Ferritin is a complex of a hollow, spherical protein and a hydrous, ferric oxide core of ≤4500 iron atoms inside the apoprotein coat; the apoprotein has multiple (ca. 12) binding sites for monoatomic metal ions, e.g., Fe(II), V(IV), Tb(III), that may be important in the initiation of iron core formation. In an earlier study we observed that the oxidation of Fe(II) vacated some, but not all, of the metal-binding sites, suggesting migration of some Fe during oxidation, possibly to form nucleation clusters; some Fe(III) remained bound to the protein. Preliminary extended X-ray absorbance fine structure (EXAFS) analysis of the same Fe(III)–apoferritin complex showed an environment distinct from ferritin cores, but the data did not allow a test of the Fe cluster hypothesis. In this paper, with improved EXAFS data and with Mössbauer data on the same complex formed with ^{57}Fe, we clearly show that the Fe(III) in the distinctive environment is polynuclear (Fe atoms with Fe–Fe = 3.5 Å and T_B = 7 K). Moreover, the arrangement of atoms is such that Fe(III) atoms appear to have both carboxylate-like ligands, presumably from apoferritin, and oxo bridges to the other iron atoms. Thus the protein provides sites not only for initiation but also for nucleation of the iron core. Sites commodious enough and with sufficient conserved carboxylate ligands to accommodate such a nucleus occur inside the protein coat at the subunit dimer interfaces. Such Fe(III)–apoferritin nucleation complexes can be used to study the properties of the several members of the apoferritin family.

Ferritin maintains iron in a form available to cells for such crucial uses as oxygen transport (the globins), electron transfer and oxygen activation (the cytochromes), nitrogen reduction (nitrogenase), and DNA synthesis (ribonucleotide reductase). The need for ferritin appears to have coincided with the evolution of dioxygen as a byproduct of photosynthesis. Oxygen in the environment prohibits life forms from using the facile metabolism of soluble ferrous and requires the complex uptake, distribution, storage, and recycling of relatively insoluble ferric [the solubility of Fe(III) is approximately 10^{-9} times that of Fe(II) in air at physiological pH (Biederman & Schindler, 1957)].

Thousands (≤4500) of iron atoms may be accommodated in a single ferritin molecule as a polynuclear, hydrous, ferric oxide core inside the hollow apoprotein shell that is formed by the 24 subunits of apoferritin (inner diameter ca. 80 Å in mammalian ferritins [reviewed in Theil (1983, 1987)]). However, in any population of ferritin molecules, a range of core sizes is present. Channels piercing the apoprotein shell appear to provide sites for the entrance and egress of iron. The

† This work was supported in part by Grant AM20251 from the National Institutes of Health (E.C.T. and D.E.S.), Grant PCM-8305995 from the National Science Foundation (B.H.H.), and NIH Research Career Development Award AM01135 (B.H.H.). The work reported herein was partially carried out at the Stanford Synchrotron Radiation Laboratory, which is supported by the Department of Energy Office of Basic Energy Services and the National Institutes of Health Biotechnology Research Program, Division of Research Resources. This is a contribution from the Department of Biochemistry, School of Agriculture and Life Sciences and School of Physical and Mathematical Sciences. This is paper 10315 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601. The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned.
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0006-2960/87/0426-0497$01.50/0

importance of apoferritin in core formation is to promote growth of the core crystallites inside the hollow center of the protein shell, since polynuclear iron complexes with similar (although not identical) properties can form without the protein. Storage of iron inside the protein shell minimizes the effect of reactions of iron that are deleterious to the cell, e.g., productions of free radicals. In addition, the protein can control the rate of iron release (Jones et al., 1978; Mertz & Theil, 1983). Apoferritin structure is highly conserved, at least among vertebrates (Wustefeld & Crichton, 1982; Addison et al., 1983; Boyd et al., 1985; Dorner et al., 1985; Didsbury et al., 1986). Posttranslational modifications of apoferritin also occur, e.g., the covalent cross-linking of macrophage ferritin subunit dimers, which alter ferritin iron uptake and release in vivo and in vitro (Mertz & Theil, 1983). Such variations in function and structure may reflect differences in the iron–protein interactions during core formation and/or differences in the structure of the iron core itself.[1]

The formation of ferritin may be subdivided into several steps, beginning with the assembly of apoferritin subunits into the hollow sphere. Next, Fe(II) binds to multiple sites (Harrison et al., 1974; Chasteen & Theil, 1982) and is oxidized to Fe(III), which is also bound to the protein (Chasteen & Theil, 1982; Sayers et al., 1983); however, some of the metal-binding sites are vacated, suggesting migration of Fe atoms during oxidation to form clusters (Chasteen & Theil, 1982). The hydrolysis of hydrated Fe(III) and the formation of core crystallites from oxo-bridged Fe(III) atoms lead to the final iron core. How the protein orients the growth of the crystallites toward the hollow center of the protein and whether the protein has any role beyond binding monoatomic Fe(II) and Fe(III) are not known.

In this paper we describe the continuation of our studies on the early stages of ferritin iron core formation. We are testing the hypothesis that during oxidation of Fe(II) bound to the protein, i.e., <12 Fe(II)/molecule, some Fe atoms migrate to form clusters. Since we already know that Fe(III) formed by the oxidation of bound Fe(II) is also bound to the protein at multiple sites (Chasteen & Theil, 1982), we hypothesize further that such Fe(III) clusters or nuclei would also be attached to the protein. An Fe(III)–apoferritin complex was formed by stripping the protein of iron (≤0.05/subunit), adding back small numbers of ferrous ions (0.4/subunit), and allowing oxidation to occur in situ; the iron/protein ratio was selected to be below the saturation of the protein estimated by competition with the binding of vanadyl (Chasteen & Theil, 1982). Apoferritin was used from horse spleen ferritin because it is relatively simple compared to apoferritin from other tissues (≥90% of the subunits are identical), because a large amount of structural information is available [amino acid sequence (Heusterspreute & Crichton, 1981), three-dimensional structure (Rice et al., 1983)], and because it is abundant. The complex was further examined by X-ray absorption spectroscopy, this time with data allowing analysis beyond 2.6 Å, and also by the Mössbauer technique [using ^{57}Fe(III)]. We found evidence, from both spectroscopic analyses, for the presence of polynuclear Fe(III); each Fe(III) atom had both carboxylate-like ligands and oxo bridges to other Fe(III), indicating that migration of Fe atoms did occur during oxidation and that nucleation of the ferritin core crystallites occurs on the protein.

Thus, the protein not only binds solitary iron atoms but also provides sites for clusters of iron atoms to nucleate the ferritin core crystallites. Such sites on the protein could provide the orientation necessary to promote core growth toward the hollow center of the protein. The availability of nucleation complexes [apoferritin binding Fe(III) clusters] provides the opportunity to determine the influence of variations in apoferritin structure on ferritin core formation.

Experimental Procedures

Preparation of Fe(III)–Apoferritin Complex. Horse spleen ferritin 2 times crystallized and cadmium free, obtained from Miles Laboratories, was stripped of iron by dialysis against 0.14 M thioglycolic acid/0.05 M sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes·Na), pH 7, first in the presence of air and then under nitrogen (Chasteen & Theil, 1982); the iron content of the apoprotein was ≤0.03/subunit or ≤0.72/molecule. Solutions of $FeSO_4$ freshly prepared in oxygen-free water or ^{57}Fe wire dissolved at 50 °C in 0.1 N HCl, assayed for concentration as the *o*-phenanthroline complex, were purged (either with a stream of argon or 95%/5% N_2/H_2 that had been passed through heated copper mesh) and added to solutions of apoferritin in 0.05 M Hepes·Na that had been similarly purged. The iron/protein ratio achieved was 0.4/subunit. [Saturation appears to be achieved at ca. 0.5 Fe/subunit (Chasteen & Theil, 1982).] After the sample was allowed to bind for 30 min at 4 °C with purging, air was admitted to the sample and oxidation allowed to occur overnight at 4 °C before measurement of the X-ray absorption spectra. Samples for Mössbauer spectroscopy were frozen in liquid nitrogen. Spectral data were collected for ^{57}Fe(II)–apoferritin before thawing, followed by the admission of air and incubation overnight at 4 °C before refreezing, to collect data on ^{57}Fe(III)–apoferritin.

X-ray Absorption Spectroscopy. Measurements were made at room temperature, in the fluorescence mode, at the Stanford Synchrotron Radiation Laboratory, with a dedicated beam (line II-4) unfocused, at 3.0 GeV and 30–70 mA, and a Si(111) crystal. Two independently prepared samples were each measured over a period of ca. 8 h. Additional measurements were also made with two independent samples during a parasitic run. Protein subunit integrity, monitored by electrophoresis in sodium dodecyl sulfate (SDS) gels, indicated that no damage had occurred to the protein during the measurements. The data were analyzed as previously described [Theil et al., 1979; Sayers et al., 1983; Mansour et al., 1985; see also Heald et al. (1979)] with ferritin and a μ-oxo-bridged Fe(III)–Fe(III) complex (Armstrong et al., 1984), kindly provided by Stephan J. Lippard, as models.

Mössbauer Spectroscopy. Standard transmission measurements were made with a 50-mCi ^{57}Co(Rh) source at room temperature. The source was driven by a Ranger VT-700 Mössbauer velocity transducer operating at the linear acceleration mode. A Janis 8DT SuperVaritemp cryostat was used to provide absorber temperatures between 1.5 and 240 K. Velocity calibration was performed on room temperature spectra of a thin metallic iron foil, relative to which all isomer shifts are quoted. The high-field spectrum was recorded in Dr. E. Münck's laboratory at the Gray Freshwater Biological Institute, University of Minnesota. The high-field spectrometer has been described elsewhere (Zimmermann et al., 1978).

Results

Extended X-ray Absorbance Fine Structure (EXAFS) Analysis of Fe–Fe Interaction in Fe(III)–Apoferritin. The earliest stage of iron core formation in ferritin appears to be the binding of Fe(II) to sites on the apoprotein [reviewed in

[1] Studies to be published separately (Yang et al., 1986) indicate that the local structure of hydrous ferric oxide cores can vary, e.g., if sulfate (chondroitin sulfate) is present during nucleation and core formation.

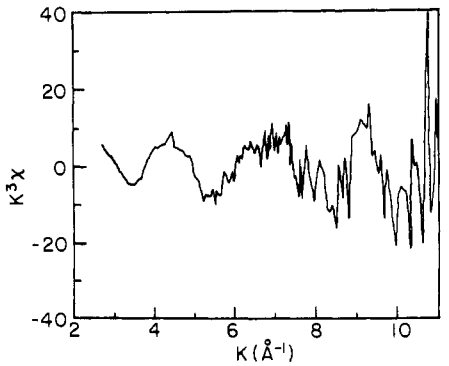

FIGURE 1: X-ray absorption spectrum (region of extended fine structure) for Fe(III)–apoferritin complex, k^3 weighting. The complex was prepared by adding 0.4 Fe(II)/subunit (10/molecule), followed by oxidation in situ; the measurements were made in the fluorescence mode (see Experimental Procedures).

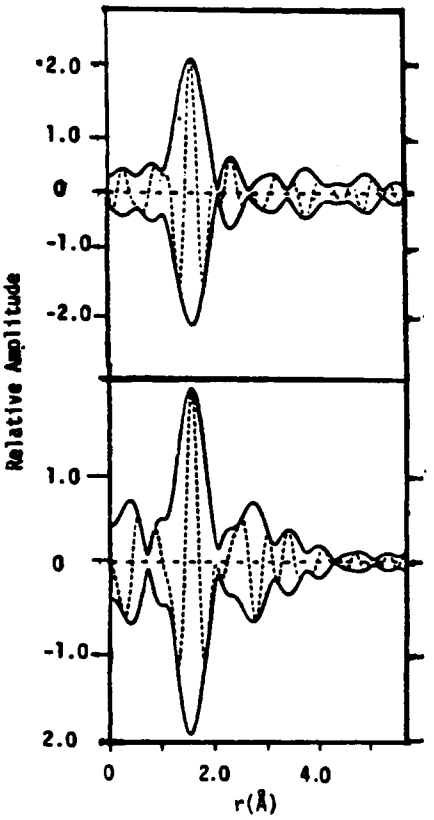

FIGURE 2: Fourier transform (3.0–10.2 kÅ^{-1}) of Fe(III)–apoferritin complex (10 Fe/molecule). Ferritin (ca. 2000 Fe/molecule) is included for comparison. (Upper panel) Fe(III)–apoferritin complex; (lower panel) ferritin; k^3 weighting, not corrected for the phase shift. The different combinations of ranges used in modeling the data were 0.52–2.06, 0.52–2.64, 0.52–3.38, 0.52–4.19, 2.64–3.38, and 2.64–4.19 Å.

Theil (1983, 1987)]. Fe(II) and vanadyl saturate the protein at a stoichiometry of ≤0.5 atom/subunit (≤12/molecule). For further study, we selected a stoichiometry where most of the binding sites are saturated by Fe(II) i.e., 0.4/subunit. Oxidation of Fe(II) to Fe(III) in situ yields a complex with Fe(III) also bound to the protein, apparently to carboxylate-like ligands (Chasteen & Theil, 1982; Sayers et al., 1983); however, the data did not permit an assessment of whether or not the Fe absorber had Fe neighbors. If the Fe absorber were part of a polynuclear complex, the results would indicate that the protein participates in the formation of the nuclei for the core crystallites. Accordingly, we collected X-ray absorption data with a higher signal-to-noise ratio than previously (Sayers et

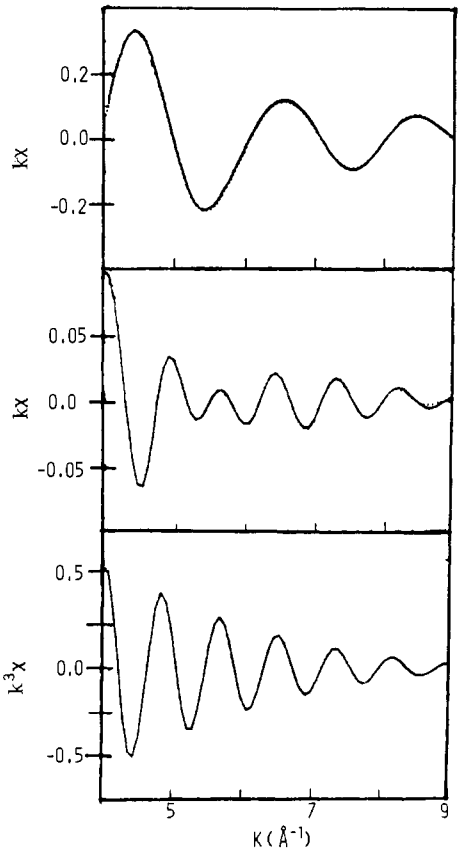

FIGURE 3: Comparison of $k\chi$ for filtered experimental data and numerical fits for selected ranges [viz., 0.52–2.64 Å (top), 2.64–4.19 Å (middle), and 3.38–4.19 Å (bottom)] of Fourier transform of Fe(III)–apoferritin complex (see Figure 2). The ranges were chosen to show the nearest Fe–O and Fe–C interactions (top), the Fe–Fe and distant Fe–O interactions (middle), and the distant Fe–O interaction alone (bottom). Note that the bottom panel is displayed after analysis, with k^3 weighting to emphasize the absence of the high Z (Fe) component at the longer distances. (Solid line) Experimental data, (dashed line) modeling fits. The difference can only be observed at $k \geq 8$ because the variance is so small. The variance is 3.05×10^{-5} for Fe–O and Fe–C interactions, 1.60×10^{-5} for the Fe–Fe and distant Fe–O interactions, and 8.56×10^{-10} for the distant Fe–O interaction.

al., 1983). EXAFS was analyzed to probe the environment 3–4 Å from the absorbing Fe atom for the presence of a high-Z atom (Fe). Note that we prepare complexes to study the Fe–apoferritin interaction using 0.4 Fe/subunit (9.6/molecule) to ensure that, when the metal is added as Fe(II), it is all bound to the protein. The possibility that unbound iron is present after oxidation is eliminated by the facts that Fe(III) has a solubility of 10^{-18} M at pH 7 (Biederman & Schindler, 1957) and that no precipitate of iron was observed.

In four sets of data collected with independent samples, a feature was observed in the Fourier transform at 3–4 Å (Figure 1) that, with previously described procedures (Theil et al., 1979) as outlined below, we have identified as a high Z atom (Fe). Only the data collected for two samples had a sufficiently high signal-to-noise ratio to be adequate for modeling. The Fe environment was analyzed with data from the ranges 0.52–2.06, 0.52–2.64, 0.52–3.38, 0.52–4.19, 2.64–3.38, 2.64–4.19, and 3.38–4.19 Å in the Fourier transform (Figure 2) and various combinations of single- and multiple-shell fits. Over 100 different fits were tested.

In the case of the Fe–Fe interaction, the effect of using the different ranges is illustrated in Figure 3. When the window 2.64–4.19 Å (Figure 2) was back-transformed into k space, the curve produced clearly shows the contribution of a high-Z

Table I: EXAFS Analysis of Low Z Nearest (O, C) Neighbors in Fe(III)–Apoferritin

transform range	model	CN	r (Å)	$\Delta\sigma^2$ ($\times 10^3$ Å^2)	V ($\times 10^5$)
0.52–2.06	Fe–O	5.0	1.95	−5	5.8
2.06–2.64	Fe–C (O)[b]	1.6	2.72	−5	0.36
0.52–2.64	Fe–O	5.2	1.94	−5	3.16
	Fe–C (O)[b]	1.3	2.79	−5	

[a] Designations: CN, coordination number; r, interatomic distance; $\Delta\sigma^2$, relative mean square disorder between the sample and the standard (Debye–Waller factor); V, variance between the modeled data and the experimental data. Transform ranges indicated are from the Fourier transform of the data as shown in Figure 2. Note that either single-shell models or a two-shell model gives comparable fits to the experimental data. [b] EXAFS analysis cannot distinguish between C and O. However, Fe–C is the more likely because of the good model provided by iron oxalate and because esterification of carboxylates on apoferritin blocks oxidation of Fe(II) (Wetz & Crichton, 1976).

Table II: EXAFS Parameters for Fe(III)–Apoferritin [10 Fe(III)/Molecule] Compared to Ferritin [2000 Fe(III)/Molecule]

	CN	r (Å)	$\Delta\sigma^2$ ($\times 10^3$ Å^2)
Fe–O (N?)[b]			
ferritin[d]	6.0	1.95	0
Fe(III)–apo	5.2 ± 0.5	1.94 ± 0.02	−4.9 ± 0.5
Fe–C (O?)[b]			
ferritin[d]			
Fe(III)–apo	1.3 ± 0.5	2.79 ± 0.10	−5.4 ± 1.0
Fe–Fe[c]			
ferritin[d]	6.0	3.34	0
Fe(III)–apo	2.4 ± 0.5	3.57 ± 0.10	1.1 ± 1.0

[a] Designations: CN, coordination number; r, interatomic distance; $\Delta\sigma^2$, relative mean square disorder between the sample and the standard (Debye–Waller factor). The data presented are fits over the range 0.52–4.19 Å in the Fourier transform (Figure 2) and are representative of over 100 fits, and thus no variance is included. The estimate of the error is based upon the variation among fitting results with different parameters, e.g., k weighting, range of transform, range of filter, number of parameters varied, and numbers of absorber/scatterer pairs in the model. Quantitative data for the distance Fe–O interactions (3.38–4.19 Å, Figure 3) are not included because of the unreliability of the analyses at such distances; e.g., it is difficult to eliminate possible effects of multiple scattering. [b] Fitted well by iron(III) oxalate (Sayers et al., 1983); transform ranges used were 0.52–2.06, 0.52–2.64, and 2.06–2.64 Å. [c] Ferritin was used as a model; transform ranges were 2.64–4.19 and 2.64–3.38 Å. [d] Data from Sayers et al. (1983).

(Fe) atom (Figure 3, middle) [see Stern et al. (1975)]. In contrast, the contribution of the Fe–Fe interaction is absent from the back-transform of the data in the ranges 0.52–2.64 Å (Fe–O, Fe–C; Figure 3, top) and 3.38–4.19 Å (Fe–O; Figure 3, bottom). The overlapping interactions in the range 2.64–3.38 Å are such that it is not possible to display a single, clean Fe shell.

The analysis of nearest-neighbor low Z atoms was straightforward. Comparable quantitative results were obtained whether each shell was analyzed singly (transform ranges 0.52–2.06 and 2.06–2.64 Å, respectively, Figure 2) or together (transform range 0.52–2.64 Å, Figure 2; Table I). The analysis thus contrasts with that obtained in a preliminary data report (Sayers et al., 1983), where the statistical uncertainty of the data necessitated the use of a smaller range in the original transform. As a consequence, each shell in the earlier data could not be filtered separately but could only be fit reasonably with a two-shell model; the results were important mainly because an Fe(III)–apoferritin interaction, distinct from ferritin, could be demonstrated. The lower reliability of the quantitative results in the preliminary report was indicated by the large value given to the uncertainty in the tabulated data (Sayers et al., 1983).

Quantitative data are presented (Tables I and II) for the interactions between the absorber and scatterers up to and including the Fe–Fe interaction. Although low Z interactions further away were readily observed (Figure 3, bottom), quantitation of the interaction was not sufficiently accurate to tabulate, because of the difficulty of evaluating multiple scattering effects. The Fe–Fe distance obtained from the analysis is 3.57 Å (Table I), similar to and possibly identical with the Fe–Fe distance in the iron core of ferritin. The size of $\Delta\sigma^2$, the Debye–Waller factor, suggests that the order is also similar to that of ferritin. However, the coordination number is much lower than for atoms in the core of ferritin, indicating that the cluster is incomplete. Since only a weak $g' = 4.3$ electron paramagnetic resonance (EPR) signal was detected at 4 K, few if any of the iron atoms exist as monoatomic species.[2]

In addition to the Fe–Fe interactions, each Fe atom interacts with five to six low Z atoms at 1.95 Å and one to two low Z atoms at 2.79 Å. The atoms are designated O and C, respectively (rather than other low Z atoms),[3] because carboxylate ligands are important in the initiation of core formation (Wetz & crichton, 1976), and because Fe(ox)·$2H_2O$ (ox = oxalate) is a good model for the first two shells of the Fe(III)–apoferritin complex. The interatomic distances coordination numbers, and Debye–Waller factor were little affected by the different models. Similar results were obtained for the Fe(III)–apoferritin complex whether Fe–O was used from ferritin or from a μ-oxo-bridged dimer (Armstrong et al., 1984) as a standard.

The existence of Fe(III) clusters observed by EXAFS explains the regeneration of metal-binding sites previously observed when saturating levels of Fe(II) were oxidized to Fe(III) (Chasteen & Theil, 1982); during oxidation some iron atoms move from solitary sites to clustered sites. Analysis of the Mössbauer spectrum of the ^{57}Fe(II) complex (see below), which is the precursor of the Fe(III) complex, indicates that the Fe(II) is most likely a monoatomic species octahedrally coordinated to water and carboxylate-like ligands.

The Fe(III)–apoferritin complex described is likely to contain a relatively small distribution of sizes of iron clusters because both the iron and protein sediment in a zone characteristic of (low-iron) apoferritin, because few, if any, Fe(III) atoms are monoatomic (only a weak $g' = 4.3$ EPR signal), and because metal-binding studies show that ca. two to three of the protein-binding sites are occupied by Fe(III) when 10 atoms of Fe are added per molecule (Chasteen & Theil, 1982). The Fe(III)–apoferritin complex is likely to be a precursor of ferritin, since the addition of 480 Fe(II) atoms to apoferritin under conditions similar to those forming the Fe(III)–apoferritin complex yields ferritin with normal iron cores, as judged by sedimentation (Mertz & Theil, 1983).

Mössbauer Studies of Fe(III)–Apoferritin Complex. In order to confirm the presence of multinuclear Fe(III) clusters indicated by the EXAFS analysis of the Fe(III)–apoferritin

[2] The dimeric Fe(II)–Fe(III)–apoferritin complex previously detected by EPR (Chasteen & Aisen, 1985) and representing a small fraction of the iron atoms present may be a transient precursor to the Fe(III) clusters we have observed; the inability to observe the dimers by Mössbauer spectroscopy of combinations of ^{57}Fe(II)/^{56}Fe(III) and ^{57}Fe(III)/^{56}Fe(II) suggests that their contribution was below the limits of sensitivity (ca. 10% of the Fe).

[3] Note that the possibility of an Fe–O–O group rather than an Fe–O–C group cannot be excluded by the data, but the instability of such a peroxo derivative renders it unlikely. The possibility of Fe–N–C also exists.

Table III: Mössbauer Parameters for Fe(III)–Apoferritin, Horse Spleen Ferritin, and Model Compounds

sample	QS (mm/s)	IS (mm/s)	T_B (K)	$B(T = 0)$ (T)
Fe(III)–apoferritin[a]	0.78[b]	0.48[b]	7	44.7
ferritin	0.70[b]	0.46[b]	38	48.7
ferric ammonium citrate	0.64[c]	0.50[c]		
ferric citrate	0.62[c]	0.51[c]		

[a] Extrapolation of the data to 0 K results in the disappearance of the quadrupole doublet. [b] Values at 96 K. [c] Values at 100 K.

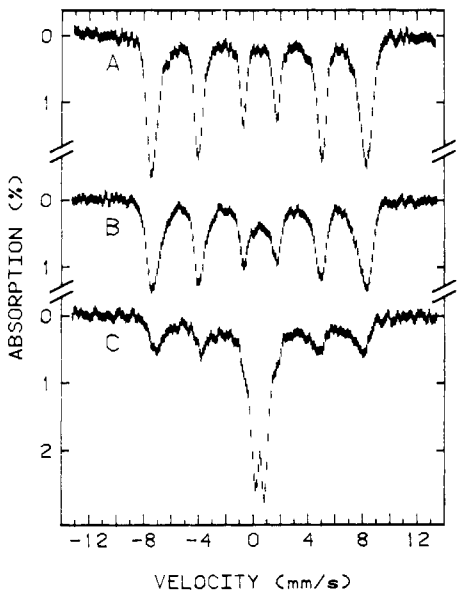

FIGURE 4: Mösbauer spectra of horse spleen ferritin in zero field: (A) 4.2, (B) 11, and (C) 32 K.

complex and to further study the properties of such nucleation complexes, Mössbauer spectra of ^{57}Fe(III)–apoferritin, prepared by oxidizing ^{57}Fe(II) added at 0.4/subunit, were obtained. At 96 K, the Fe(III)–apoferritin complex exhibits a single quadrupole doublet. The parameters (IS and QS) are listed in Table III and are compared with those of horse spleen ferritin and selected ferric model compounds. The QS (0.78 mm/s) for the Fe(III)–apoferritin is significantly larger than that of ferritin (0.70 mm/S), indicating different iron environments between these two samples. It should be noted that a ^{57}Fe(III)–apoferritin complex prepared by oxidizing ^{57}Fe(II) added at 0.8/molecule [exceeding the binding capacity of the protein (Chasteen & Theil, 1982)] appeared to be much more similar to ferritin than the complex prepared by oxidizing ^{57}Fe(II) at 0.4/subunit [all Fe(II) bound (Chasteen & Theil, 1982)].

At temperatures lower than 96 K, the Mössbauer spectra evolve from a quadrupole doublet into a magnetic sextet for both horse spleen ferritin (Figure 4) and the Fe(III)–apoferritin complex (Figure 5). This evolution is a typical phenomenon observed for magnetically ordered ultrafine particles due to the blocking of superparamagnetic fluctuations (Rancourt & Daniels, 1984).[4] In general, small magnetic particles can be characterized by two parameters: the superparamagnetic blocking temperature, T_B, which is defined as the temperature where half of the iron absorption is in a magnetic sextet and half is in a superparamagnetic doublet, and the hyperfine field extrapolated to zero temperature $B(T = 0)$. These parameters for both the Fe(III)–apoferritin and the horse spleen ferritin are listed in Table III. The smaller $B(T = 0)$ value for the Fe(III)–apoferritin complex again suggests a distinct iron environment and may indicate a somewhat lower magnetic dimensionality relative to ferritin; Johnson (1984) has found that a quasi one-dimensional antiferromagnet has a $B(T = 0)$ 37% lower than that of the corresponding three-dimensional material.

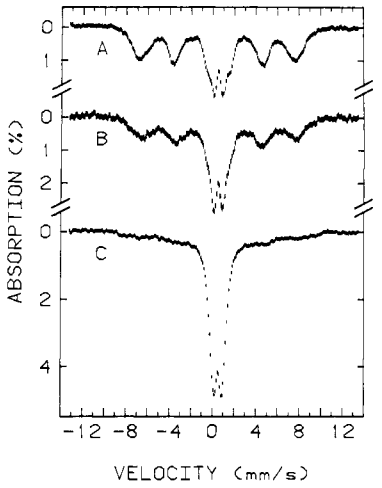

FIGURE 5: Mössbauer spectra of ^{57}Fe(III)–apoferritin complex in zero field: (A) 1.5, (B) 4.2, and (C) 10 K.

The theory of superparamagnetism [Coey et al. (1984) and references cited therein] indicates that the blocking temperature T_B is linearly proportional to KV, where V is the volume of the particle and K is the magnetic anisotropy constant, which may be different for different materials. Assuming the same K for both the Fe(III)–apoferritin complex and ferritin [which contains a core size of approximately 2000 iron atoms (Smith et al., 1985)], the T_B values, 7 and 38 K, observed for the Fe(III)–apoferritin and ferritin, respectively, would then indicate a particle size of approximately 400 iron atoms in the Fe–apoferritin. This value appears to be too large in view of the EXAFS result presented in the previous section. Could the anisotropy constant K be much larger for the Fe(III)–apoferritin complex than for ferritin [for which $K = 6.7 \times 10^3$ J/m^3 (Williams et al., 1978)], leading to a much smaller particle-size estimate. By use of a zero-field splitting parameter $D = 0.7$ cm^{-1} typically found for Fe(III) in biological materials (Huynh & Kent, 1984), the estimated K value varies between 0 and 2×10^6 J/m^3, depending on whether the contributions from different ions add constructively or destructively. By use of the largest value for K, which is 200 times larger than that for ferritin, the lower limit of the particle size in the Fe(III)–apoferritin complex would be reduced from 400 to just a few. Thus, while the Mössbauer superparamagnetic behavior demonstrates that the iron is in polynuclear clusters, it does not provide a reliable estimate of the cluster size.

Mössbauer Studies of Fe(II) Precursor of Fe(III)–Apoferritin Complex. The Fe(II) precursor of the Fe(III) complex has not been characterized directly before. Advantage was taken of the relative stability of Fe(II) in oxygen-free solutions at low temperatures to examine the properties of the ^{57}Fe(II)–apoferritin complex. A spectrum of the complex recorded at 4.2 K is shown in Figure 6A. The major spectral com-

[4] The transformation from a doublet to a sextet upon lowering of temperature is also consistent with a mononuclear high-spin ferric center. However, such a center generally yields an intense and characteristic EPR signal at $g \sim 4.3$. The Fe(III)–apoferritin, on the other hand, exhibits an extremely weak $g = 4.3$ signal that does not account for all the iron atoms in the sample.

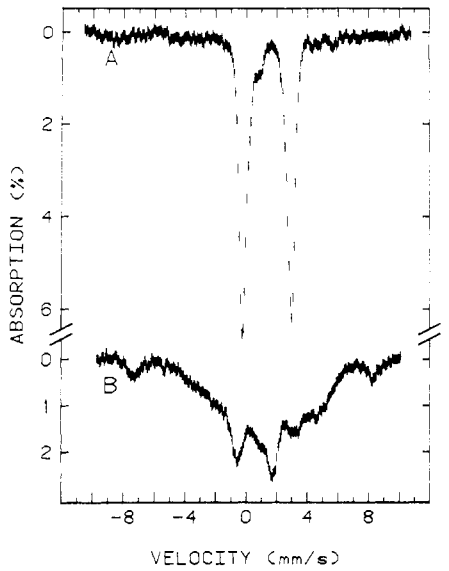

FIGURE 6: Mössbauer spectra of ^{57}Fe(II)–apoferritin complex at 4.2 K. Spectrum A was recorded in zero field and spectrum B with a magnetic field of 6 T applied parallel to the γ-beam.

ponent is a quadrupole doublet with isomer shift (IS = 1.34 mm/s) and quadrupole splitting (QS = 3.08 mm/s) characteristic of high-spin Fe(II) complexes.[5] These observed parameters are very similar to those for $FeCl_2 \cdot 4H_2O$ (IS = 1.36 mm/s and QS = 3.12 mm/s) (Grant et al., 1966) or site II of $Fe_3(PO_4)_2 \cdot 8H_2O$ (IS = 1.34 mm/s) and QS = 3.18 mm/s) (Gonser & Grant, 1967), which contain *trans*-$Fe(H_2O)_4Cl_2$ or *cis*-$FeO_4(H_2O)_2$ units, respectively. Also, the observed isomer shift, 1.34 mm/s, is within the range of those of octahedrally coordinated Fe(II) complexes (1.2–1.5 mm/s), which are generally larger than that of tetrahedral or square-planar coordinated compounds (Menil, 1985). Consequently, these data suggest that in Fe(II)–apoferritin the iron is octahedrally coordinated, probably by both water and anions. The intermediate value of the isomer shift between that for octahedral coordination by six water molecules (1.40 mm/s) (Grant et al., 1966) and six carboxylate groups (1.33 mm/s) (Hoy & Barros, 1965) further suggests that the ligands in the Fe(II)–apoferritin complex are water and carboxylate, as has been indicated by the EXAFS analysis of the Fe(III) complex.

Minor spectral components are also observed in spectrum of Figure 6, as indicated by a shoulder at 0.90 mm/s and weak absorption distributed over the whole velocity range. At higher temperatures (e.g., 100 K), these components collapse into a quadrupole doublet with parameters indicative of high-spin Fe(III). These Fe(III) impurities were estimated to be less than 15% of the total iron content and probably arose after Fe(II) binding during transfer to the sample cup and freezing preparation.

It has been suggested that paired iron atoms may participate in ferritin iron core formation (Banyard et al., 1978). To test the possibility that the Fe(II) atoms were paired, the magnetic behavior of the Fe(II)–apoferritin was measured. A spectrum was recorded at 4.2 K with a strong magnetic field of 6 T applied parallel to the γ-beam (Figure 6B). If the Fe(II) atoms were antiferromagnetically coupled in pairs as in hemerythrin (Stenkamp et al., 1981), a diamagnetic spectrum with a well-defined pattern would be observed. Instead, a broad and rather featureless spectrum is obtained, indicating that Fe(II) is most likely in a solitary environment; if the Fe(II) atoms were paired, the exchange coupling constant would have to be much smaller than 5 cm^{-1} in order for the 6-T applied field to break the coupling. The Fe(II)–Fe(III)–apoferritin complex previously observed by EPR (Chasteen & Aisen, 1985) was not observed in the Mössbauer spectrum, suggesting that, if present, it constituted <10% of the iron.

Discussion

The dynamics of turnover of iron stored in ferritin can be influenced by the cell type as in the comparison of red cell and liver or reticuloendothelial cells (Yamada & Gabuzda, 1974; Brown & Theil, 1978) or of apoferritin with and without natural or synthetic subunit dimer cross-links (Mertz & Theil, 1983; Theil, 1986) and by the size of the iron core (Yamada & Gabuzda, 1974). Iron core size may be altered by small ions such as Zn(II) and inorganic phosphate in vivo and/or in vitro [Coleman & Matrone, 1969; Treffry & Harrison, 1978; Coey et al. (1984) and references cited therein; Rancourt & Daniels, 1984; also see Theil (1983, 1987) for reviews]. In spite of the effects associated with alterations in storage iron turnover, e.g., the iron content of ferritin (iron uptake and release) in copper poisoning (Mertz & Theil, 1983) and iron overload (Van Wyck et al., 1971), only a few studies have probed the molecular basis for such alterations. Moreover, even information about mechanism(s) of normal iron uptake and release by ferritin is incomplete.

As part of the investigation of the early stages of ferritin core formation and of the sites for nucleation of the iron core crystallites occurring on the protein, we prepared an Fe(III)–apoferritin complex. EXAFS and Mössbauer studies show that the iron atoms are apparently attached to the protein and bridged to each other in an environment distinct from iron in the ferritin core (Tables I and II; Figures 1, 3, and 4). For example, Mössbauer analysis showed the iron in the Fe(III)–apoferritin complex was in a cluster of multiple iron atoms with magnetic properties similar to ferritin but with a lower blocking temperature (7 K) and $B(T = 0)$ (Table III), suggesting a smaller particle size and probably lower magnetic dimensionality. Quantitative analysis of the iron environment in the Fe(III)–apoferritin complex using the EXAFS showed the presence of an Fe–Fe interaction at 3.57 Å. Since the coordination number for the Fe atoms is much lower (N = 2–3) than for ferritin (N = 6), there are only a small number of Fe atoms at the sites in the Fe(III)–apoferritin complex. The results also confirmed the preliminary studies that showed a distinctive environment for Fe(III) in the complex, compared to ferritin (Sayers et al., 1983); in the complex, Fe(III) interacts with two shells of low Z atoms instead of the one low Z shell in ferritin. The two low Z shells are most likely contributed by carboxylate side chains of the protein, since the interatomic distances are in the range of those from crystallographic analysis of an Fe–carboxylate complex [see Sayers et al. (1983)] and because modification of apoferritin has shown that carboxylates are important for iron core formation (Wetz & Crichton, 1976). Since Fe(III) interacts both with protein ligands (probably carboxylate) and with iron neighbors, the oxo-bridged Fe(III) cluster is bound to the protein, as suggested by the results of competitive metal-binding studies (Chasteen & Theil, 1982).

Thus, the protein not only binds single iron atoms but also participates in forming the nuclei for the crystallites of the

[5] Note that the quadrupole splitting for the Fe(II)–apoferritin complex is temperature-independent, with a value of 3.05 mm/s at 180 K compared with 3.08 mm/s at 4.2 K. The only reported Mössbauer analysis of Fe(II) in ferritin is that of electrochemically reduced horse spleen ferritin (Watt et al., 1985) in which the quadrupole splitting is 2.88 mm/s at 100 K, distinct from the Fe(II)–apoferritin complex.

ferritin core. Where the Fe(III) clusters form on the protein is not known. There are not enough conserved, grouped carboxylate ligands on the outer surface of the protein to accommodate the Fe(III) clusters (Rice et al., 1983). However, a site on the protein with both sufficient numbers of conserved carboxylic groups, e.g., Glu-57, -60, and -63 (Wustefeld & Crichton, 1982; Addison et al., 1983; Boyd et al., 1985; Dorner et al., 1985; Didsbury et al., 1986), and flexibility (Rice et al., 1983) to accommodate the cluster and a groove to position and to orient core growth toward the hollow center of the protein is the inner surface of the subunit dimer interfaces. [The outer surfaces of the dimer interfaces have such closely packed amino acid side chains (Rice et al., 1983) that core growth toward the outside would be blocked.] Manipulation of conditions during the formation of the apoferritin–Fe(III) cluster will provide the opportunity to study the effects of apoferritin structure, phosphate, and cations such as zinc on the structure of the iron core and ultimately on iron storage and metabolism.

Acknowledgments

The assistance of Ann McKenzie in the purification of apoferritin and of Joann Fish in typing the manuscript is gratefully acknowledged.

Registry No. Iron, 7439-89-6; ferric ammonium citrate, 1185-57-5; ferric citrate, 28633-45-6.

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