planation for the variability, particularly in proteins which have the same tyrosinate ligands, is that the C-O bond strength is influenced by the environment of the metal phenolate moiety. An example of such behavior is observed for the Fe(EHGS)(H_2O) complex, where ν(C-O) occurs at 1286 cm^{-1} in the solid material and at 1300 cm^{-1} in aqueous solution with 20% DMSO.

Potential Fe-O stretching modes have been detected in the RR spectra of a number of iron tyrosinate proteins. These include the 575-cm^{-1} vibration in purple acid phosphatase[17] as well as peaks at 578, 588, and 603 cm^{-1} in three different mutant hemoglobins with axial tyrosinate ligands.[24] Nevertheless, in a number of other iron tyrosinate proteins such as transferrin,[25] protocatechuate 3,4-dioxygenase,[26] and catalase,[16,27] there is no peak in the 600-cm^{-1} region of intensity comparable to that of the 1100-1600-cm^{-1} phenolate modes. The inability to detect a ν(Fe-O) mode in these proteins may be indicative of a fairly long Fe-O(tyrosinate) bond. This possibility is supported by the report of a 2.2-Å Fe-O bond length in *Penicillium vitale* catalase, based on the crystal structure at 2.0-Å resolution.[28]

Conversion of ferric phenolates from high-spin to low-spin states is generally accompanied by a decrease in Fe-O bond distance.[19] Thus, the low-spin complexes examined in this study had Fe-O frequencies of 622-628 cm^{-1} (Table I), and their ν(Fe-O) modes were 3.4-4.5-fold more intense than the ~1600-cm^{-1} phenolate ν(C=C) mode (Table II). A further distinguishing characteristic of the low-spin ferric phenolates is the marked red shift of the Tyr → Fe(III) CT band (Table II) and the selective enhancement of the phenolate vibrational modes upon excitation within the ~650-nm absorption band. Thus, RR spectroscopy could provide a useful method for identifying low-spin ferric phenolate sites in proteins.

The enzyme nitrile hydratase contains a low-spin Fe(III) site.[8b] Its EPR parameters are well matched by Fe^{III} salen complexes with axial thiolate or imidazolate ligands.[8a] This finding, along with the 712-nm absorption maximum (ϵ_M = 1400 per Fe) in the enzyme, suggests the additional presence of phenolate ligands. Unfortunately, it has not yet been possible to obtain an RR spectrum of nitrile hydratase due to the large fluorescence of the protein.[29] We also observed markedly increased fluorescence for the low-spin $[Fe(EHGS)(CN)_3]^{3-}$ complex but not for the low-spin $[Fe((sal)_4trien)](PF_6)$ complex. In addition, the low-spin tricyano form of transferrin[4] has been found to be considerably more fluorescent than the native high-spin protein.[30] Thus, excessive fluorescence may interfere with the ability to detect phenolate vibrational modes in low-spin iron proteins by resonance Raman spectroscopy.

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Biomineralization of Iron: Mössbauer Spectroscopy and Electron Microscopy of Ferritin Cores from the Chiton *Acanthopleura hirtosa* and the Limpet *Patella laticostata*

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Ferritins isolated from the hemolymph of the chiton *Acanthopleura hirtosa* and the limpet *Patella laticostata* have been studied by ^{57}Fe Mössbauer spectroscopy, electron diffraction, transmission electron microscopy (TEM), and inductively coupled plasma (ICP) spectrometry. Mössbauer spectra of the samples at 78 K were quadrupole-split doublets with similar quadrupole splittings and chemical isomer shifts, characteristic of octahedral high-spin iron(III), while at 4.2 K the spectra were magnetically split sextets. The spectra yield mean superparamagnetic blocking temperatures of about 32 and 30 K for the *A. hirtosa* and *P. laticostata* ferritins, respectively, and indicate magnetic ordering temperatures of about 37 and 34 K. Core size distributions were measured for both the *A. hirtosa* and *P. laticostata* ferritins by using TEM and gave mean core size ranges of 8.0-8.5 and 7.5-8.0 nm, respectively. Diffuse lines in the electron diffraction patterns of the ferritin cores indicated the presence of ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$) of limited crystallinity. Phosphorus to iron atomic ratios were measured by ICP spectrometry. The phosphorus levels were close to the limit of detection, giving approximate mean values of P:Fe of 1:44 for the *P. laticostata* ferritin and 1:36 for the *A. hirtosa* ferritin. These levels of phosphate are significantly less than those for the crystalline cores of mammalian ferritins and considerably less than those for the noncrystalline core of bacterioferritins.

Introduction

Chitons and limpets are marine invertebrates that incorporate iron minerals into composite materials that make up the hard structural components of the teeth on their radulas.[2,3] The radulas are used to scrape algae from the rocks in the intertidal regions and, in the process, are continuously worn away, thus necessitating the continual production of iron minerals to replace those lost with the discarded teeth. It has been suggested that this need for a high turnover of iron is related to the exceptionally high concentration (approximately 400 $\mu g \cdot mL^{-1}$) of ferritin found in the

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hemolymph of the chiton *Acanthopleura hirtosa*,[4,5] previously named *Clavarizona hirtosa*.[6]

Ferritin is a protein that is widespread in Nature, including bacteria, plants, and animals, and its function is usually associated with iron storage. It consists of an approximately spherical polypeptide shell composed of subunits. In the case of horse spleen ferritin, whose X-ray structure has been reported,[7] the shell has channels which allow certain ions to pass into and out of the central cavity. The cavity, which has a diameter of about 8.0 nm,[7] holds an iron-containing mineral core. For horse spleen ferritin, the core is composed of the mineral ferrihydrite, $5Fe_2O_3{\cdot}9H_2O$,[7,8] with some phosphorus, present as phosphate, to give an analytical formula of $(FeOOH)_8(FeO{\cdot}OPO_3H_2)$.[9]

The mineral cores of ferritin are well within the size range required for superparamagnetic phenomena. Mammalian ferritins such as those isolated from thalassemic human spleen[10,11] and horse spleen[12-14] exhibit superparamagnetic behavior in their Mössbauer spectra. However, some bacterial ferritins such as those isolated from *Pseudomonas aeruginosa*[11] and *Escherichia coli*[15] have a magnetic ordering transition at a very low temperature (about 3 K) so that above this temperature their spectra consist of a quadrupole-split doublet only. This eliminates the possibility of observing superparamagnetic phenomena in the Mössbauer spectra. Ferritin from the bacterium *Azotobacter vinelandii*, however, does exhibit superparamagnetism.[16] The ferritin isolated from the hemolymph of the limpet *Patella vulgata* was found to give Mössbauer spectra that showed characteristics of both a superparamagnetic transition and a transition from a magnetically ordered to a paramagnetic state.[11,17]

In this paper we describe the magnetic properties, crystallographic structure, and the composition of the iron cores of two ferritins from marine invertebrates using ^{57}Fe Mössbauer spectroscopy, electron diffraction, and transmission electron microscopy (TEM). These properties are important factors in the rapid transport of iron required in the extensive mineralization of the radula teeth. One of these ferritins (isolated from the hemolymph of the chiton *Acanthopleura hirtosa*) has been shown to take up iron almost 10 times more rapidly than does horse spleen ferritin.[4]

Materials and Methods

Ferritin was isolated from the hemolymph of specimens of the chiton *A. hirtosa* and the limpet *P. laticostata* as described elsewhere.[4,18]

^{57}Fe Mössbauer spectroscopy was carried out with a ^{57}Co in rhodium source and a conventional constant-acceleration spectrometer. The spectra of the samples at temperatures above 4.2 K were obtained with a continuous-flow cryostat (Oxford Instruments), calibrated at 4.2 and 77 K. The spectra of the samples at 4.2 K and below were obtained with a pumped liquid helium bath cryostat. The spectrometers were calibrated by recording the Mössbauer spectrum of a thin α-Fe foil at room temperature. The positions of the lines were taken to be those reported by the U.S. National Bureau of Standards.[19] The center of the Fe-foil spectrum was taken to be zero velocity by convention.

Table I. Quadrupole Splitting at 78 K (or Quadrupole Perturbation on Magnetic Splitting at 4.2 K), Δ_Q, Chemical Isomer Shift, δ, and the Magnetic Hyperfine Field, B_{hf}, from the Mössbauer Spectra of the Hemolymph Ferritins from *A. hirtosa* and *P. laticostata* at 78 and 4.2 K

	A. hirtosa			*P. laticostata*		
T, K	Δ_Q, mm/s	δ, mm/s	B_{hf}, T	Δ_Q, mm/s	δ, mm/s	B_{hf}, T
78	0.78	0.47		0.78	0.47	
4.2	−0.06	0.48	47.9	−0.02	0.47	47.8

The Mössbauer spectra were least-squares computer-fitted with a combination of quadrupole-split doublet and magnetically split sextet components. For spectra showing superparamagnetic behavior, one doublet and one sextet component were used, with the latter having the line widths and intensities of the various pairs of lines constrained to simulate a distribution of magnetic hyperfine fields and a random distribution of crystallite orientations. For spectra showing the transition from a magnetically ordered to a paramagnetic state, the magnetically split sextet component was fitted with three separate doublets in order to reflect the different relaxation behavior appropriate to the outer, middle, and inner pairs of lines of the magnetically split sextet as the transition is approached.[20]

For electron microscopy the ferritins were stained with phosphotungstic acid (2%, pH 6.0) for 5 min and were air-dried onto Formvar-coated copper electron microscope grids. Micrographs were obtained with a Philips 301 transmission electron microscope operating at 80 keV. Unstained samples were used for electron diffraction on a JEOL CX-100 microscope. Elemental analyses for iron and phosphorus were carried out by using inductively coupled plasma (ICP) spectrophotometry.

Interpretation of Spectral Data

The ^{57}Fe Mössbauer spectrum of a material as the temperature is raised from below to above its magnetic ordering temperature consists of a sextet, the splitting of which decreases as the temperature is raised until the spectrum becomes a doublet at the *magnetic ordering temperature*. During the collapse of the sextet, broadening of the lines is often observed. The *magnetic ordering temperature*, T_{ord}, of the material can be determined by the temperature at which the spectrum becomes a doublet. At temperatures above T_{ord}, the material is paramagnetic.

A superparamagnetic material consists of small particles (typically less than 20 nm), each of which is magnetically ordered such that the atomic magnetic moments are all aligned within the particle. However, this system of aligned moments may fluctuate in direction with respect to the crystal axes. This phenomenon is known as superparamagnetic relaxation. If the direction of the spin system is represented as a point on a sphere, then the superparamagnetic relaxation of the spin system can be represented by a random walk of the point on the sphere. The rate of fluctuation increases with temperature.

The Mössbauer spectrum of a slow-relaxing system is a sextet, while that of a fast-relaxing system is a doublet. The temperature at which the spectrum rapidly collapses from a sextet to a doublet for a superparamagnetic material is the spectral superparamagnetic transition temperature often termed *the blocking temperature*, T_B. This temperature is different for different particle sizes. In all real samples there is a particle size distribution that leads to a distribution of blocking temperatures. Thus a superparamagnetic material spectrum often consists of a superposition of a sextet and a doublet corresponding to the slow- (large) and fast- (small) relaxing particles, respectively. The fraction of particles that have a blocking temperature at the ambient temperature is small. So the spectrum of a superparamagnetic material at low temperatures is a sextet. As the temperature is raised, a central

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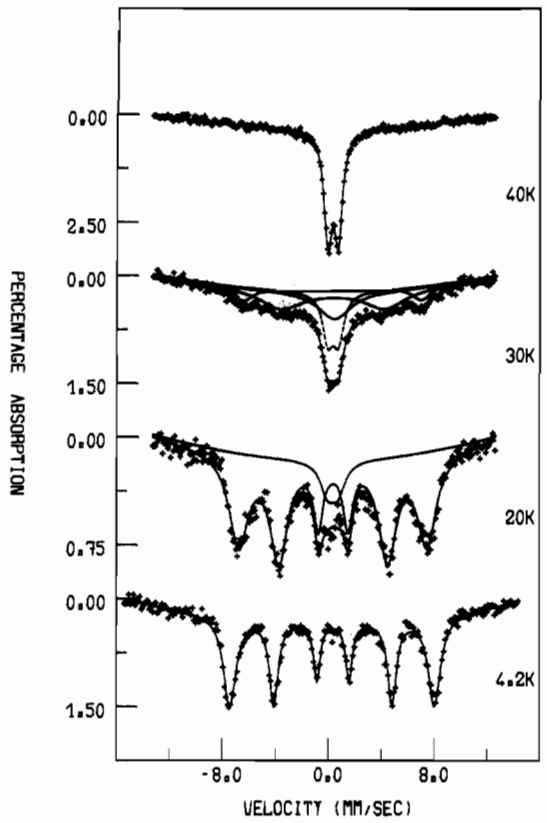

Figure 1. A selection of Mössbauer spectra of the ferritin isolated from the hemolymph of the chiton *A. hirtosa* at temperatures between 4.2 and 40 K illustrating the variation with temperature.

doublet appears and grows in intensity at the expense of the sextet. The sextet disappears in the wings of the spectrum. The mean blocking temperature of the sample is determined by the temperature at which the doublet and sextet are of equal spectral area. Thus the Mössbauer spectrum of a superparamagnetic material may consist of a doublet even though there is still magnetic order within the particles; i.e., the blocking temperature is less than the magnetic ordering temperature.

All the Mössbauer spectra shown in this paper have a parabolic background, which is a standard instrumental effect[21] caused by the variation in the solid angle between the source and the detector window as the source scans through the velocities.

Results

At temperatures of 40 K and above, the Mössbauer spectra of the ferritins from both *A. hirtosa* and *P. laticostata* are quadrupole-split doublets with broad lines (Figures 1 and 2). The spectral parameters of the samples at 78 K, which are given in Table I, indicate that the oxidation state of the iron is Fe(III) and that the iron is high-spin, octahedrally coordinated. At temperatures of 4.2 K and below, the Mössbauer spectra of the two samples are magnetically split sextets with broad lines (Figures 1 and 2). As with the 78 K data, the spectral parameters of the samples at 4.2 K are very similar for the two ferritins (Table I).

As the temperature is raised above 4.2 K, there is a coexistence of a central spectral component due to fast-relaxing particles and a sextet spectral component due to the slow-relaxing particles. The spectral area of the central component grows at the expense of the sextet with increasing temperature, which is characteristic of a superparamagnetic material. However, as the temperature is increased further, there is a considerable decrease in the magnetic hyperfine field splitting of the sextet and an increase in the line widths of the sextet, significantly more than is seen in the spectra of thalassemic human spleen[10,11] and horse spleen ferritins.[12-14] The 30 K spectrum of *P. laticostata* and the 30 and 35 K spectra of *A. hirtosa* show that the sextet is collapsing into the central doublet rather than disappearing in the wings of the spectrum as the temperature is raised. Also, the central component is sharpening up into a doublet at these temperatures. Both these effects are more characteristic of the Mössbauer spectra of a solid passing from its magnetically ordered phase to a paramagnetic phase.[11,22]

Table II. Electron Diffraction Pattern Data (*d* Spacings in Å) of the Hemolymph Ferritins from *A. hirtosa* and *P. laticostata*[a]

A. hirtosa ferritin	*P. laticostata* ferritin	ferrihydrite
	2.55	2.52
2.39		
2.25	2.25	2.25
		1.97
		1.72
1.46	1.48	1.48

[a] Five diffraction maxima characteristic of ferrihydrite are included for comparison[7].

Table III. Phosphorus to Iron Atomic Ratios for Ferritins Measured by Inductively Coupled Plasma Spectrometry

source of ferritin	P:Fe atomic ratio		no. of determinations
	mean	range	
limpet hemolymph (*P. laticostata*)	1:44	1:41–1:47	2
chiton hemolymph (*A. hirtosa*)	1:36	1:24–1:58	3
horse spleen	1:13	1:8–1:16	3

The mean blocking temperatures, $\langle T_B \rangle$, of the samples were determined from the plot of percentage doublet spectral area versus temperature (spectra were recorded at 5 K intervals). $\langle T_B \rangle$ is taken to be the temperature at which the doublet is 50% of the spectral area. The magnetic ordering temperatures, T_{ord}, were determined from the temperature at which the spectrum became a quadrupole-split doublet. The accuracy of this determination was limited by the 5 K temperature intervals at which the spectra were recorded. The data indicate $\langle T_B \rangle$ of 30 ± 1.5 and 32 ± 1.5 K and T_{ord} of $34 \pm \frac{2}{3}$ and $37 \pm \frac{3}{2}$ K for the ferritins from *P. laticostata* and *A. hirtosa*, respectively. The error notation for the values of T_{ord} indicates different uncertainties above and below the quoted values due to the spectra being recorded at discrete temperature intervals.

Electron micrographs of the hemolymph ferritins extracted from the two species are shown in Figure 3. The electron dense cores of the ferritin molecules are imaged in the micrographs. The particle size distribution is shown in Figure 4. The mean core size range of the chiton hemolymph ferritin was 8.0–8.5 nm, which was larger than that of the limpet hemolymph ferritin (7.5–8.0 nm). Both of these invertebrate ferritin cores are appreciably larger than that of thalassemic human spleen ferritin, which has a mean core size range of 5.5–6.0 nm.[23]

The crystallinity of the cores was determined by electron diffraction (Table II). The ferritin cores from both the chiton and the limpet gave patterns that showed limited crystallinity, with diffuse diffraction lines having *d* spacings corresponding to those of the mineral ferrihydrite.[7,8]

Inductively coupled plasma spectrometry was used to measure the phosphorus to iron atomic ratios in the limpet and chiton ferritins. Measurements were also made on a sample of commercial horse spleen ferritin (Sigma) for comparison. The results are given in Table III. The phosphate levels for the molluscan ferritins are very low. The values are significantly lower than values reported for mammalian ferritins, which have P:Fe ratios between 1:4.5 and 1:21[23-25] and are considerably lower than values

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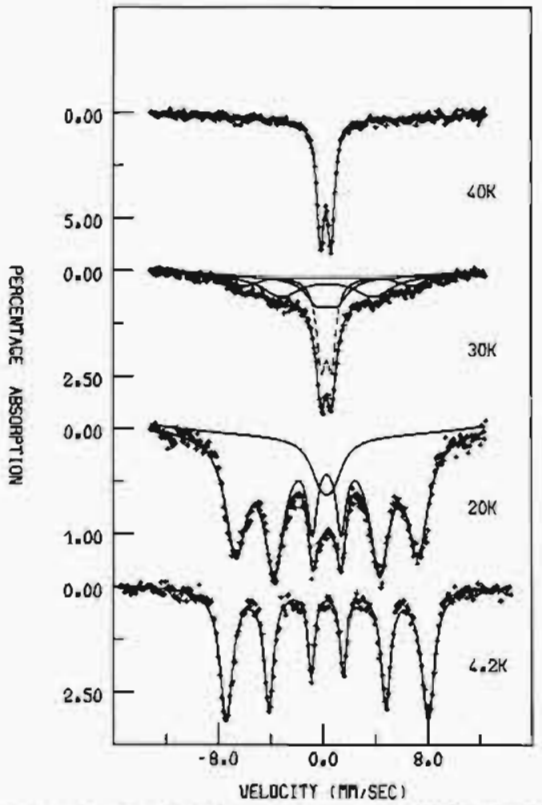

Figure 2. A selection of Mössbauer spectra of the ferritin isolated from the hemolymph of the limpet *P. laticostata* at temperatures between 4.2 and 40 K illustrating the variation with temperature.

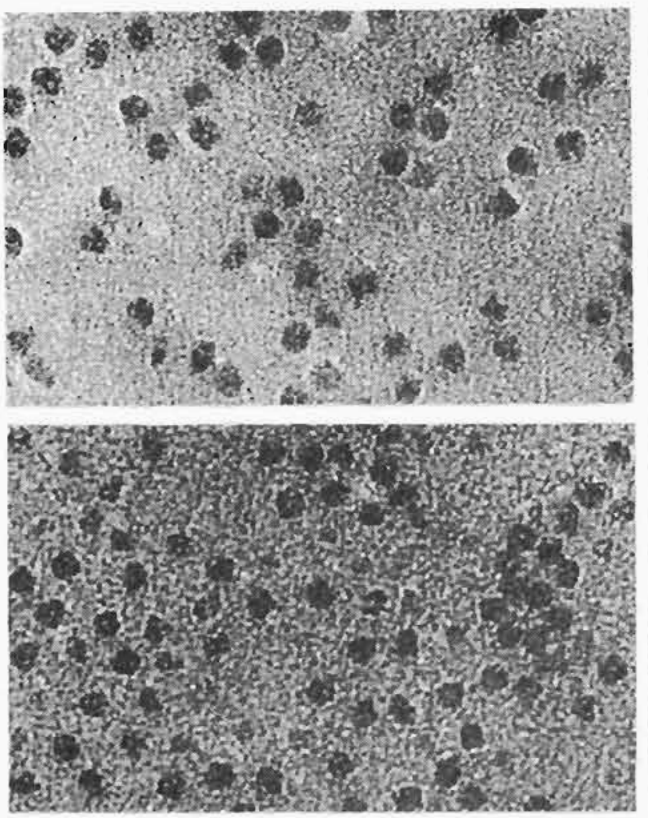

Figure 3. Electron micrographs of unstained ferritin molecules (×483 500) from (top) *A. hirtosa* and (bottom) *P. laticostata*.

reported for bacterioferritins (1:1.4 and 1:1.7).[26,23]

Discussion

The iron content of the hemolymph ferritins from *A. hirtosa* and *P. laticostata* are 1500–2500 Fe atoms per molecule and ca. 2000 Fe atoms per molecule, respectively.[4,18] These values are appreciably less than that of approximately 3000 Fe atoms per molecule reported for horse spleen ferritin,[7,27] but rather more than the approximate 1000 Fe atoms per molecule reported for bacterial ferritins.[28]

These data in conjunction with the electron microscopy, electron diffraction, and ICP results indicate that the molecular structure of these ferritin cores would need to accommodate, in a volume that is slightly larger than that of horse spleen ferritin, somewhat fewer Fe atoms and many fewer P groups. This suggests that the core structures are relatively low in density and probably comprise localized domains of partially ordered ferrihydrite together with more disordered regions.

This structural model is supported by the Mössbauer spectral data. The broad spectral lines suggest some variation in the iron site environments. The mean blocking temperatures for these invertebrate ferritins are lower than those for horse and human spleen ferritins. This is consistent with the poorer crystallinity as well as with the lower number of Fe atoms in the core, giving overall a lower magnetic anisotropy field. The open structure and poor crystallinity result in reduced superexchange interactions reflected in the low magnetic ordering temperature indicated by the spectra. A higher phosphate content may be expected to lower this ordering temperature even further. The bacterioferritin isolated from *P. aeruginosa*[11,29] has a very high phosphate:iron ratio of 1:1.7 and a magnetic ordering temperature of less than 4 K.

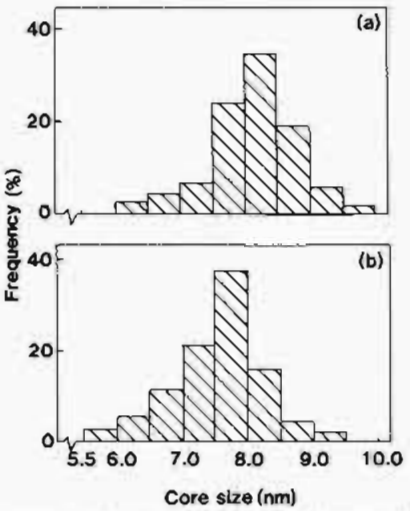

Figure 4. Core size distribution of the ferritins from (a) *A. hirtosa* and (b) *P. laticostata*. Each distribution was obtained by measuring the largest diameter of more than 200 cores.

It is recognized that phosphate plays an important role in iron biomineralization.[30] Previous studies of ferritins and bacterioferritins have indicated that the presence of high levels of phosphate in the cores, such as those found in the bacterial ferritins from *P. aeruginosa*[23] and *A. vinelandii*,[31] are associated with noncrystalline core structure. Other iron biomineral systems with high phosphate levels also appear to be noncrystalline.[32-35] Small

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numbers of PO_4^{3-} ions in an iron oxyhydroxide crystal may be expected to disrupt the regularity of the lattice due to the larger dimensions of the PO_4^{3-} ion relative to O^{2-} and OH^- ions. However, the phosphate content in these materials is of relative proportions high enough for the compounds to be described as hydrated ferric phosphates, and thus they have the potential for a different lattice structure. This indicates that some other mechanism besides the ionic dimensions of PO_4^{3-} may be responsible for their disordered structure.

The two invertebrate proteins in the present study have extremely low inorganic phosphate contents, and yet the electron diffraction indicates only limited crystallinity compared to the cores of human thalassemic spleen[23] and horse spleen ferritins.[8,36,37] These latter ferritins have higher levels of phosphate associated with their cores, although not the very high levels found in the bacterioferritins. It appears, therefore, that for cores with lower levels of phosphate there is not a direct correlation between phosphate content and crystallinity. It is only at higher levels of phosphate that noncrystallinity is ensured. Other factors that determine core structure include the molecular nature of the inner surface of the protein shell, which may influence the rate of Fe(II) oxidation, nucleation and growth of the core,[28] and the rate of ion diffusion and incorporation into the core. Slower rates would give a more crystalline core. The rate of formation of the core may be related to the turnover of iron in the organism. Limpets and chitons have a high turnover of iron necessitated by the need to continuously form iron minerals for structural components of their radulas.[4,5] This could explain the limited crystallinity found in these ferritins and the ferritin of *P. vulgata*.[23]

A further consideration that may account for the weak dependence of the magnetic ordering temperature on phosphate content at low to medium phosphate levels concerns the structural localization of phosphate in the ferritin cores. It is probably only when the phosphate is bound within the core structure that magnetic exchange interactions are diminished, so affecting the magnetic ordering temperature. Weak adsorption of phosphate to the surface sites of the ferrihydrite cores is suggested by the observation that about 77% of the phosphate of horse spleen ferritin cores is lost during alkali treatment,[9] which removes the protein shells from the cores. Furthermore, the P:Fe ratio is rather higher than average in cores with low iron content and lower than average in cores of high iron content,[38] again suggesting surface adsorption of phosphate. Such weak absorption would have minimal influence on both the crystallographic order and the magnetic ordering temperature.

Furthermore, as shown by the lower redox potential required for the reductive dissolution of bacterioferritin cores of *P. aeruginosa* compared with mammalian ferritin,[39] ferritin cores free of phosphate will have increased solubility over those containing phosphate as an integral component of the bulk structure.

This enhanced solubility may be of functional significance in the requirement for rapid iron mobilization and mineralization within the radula tissue. Ferritin from the chiton *A. hirtosa* is known to take up iron very much more rapidly than horse spleen ferritin,[4] and this reactivity is thus consistent with the structural features of the core reported here.

Registry No. Fe, 7439-89-6; PO_4, 14265-44-2; ferrihydrite, 39473-89-7.

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^{1}H NMR Studies of the Oxidized and Partially Reduced 2(4Fe-4S) Ferredoxin from *Clostridium pasteurianum* §

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^{1}H NMR spectra of the oxidized ferredoxin from *Clostridium pasteurianum*, which contains two weakly paramagnetic $Fe_4S_4^{2-}$ clusters, have been recorded and ^{1}H nuclear Overhauser effects have been used to determine proton pairs of β-CH_2 of the cluster-coordinated cysteines. The shift dependence on the dihedral angles between the Fe-S-C and S-C-H planes has been discussed. The size of the hyperfine shifts and their temperature dependence can be reproduced with a model in which some of the antiferromagnetic coupling constants between Fe(III) and Fe(II) ions are smaller than the other antiferromagnetic coupling constants. ^{1}H saturation transfer experiments on the partially reduced protein provides an estimate of the lower limit of the intermolecular electron transfer between the fully oxidized, intermediate, and fully reduced species.

Introduction

During the last years iron-sulfur proteins have attracted the interest of researchers owing to their role in electron-transfer processes during mitochondrial respiration, nitrogen fixation, and photosynthesis.[1-4] These systems are able to reversibly transfer one or more electrons, undergoing changes in NMR,[5,6] EPR,[7,8] Mössbauer,[7] magnetic susceptibility, and optical properties.[8,9] At least one of the oxidation states of all the known Fe-S proteins

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∥ Abbreviations: *C.p.*, *Clostridium pasteurianum*; DEAE-, (diethylamino)ethyl-; EPR, electron paramagnetic resonance, Fd, ferredoxin; MODEFT, modified driven equilibrium Fourier transform; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; P_i, phosphate; ppm, parts per million; WEFT, water-eliminated Fourier transform.

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