Structural Variations in Soluble Iron Complexes of Models for Ferritin: An X-Ray Absorption and Mössbauer Spectroscopy Comparison of Horse Spleen Ferritin to Blutal (Iron-Chondroitin Sulfate) and Imferon (Iron-Dextran)

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ABSTRACT

Variations in the turnover of storage iron have been attributed to differences in apoferritin and in the cytoplasm but rarely to differences in the structure of the iron core (except size). To explore the idea that the iron environment in soluble iron complexes could vary, we compared horse spleen ferritin to pharmaceutically important model complexes of hydrous ferric oxide formed from $FeCl_3$ and dextran (Imferon) or chondroitin sulfate (Blutal), using x-ray absorption (EXAFS) and Mössbauer spectroscopy. The results show that the iron in the chondroitin sulfate complex was more ordered than in either horse spleen ferritin or the dextran complex (EXAFS), with two magnetic environments (Mössbauer), one (80%–85%) like $Fe_2O_3 \cdot nH_2O$ (ferritinlike) and one (15%–20%) like Fe_2O_3 (hematite); since sulfate promotes the formation of inorganic hematite, the sulfate in the chondroitin sulfate most likely nucleated Fe_2O_3 and hydroxyl/carboxyls, which are ligands common to chondroitin sulfate, ferritin and dextran most likely nucleated $Fe_2O_3 \cdot nH_2O$. Differences in the structure of the iron complexed with chondroitin sulfate or dextran coincide with altered rates of iron release in vivo and in vitro and provide the first example relating function to local iron structure. Differences might also occur among ferritins in vivo, depending on the apoferritin (variations in anion-binding sites) or the cytoplasm (anion concentration).

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INTRODUCTION

Iron is needed for DNA synthesis (ribonucleotide reductase), electron transport (cytochromes), oxygen activation (cytochrome P-450s), and oxygen transport (hemoglobin). However, iron is very insoluble at physiological conditions (10^{-18} *M*) [1]; the problem is overcome by storing iron in reservoirs such as ferritin. Since the turnover of stored iron varies from one cell type to another [2, 3], and since ferritin has cell-specific features of structure [3–6], the differences in turnover of stored iron could reflect the cell-specific features of the protein, the influence of cytoplasmic constituents, e.g., zinc [7], or properties of the stored iron itself.

In general, the structure of stored iron has been assumed to be constant, varying only in size [8, 9]. However, variations in the physiological availability of iron in soluble iron complexes formed with dextran (Imferon) or chondroitin sulfate (Blutal) [10] suggest that differences could occur in the structure of the iron in polysaccharide complexes as well as in ferritin. To determine if the local environment of iron cores can differ in ferritin and in the soluble complexes with dextran or with chondroitin sulfate, we used EXAFS (extended x-ray absorption fine structure) analysis and Mössbauer spectroscopy. The potential nucleation sites for iron polymerization differ for horse spleen ferritin (protein carboxylate), dextran (sugar hydroxyl), and chondroitin sulfate (sugar sulfate/hydroxyl/carboxylate). The results from Mössbauer spectra confirm earlier EXAFS data [20] on the similarity of the iron in horse spleen ferritin and in the iron-dextran complex, suggesting that protein carboxylate or sugar hydroxyl lead to very similar hydrous ferric oxide polymers, except for particle size. In contrast, when sugar sulfate/hydroxyl/carboxylate ligands are present, the average iron atom is in a more ordered environment, distributed among two types of structures, one like hematite, Fe_2O_3, and the other, $Fe_2O_3 \cdot nH_2O$. The data are the first, as far as we know, that demonstrate short-range structural differences related to the biopolymer present during the formation of soluble iron complexes; the structural differences coincide with different rates of reduction in vitro [29]. Such variations in structure and iron reduction in synthetic model complexes provide a possible explanation for cell-specific variations in ferritin protein and iron turnover in vivo [2, 3].

MATERIALS AND METHODS

Materials

Horse spleen ferritin was obtained from Miles Laboratories and used as is. Both the iron-dextran and iron-chondroitin sulfate complexes were prepared by adding $FeCl_3$ to solutions of the polysaccharide and neutralizing with NaOH, pH 7, and sterilizing by heating to 100°C (iron-chondroitin sulfate) or autoclaving (iron-dextran). Iron-dextran (Imferon) was the generous gift of Merrill-National Laboratories, Cincinnati, and consists of a sterile solution of the complex with 40 mg/ml of iron, 160 mg/ml Dextran C, pH ca. 7, and NaCl at 0.14 *M*. EXAFS experiments used the solutions as provided, diluted to 50 mM with water; Mössbauer and powder x-ray diffraction experiments were performed on lyophilized material. Iron-chondroitin sulfate (Blutal) was kindly donated by Dainippon Pharmaceutical Co. Ltd., Osaka, and consisted of 4 mg/ml of iron, 20 mg/ml chondroitin sulfate, pH 7–7.3, 0.14 *M* NaCl. Because of lower iron concentration in solutions of the iron-chondroitin sulfate complex and the need to lyophilize larger volumes, the samples were dialyzed against distilled water before

lyophilization for the x-ray diffraction study; no reduction in iron concentration was observed.

X-Ray Absorption Spectroscopy

Measurements were made at the Stanford Synchrotron Radiation Laboratory on beam line II-3, using a fluorescence detection system and Si 111 crystal as previously described [33]. Samples were measured at room temperature in solution. Examination of ferritin subunits using electrophoresis in denaturing gels (sodium dodecyl sulfate) shows no degradation as a result of exposure to x-rays. EXAFS analysis followed the methods used previously [20]; in particular, the contributions from more distant neighbors were analyzed by fitting of multiple shells because of the difficulty of accurately filtering the data for each individual shell.

Power X-Ray Diffraction

Powder diffraction patterns were obtained using Cu K_α radiation and a Diano XRD 700 diffractometer equipped with graphite diffracted beam monochromater.

Mössbauer Spectra

Absorbers contained ~ 10 mg Fe/cm^2, mixed with powdered sugar and pressed to uniform thickness against an aluminum foil. The absorbers were sealed with a benzene–styrofoam suspension, air-dried, and mounted in a closed-cycle cryostat which could maintain absorber temperatures down to 12 K within ~ 0.1 K. The source was ~ 10 mCi ^{57}Co(Rh) at room temperature (RT). Counting data were collected simultaneously with velocity calibration data from a laser interferometer. This system has been described in the literature [34].

Data were converted to counts versus velocity (432 points per spectrum), corrected for the small absorption due to iron in the aluminum foil and beryllium windows, and analyzed by least squares using the IBM 3081 of the Triangle Universities Computing Center. Two programs were used for data analysis. In the intermediate temperature range (20 K $< T <$ 100 K), the spectra were fitted to a sum of 1–3 magnetic sextets plus a central doublet. For spectra at RT (~ 295 K) and ≤ 20 K, a distribution program was used [35] which is a modification of the one reported by Wivel and Mørup [36]. This program fits the observed data to a broad distribution of quadrupole splittings and/or magnetic fields. For this program, the linewidth of each component was fixed at 0.35 mm/sec; the intensity ratios were fixed at 1:1 for the two peaks of a doublet or 3:2:1 for the outer, middle, and inner pair of peaks for a sextet. The isomer shift (IS) was allowed to vary. For the sextet distributions, the quadrupole splitting (QS) was also allowed to vary, where QS is 1/2 the difference in separation of the two highest minus the two lowest velocity peaks. The output of this program is a probability histogram for the distribution which best fits the experimental data. The histogram can be characterized by a mean value, a standard deviation, a field, H(max), or QS(max), corresponding to maximum probability, and upper, UHW, and lower, LHW, half-widths for the distribution. All individual lines in both fitting procedures were assumed to have Lorentzian line shape.

RESULTS

X-Ray Absorption Spectroscopy

Analysis of the extended x-ray absorption fine structure (EXAFS) can provide quantitative information about average interatomic distances, coordination number, the type of atom (high or low atomic number), and the relative degree of order of the atoms near the absorbing atom [19]. In the case of ferritin, iron-dextran, and iron-chondroitin sulfate, the absorbing atom is known to be Fe(III), the nearest neighbor (first shell) is O, the next neighbor (second shell) is Fe(III), and the next neighbor (third shell) is O.

Both the EXAFS (Fig. 1b, 1e) and the Fourier transform of the EXAFS portion of the spectra (Figs. 1c, 1f) show that the Fe(III)-chondroitin sulfate complex is distinct from horse spleen ferritin (previous EXAFS data showed the Fe(III)–dextran complex to be very similar to horse spleen ferritin [20]). In the Fourier transform, for example, the amplitude of the contribution of atoms in the layers of atoms second (Fe) and third (O) from the absorbing Fe atom is much larger for iron-chondroitin sulfate than for horse spleen ferritin. Such an observation suggests that the iron in the chondroitin

FIGURE 1. X-ray absorption spectra for horse spleen ferritin and iron-chondroitin sulfate. Top, horse spleen ferritin; bottom, iron-chondroitin sulfate. Left to right: x-ray absorption spectrum, normalized EXAFS, Fourier transform of the EXAFS. Note the extra "beat" at ca. 5.5 $Å^{-1}$ for the EXAFS and the greater amplitude of the Fe–Fe interaction (r = 2.9 Å, not corrected for the phase shift) in the Fourier transform of the iron-chondroitin sulfate complex, which indicate larger contributions from iron (more order) compared to horse spleen ferritin.

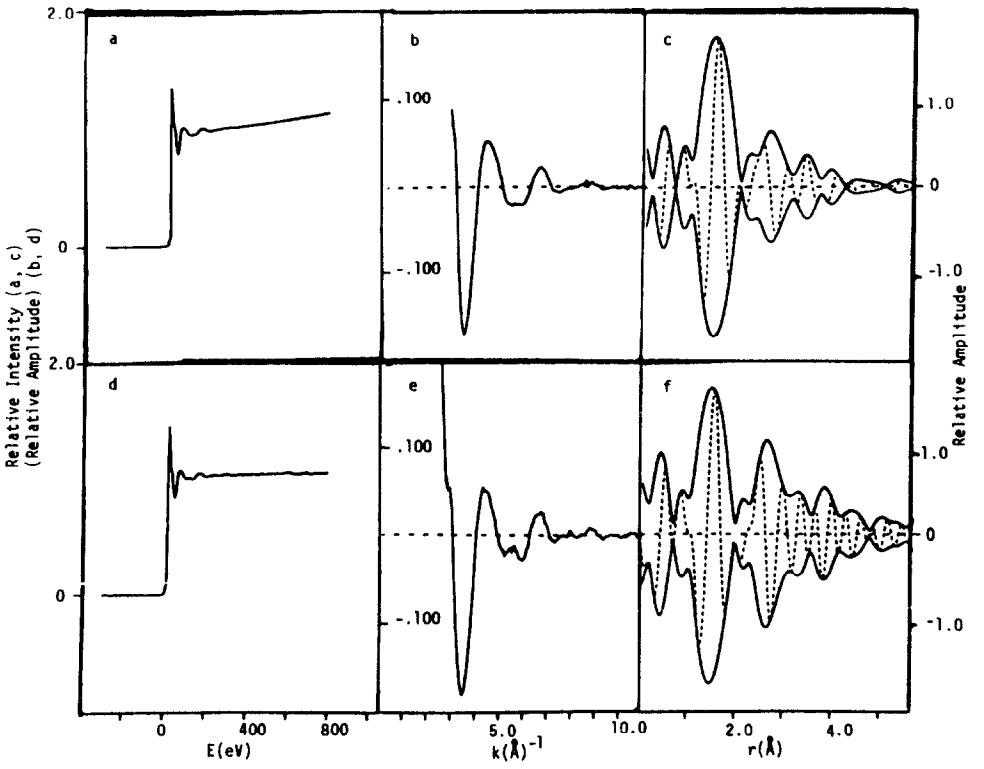


TABLE 1. EXAFS Analysis of Polynuclear $Fe_2O_3 \cdot nH_2O$: The Influence of Polymeric Complexing Agents on Synthetic Ferritin Cores[a]

Complex	N	r	$\Delta\sigma^2 \times 10^{+3}$
First shell (O, N?)			
Ferritin $(Fe_2O_3 \cdot nH_2O)$-protein	6.0	1.95	0
Imferon $(Fe_2O_3 \cdot nH_2O)$-dextran	6.0	1.95	0.20
Blutal $(Fe_2O_3 \cdot nH_2O)$-chondroitin sulfate	5.9	1.93	0.76
Second shell (Fe)			
Ferritin $(Fe_2O_3 \cdot nH_2O)$-protein	6.0	3.34	0
Imferon $(Fe_2O_3 \cdot nH_2O)$-dextran	6.0	3.34	−1.6
Blutal $(Fe_2O_3 \cdot nH_2O)$-chondroitin sulfate	6.0	3.34	−7.8

[a] Note the large negative Debye-Waller factor for iron in the chrondroitin sulfate complex indicating greater order.

sulfate complex is more ordered than in ferritin (or iron-dextran) and that the Debye-Waller factor [19] should be much smaller. The data in Table 1 support such a prediction. Using ferritin as a model to fit the data for the iron-chondroitin sulfate complex, parameters very similar to ferritin are obtained for the first shell of atoms. However, for Fe and O further away from the absorbing atom (the second and third shells), the iron core of the chondroitin sulfate complex is different from the iron core in horse spleen ferritin because the Fe-Fe interaction is more highly ordered (large negative Debye-Waller ($\Delta\sigma^2$, Table 1). The goodness of the fit is indicated by

TABLE 2. Mössbauer Data at Room Temperature (~295 K)[a]

Sample	IS[b] (mm/sec)	QS(mean) (mm/sec)	σ(QS) (mm/sec)	QS(max) (mm/sec)	LHW (mm/sec)	UHW (mm/sec)
Ferritin	0.36	0.64	0.22	0.62	0.21	0.25
Imferon[c]	0.36	0.72	0.26	0.61	0.24	0.32
Blutal	0.36	0.70	0.28	0.56	0.26	0.40
Hematite[d]	0.30	0.68	—	—	—	—
	0.32	0.98				

[a] The range of quadrupole splittings was 0.2–1.6 mm/sec in steps of 0.1 mm/sec. QS(mean) is the mean of the distribution, σ(QS) the standard deviation of the distribution, QS(max) the quadrupole splitting of maximum probability, and LHW, UHW the lower and upper half-widths of the distribution.

[b] Isomer shifts relative to metallic iron.

[c] The results for IS and QS confirm those observed by Marshall and Rutherford [40].

[d] Small particles (< 100 Å) showing only a doublet at RT for two different samples [26].

calculating the variance between the modeled and experimental data over the actual fitting range used (4.0–9.0); for all the fits the variance was between 1.63 and 3.45 $\times$ 10^{-5}. As the Mössbauer data will show (see Tables 2, 3 and Figs. 2–5), the iron-chondroitin sulfate complex contains both ferritin and hematitelike environments. However, the local structures of ferritin and hematite are sufficiently similar [20] that single shell models for iron and oxygen neighbors in the chondroitin sulfate complex fit the data well.

The possible presence of sulfur (from sulfate) was examined in the Fe(III)–chondroitin sulfate complex using Fe dithiodicarbamate as an Fe–S model; the Fe-dithiodicarbamate was synthesized according to the procedure of Leipoldt and Coppens [21]. No sulfur was detected in the Fe environment, suggesting that sulfate ligands involve less than 20% of the total iron atoms; such a situation could occur if sulfate were involved only at the nucleation site.

Mössbauer Analysis and Powder X-Ray Diffraction

Properties of the magnetic domains in ferritin and other polynuclear complexes can vary, depending, e.g., on hydration [22], the presence of phosphate [8], and, possibly in the case of ferritin, the protein shell itself. The protein appears to provide carboxylate ligands that serve as a nucleation site for formation of the iron core. In order to assess the effect of different types of nucleation sites on the superparamagnetic behavior of iron cores, three iron complexes were analyzed: ferritin from horse spleen, in which all the protein subunits are identical, iron-dextran, and iron-chondroitin sulfate.

At RT the spectra of all three samples consist of doublets (Fig. 2). However, the

TABLE 3. Mössbauer Data at Low Temperature[a]

Sample	IS[b] (mm/sec)	QS (mm/sec)	H(mean) (kOe)	$\sigma(H)$ (kOe)	H(max) (kOe)	LHW (kOe)	UHW (kOe)	RA (%)
T = 12 K:								
Ferritin	0.50	−0.08	434	100	489	34	26	92
Imferon	0.48	−0.07	428	88	475	41	31	97
Blutal	0.48	−0.01	449	67	484	37	28	80
	0.47	−0.14	527	—	—	—	—	20
T = 20 K								
Ferritin	0.49	0.00	409	111	479	34	28	80
Imferon	0.48	−0.08	373	116	466	51	35	87
Blutal	0.47	−0.04	398	106	477	54	33	77
	0.49	−0.18	527	—	—	—	—	19
Hematite[c]	—	−0.19	531	—	—	—	—	—

[a] Field range 70–520 kOe in steps of 10 kOe for Imferon, 70–530 for the other samples except 150–530 for Blutal at 12 K. A doublet component was also present in those spectra except for Blutal at 12 K. H(mean) is the mean of the distribution, $\sigma(H)$ the standard deviation of the distribution, H(max) the field of maximum probability, and LHW, UHW the lower and upper half-widths of the distribution. RA is the relative absorption due to that component.

[b] Isomer shifts relative to metallic iron at RT.

[c] Small particles (ca. 180 Å) supported on silica [26] at 4 K.

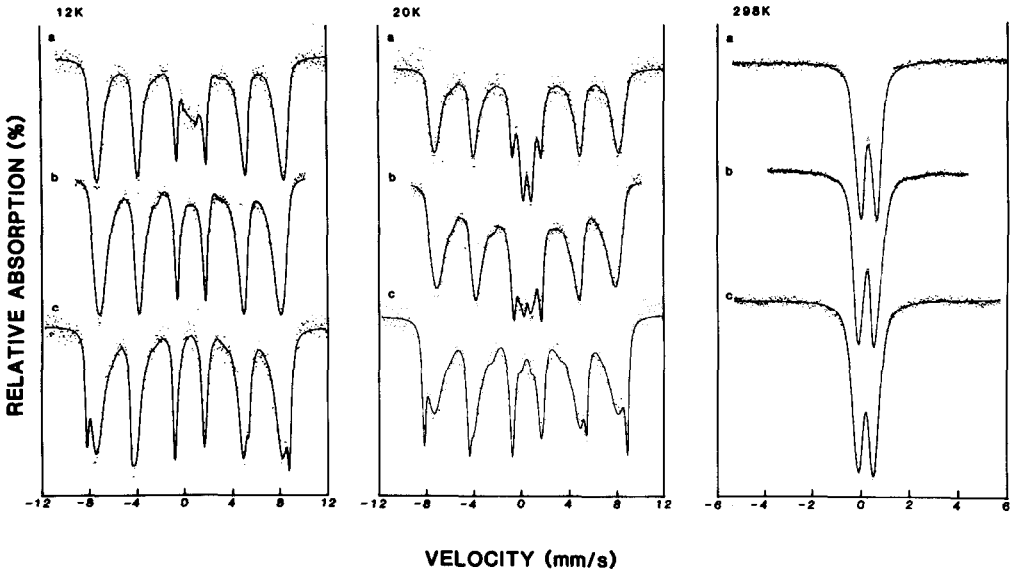


FIGURE 2. The effect of temperature on the Mössbauer spectra of iron cores in horse spleen ferritin and complexed with chondroitin sulfate or dextran. a, ferritin; b, iron-dextran; c, iron-chondroitin sulfate. Note the extra peaks at ca. ± 9 mm/sec in the iron-chondroitin sulfate complex.

spectra were much better fitted by a distribution of quadrupole splittings than by a single doublet; the distributions show identical isomer shifts for all the samples (Table 2) which correspond to high-spin Fe(III).

Significant differences occur in the quadrupole distribution of the three samples. For example, ferritin has the lowest mean value, QS (mean), smallest standard deviation σ(QS), and narrowest half-width, suggesting that the paramagnetic iron sites in ferritin are less variable than in the dextran and chondroitin sulfate complexes. The variation in QS for small hydrous oxides has been attributed to surface effects from small particle size in the related mineral ferrihydrite [23]. Since QS, σ(QS), and the half-widths of the distributions all vary for the dextran and chondroitin sulfate complexes compared to horse spleen ferritin, both surface effects and a greater variety of iron sites contribute to the Mössbauer spectra of the Fe(III)–polysaccharide complexes. Consideration of the relatively small value of the quadrupole splitting of maximum probability QS(max) with the apparent variety in the types of iron sites of the Fe(III)–chondroitin sulfate complex suggests that the complex has an important site with a more symmetrical geometry of oxygen neighbors than the other samples.

In order to explore in more detail the unique features of the iron environment in the chondroitin sulfate complex, Mössbauer spectra were obtained at low temperature where magnetic ordering in polynuclear iron complexes converts the doublet spectrum to a sextet with broadened lines, asymmetric because of a distribution of particle sizes [8].

Horse spleen ferritin was used as a standard since it has been previously characterized [8, 38]. The data for Figure 2 are presented in Table 3 and, for horse spleen ferritin, agree with the results of Williams et al. [8]. For example, in ferritin the superparamagnetic blocking temperature, T_B (defined as the temperature where 50%

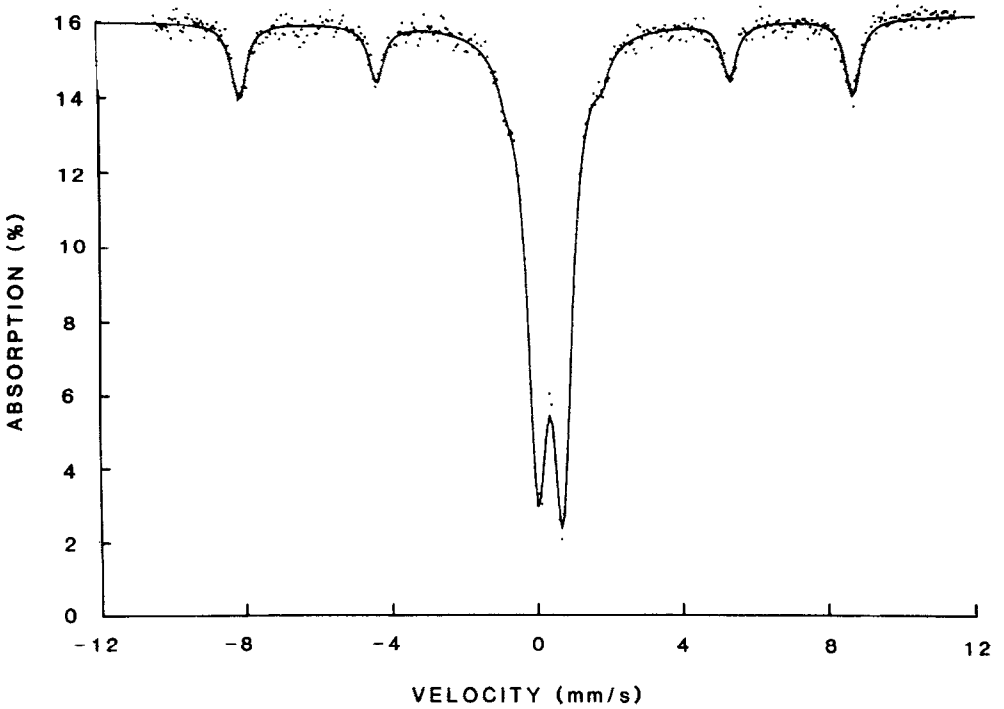
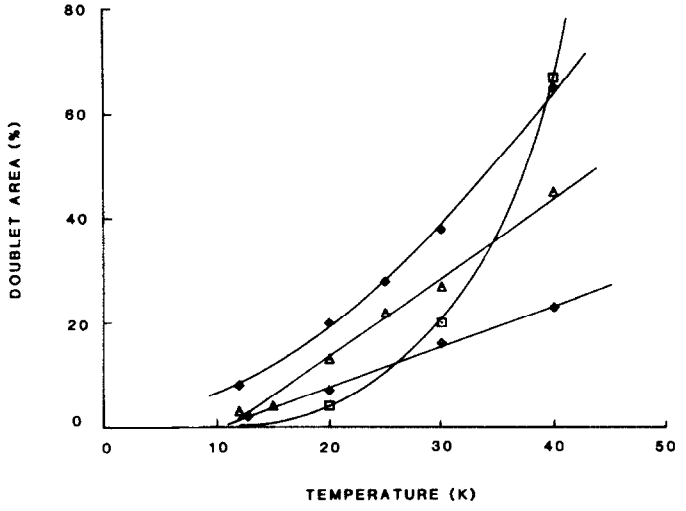


FIGURE 3. The Mössbauer spectrum of iron-chondroitin sulfate at 80 K. Note the presence of the components at ca. ±9 and 5 mm/sec; the spectra for horse spleen ferritin and iron-dextran contain only a doublet at 80 K.

FIGURE 4. Relative absorption of doublet component as a function of temperature. Ferritin (♦), iron-dextran (△), iron chondroitin sulfate (□), *d*-glucose-iron complex (◇) (data from Ref. 37). Note the much sharper rise in the doublet for iron-chondroitin sulfate over a narrower temperature range.



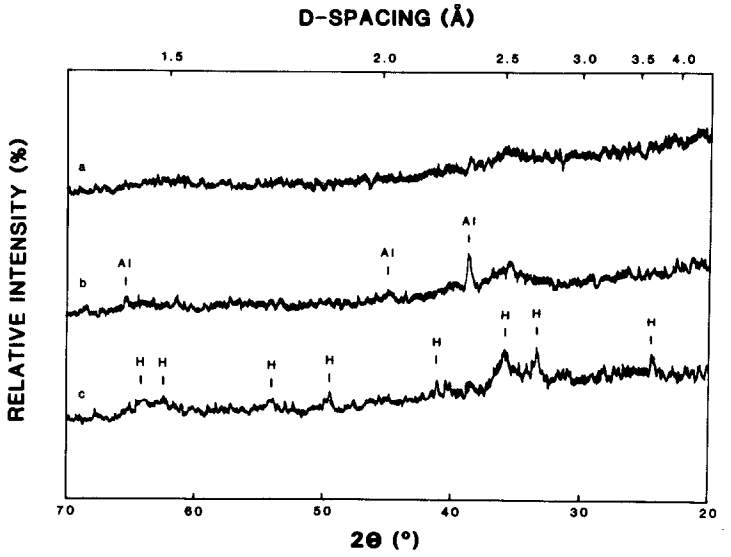


FIGURE 5. Powder x-ray diffraction patterns. a, horse spleen ferritin; b, iron-dextran; c, iron-chondroitin sulfate. Al refers to the line of aluminum contributed by the sample holder used for the iron-dextran sample. H refers to the *d*-spacing for hematite (Fe_2O_3).

of the absorption is due to the doublet), is 35 K with a range of temperature of at least 10–50 K where the doublet and sextet forms coexist (Fig. 4). In addition, the magnetic field of maximum probability at 12 K is H(max) = 489 kOe, but the broad distribution places the mean value at 434 kOe. The iron–dextran complex and iron in horse spleen ferritin are very similar, except for the small differences in H(mean), H(max), and other parameters listed in Table 3. The results indicate that the major difference between horse spleen ferritin and the iron–dextran complex is the particle size distribution. Such data combined with the larger doublet area for ferritin at low temperatures (Fig. 4) show that core sizes in horse spleen ferritin tend to be large or small, whereas for iron-dextran distribution of core sizes is more uniform.[1]

Note that previous EXAFS studies also indicated a great similarity between horse spleen ferritin and iron–dextran [20]. By electron diffraction, in contrast [24], Fe(III) dextran was more similar to β-FeOOH than to ferritin, although by Mössbauer analysis β-FeOOH is distinct from Fe(III)–dextran [25]. The major difference between electron diffraction and Mössbauer spectroscopy or EXAFS is that in electron diffraction the contributions of those atoms in ordered structures is weighted more heavily in the analysis, whereas EXAFS and Mössbauer spectroscopy weigh the contribution of all the iron atoms more equally. Thus differences in the results of the several techniques most likely reside in the relative weight in the several analyses given to the contribution of atoms in ordered and disordered regions.

The distinctly ordered iron environment detected in the RT Mössbauer spectrum of the Fe(III)–chondroitin sulfate complex, and indicated in the negative Debye-Waller factor in the EXAFS analysis, was dramatically confirmed at low temperatures (Figs.

[1] The iron in a soluble complex of hydrous ferric oxide and glucose (hydrolyzed starch from maize) is also similar to ferritin and iron-dextran, except for a somewhat larger particle size [37].

2 and 3). For example, chondroitin sulfate at 12–20 K shows the presence of a second iron component which has sharper lines and a higher magnetic field. This component comprises 20% of the absorption (Table 3), has $H = 527$ kOe, QS $= -0.15$ mm/sec at 12–20K, and persists up to $T > 80$ K (Fig. 3). The value of H is larger than that for any of the ferric oxides or oxyhydroxides, except hematite [22]. Hematite generally has fields somewhat higher than this (540 kOe), and a magnetic ordering temperature higher than RT, although small (<200 Å) particles of Fe_2O_3 (hematite) have been reported to have comparable fields at low temperature and only a doublet at RT (Tables 2 and 3) [26]. It appears that part of the iron core in chondroitin sulfate has a strongly coupled magnetic interaction similar to hematite, while part (80%) is similar to ferritin in having a broad distribution with H(max) ~ 480–490 kOe at 12 K. The latter component disappears at about the same blocking temperature as ferritin, $T_B =$ 38 K, but has a narrower range of coexistence between doublet and sextet than either ferritin or the other complexes (Fig. 4). Thus, iron–chondroitin sulfate consists of iron in two environments, both of rather uniform size distribution. The RT Mössbauer and EXAFS spectra are the average of the two domains, which, in the case of the Mössbauer data, accounts for the broad QS distribution (Table 2). The presence of a hematitelike component of the iron–chondroitin sulfate was confirmed by x-ray diffraction (Fig. 5). In concert with the apparent absence of sulfur in the iron–chondroitin sulfate complex (less than 20%), the Mössbauer and x-ray diffraction data suggest that the effect of sulfate on the iron is not direct but is more likely exerted by nucleation of a distinctive environment. The results support the notion that the structure of polynuclear iron complexes can vary, depending on the nucleation conditions.

DISCUSSION

Variations in storage iron turnover are known to occur among different cell types [2, 3] and for the same cell type under different physiological conditions [5]. The differences appear to be matched to changes in iron utilization, e.g., during normal development [3] or metabolic derangement in abnormal conditions [5]. Variations in particle size of the stored iron [9, 28] and/or the structure of the ferritin protein [4, 5, 27] have been most often considered as the cause of difference in iron turnover. However, when complexes of iron with dextran or chondroitin sulfate[2] are used as therapeutic iron supplements [10], the utilization of iron also varies, suggesting structural differences in the iron environment itself. Moreover, differences in the initial rates of reduction of Fe(III) to Fe(II) for the dextran and chondroitin sulfate complexes have been observed in vitro [29], supporting the idea that the structure of the polymeric iron is different.

The combined results of EXAFS analysis and Mössbauer spectroscopy clearly show (Tables 1–3, Figs. 1–5) that the local structure of iron in the soluble complexes depends on the biopolymer present. In the comparison of iron–chondroitin sulfate to horse spleen ferritin and iron–dextran, the presence of sulfate, known to form stable interactions with Fe(III) [30], and possibly a sugar hydroxyl or carboxyl, provides the site for the formation of the hematitelike (Fe_2O_3) structure; in a purely inorganic

[2] Chondroitin sulfate is composed of variable amounts of 4- and 6-sulfated GlcUA–GalNAc dissacharide units and is found in the extracellular matrix, particularly in tendon and cartilage [32].

system [30], sulfate has been shown to promote the formation of Fe_2O_3 compared to $Fe_2O_3 \cdot nH_2O$ (hydrous ferric oxide). No Fe–S interactions were detected in the iron–chondroitin sulfate complex, suggesting that sulfate is important in nucleating the hematitelike environment and need not coordinate to every iron atom to have an effect. Since the average iron atom in the complex with chondroitin sulfate is in a more ordered environment (Table 1), the hematitelike environment appears to influence the local environment of most of the iron atoms. Such a conclusion is supported by the 50% reduction of the initial rate of conversion of Fe(III) to Fe(II) in the chondroitin sulfate complex compared to the iron–dextran complex [29], even though only a small fraction of the Fe(III) was in the magnetically distinct environment.

The presence of Fe_2O_3 in a soluble complex has not been observed before, to our knowledge; its occurrence indicates the wide range of hydration possible in ferric oxides formed under physiological conditions. Variations in the physiological conditions during formation of storage iron, e.g., anion concentrations or apoferritin structure (anion binding), could lead to different ferritin iron cores in much the way chondroitin sulfate produced a different iron core than dextran. Thus the complete ferritin molecule would reflect variations both in the protein and the cytoplasm; the final product could be a core structure matched (normal) or disparate (pathological) to the iron needs of the cell or the organism. Recent observations support such ideas. For example, during the course of this investigation a description of the presence of varying amounts of phosphate in ferritin iron cores has been reported in bacteria and human spleens of patients with β-thalassemia [41]. In addition, studies of iron-loaded livers from patients with β-thalassemia demonstrated the presence of iron cores with unusual magnetic properties [39]. Although the presence of hematitelike environments in iron cores formed in vivo has not been reported, such complexes could form during arthritis when large amounts of iron accumulate in the joints [31] in concert with abnormal connective tissue.

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