observed, while such bathochromic shifts would be expected if simple demetalation were the dominant pathway.

We feel it is important to stress that the strong hydrolytic stability of complexes **7–9** is in marked contrast to that observed for simple, water-soluble gadolinium porphyrins (e.g. $[Gd(TPPS)]^+$ or $[Gd(TMPyP)]^{5+}$), which undergo water-induced demetalation in the course of several days when exposed to an aqueous environment[7,8] or when "challenged" by treatment with EDTA[34] (conditions under which complexes **7–9** are stable[35]). It thus appears possible that gadolinium(III) complexes derived from the new expanded porphyrin ligand **6**, or its analogues, could provide the basis for developing new paramagnetic contrast reagents for use in MRI applications. In addition, the ease of preparation and stable mononuclear nature of complexes **7–9** suggest that such expanded porphyrin ligands might provide the basis for extending further the relatively underdeveloped coordination chemistry of the lanthanides. We are currently exploring both of these exciting possibilities.

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(34) Haye, S.; Hambright, P. *J. Chem. Soc., Chem. Commun.* **1988**, 666–668.

(35) A full kinetic analysis of the rate of decomplexation of **7** in the presence of EDTA and other chelating agents will be presented elsewhere.[31] Preliminary qualitative tests, however, have been carried out and indicate that the rate of demetalation is *not* appreciable at room temperature.

Contribution from the Department of Biochemistry, North Carolina State University, Raleigh, North Carolina 27695, and Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Protein Coats of Ferritin Can Sequester Large Amounts of Ferrous Iron

J. S. Rohrer,† R. B. Frankel,‡ G. C. Papaefthymiou,§ and E. C. Theil*,†

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Maintaining iron in a soluble form compatible with life appears to have been a problem since the accumulation of dioxygen in the atmosphere began about 2.5 billion years ago.[1] Iron proteins participate in a wide variety of crucial reactions during, e.g., DNA synthesis, respiration, photosynthesis, and nitrogen fixation. Contemporary organisms solve the problem of managing iron with a protein, ferritin, that stores iron as hydrous ferric oxide inside a coat of protein (ref 2 is a review). The protein coat of ferritin is a highly conserved, spherical assemblage, 12 nm in diameter, constructed from 24 similar or identical polypeptides. In spite of the features common to all ferritins, both the protein coat and the iron core can vary. In vertebrates, for example, the protein coat of ferritin is encoded in a family of genes, used differentially by specific cell types within an organism, that is regulated by one of the more complex series of reactions to be characterized. With regard to the iron core of ferritin, variations occur in the number of Fe atoms (1–4500), the degree of order (microcrystallinity), and the ratio of P:Fe (<0.05–0.8).

Table I. Accessibility of Fe(II) to *o*-Phenanthroline during Reconstitution of Horse Spleen Ferritin from Protein Coats and $FeSO_4$[a]

time after mixing, h	% added Fe(II) native protein[b]	% added Fe(II) denatured protein[c]
0.25	52	95
0.75	45	83
2	32	77
12	3.9[d]	44

[a] Values are the means of three to seven experiments. The standard deviation is 1–7% of the mean for denatured protein and 10–14% for the native protein (Experimental Section); the percentage of Fe(II) added to buffer that was soluble without protein was 55% at 7 min, 37% at 0.25 h, 20% at 1 h, 15% at 4 h, and 0.5% at 17 h. [b] Native protein—protein in 0.15 M HepesNa, pH 7.0; see Methods. [c] Measured as the percent of total iron that formed the Fe(II)–*o*-phenanthroline complex after removing an aliquot of the Fe/protein mixture and denaturing the protein by heating in 1 N HCl at 100 °C for 30 min; other denaturants were not tested; up to 0.1 μmol of Fe was added to a solution containing 3.78 μmol of *o*-phenanthroline. Analysis of synthetic mixtures of $Fe^{III}(NO_3)_3$ and $Fe^{II}SO_4$ of 1:9, 3:7, and 1:1, analyzed under the same conditions used to denature ferritin, yielded the predicted values (within 10%) for Fe(II) (measured with *o*-phenanthroline) and Fe(III) (measured with hydroxylamine followed by *o*-phenanthroline). For commercial horse spleen ferritin, 16 ± 3% of the iron reacted as Fe(II) after denaturation, suggesting the possibility of reduction of small amounts of Fe during denaturation. [d] One experiment only.

The mechanism of ferritin iron core formation is not well understood. It is known, however, that ferritin can be reconstituted from solutions of simple Fe(II) salts and protein coats; reconstituted iron cores are like native cores by a variety of criteria (for a recent study see ref 3). Note that only small numbers of Fe(II) atoms (8–12) bind to the protein.[10] Larger numbers apparently enter the hollow center of the protein coat. Ferritin cannot be reconstituted from Fe(III) and protein coats.[4] Thus, formation of ferritin iron cores is accompanied by the release of large numbers of electrons and protons, while dissolution of the iron core could require the consumption of electrons and protons. In a cell such as a macrophage, which processes ca. 5×10^8 Fe atoms from an effete red cell in 24 h,[5] with many of the Fe atoms passing in and out of ferritin, the electron and proton flux appears to be considerable.

Recently we showed quantitatively that the complete oxidation of the bulk of Fe(II) added to protein coats of horse spleen ferritin

* To whom correspondence should be addressed.
† North Carolina State University.
‡ Current address: Department of Physics, California Polytechnic State University, San Luis Obispo, CA 93407.
§ Francis Bitter National Magnet Laboratory.

(1) Walker, J. C. G. *The Evolution of the Atmosphere*; Macmillan: New York, 1977.
(2) Theil, E. C. *Annu. Rev. Biochem.* **1987**, *56*, 289–315.
(3) Mann, S.; Bannister, J. V.; Williams, J. M., Treffry, A.; Harrison, P. M. *J. Mol. Biol.* **1987**, *198*, 405–416.
(4) Harrison, P. M.; Andrews, S. C.; Ford, G. C.; Smith, J. M. A.; Treffry, A.; White, J. L. In *Iron Transport in Microbes, Plants and Animals*; Winkelmann, G., van der Helm, D., Neilands, J. B., Eds.; VCH: New York, 1987; pp 445–447.
(5) Saito, K.; Nishisato, T.; Grasso, J.; Aisen, P. *Br. J. Haematol.* **1986**, 275–286.
(6) Rohrer, J. S.; Joo, M.-S.; Dartyge, E.; Sayers, D. E.; Fontaine, A.; Theil, E. C. *J. Biol. Chem.* **1987**, *262*, 13385–13387.
(7) Macara, I. G.; Hoy, T. G.; Harrison, P. M. *Biochem. J.* **1972**, *126*, 151–162.
(8) Bakker, G. R.; Boyer, R. F. *J. Biol. Chem.* **1986**, *261*, 13182–13185.
(9) Watt, G. D.; Frankel, R. B.; Papaefthymiou, G. C. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3640–3643.
(10) Chasteen, N. D.; Theil, E. C. *J. Biol. Chem.* **1982**, *257*, 7672–7677.
(11) Mertz, J. R.; Theil, E. C. *J. Biol. Chem.* **1983**, *258*, 11719–11726.
(12) Yang, C.-Y.; Meagher, A.; Huynh, B. H.; Sayers, D. E.; Theil, E. C. *Biochemistry* **1987**, *26*, 497–503.

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Table II. Parameters from Mössbauer Spectra of $^{57}Fe(II)$ Mixed with Protein Coats of Ferritin from Horse Spleen and Compared to XAS[a]

	time, h	temp, K		IS, mm/s	QS, mm/s	% Fe(II)
	0.75	80	Fe(II)	1.35	3.18	67
			Fe(III)	0.47	0.71	
	2	80	Fe(II)	1.40	3.18	68
			Fe(III)	0.52	0.72	
	12	80	Fe(II)	1.37	3.26	38
		80	Fe(III)	0.47	0.70	
iron(III) citrate[12]		100		0.64	0.50	
horse spleen ferritin[13]		80		0.50	0.72	
$Fe^{II}OH_2$[14]		88		1.49	3.02	

[a] Data taken from ref 6 and analyzed for percent Fe(II) and Fe(III).

was slow (ca. 66% in 12 h) when we used X-ray absorption spectroscopy (XAS) to measure the kinetics of oxidation.[6] The protein coats appeared to stabilize most of the Fe(II) in solution for many hours even when air was bubbled into the solution; most of the Fe(II) was inaccessible to *o*-phenanthroline. The numbers of Fe atoms added in the experiment were so large (480 Fe atoms/molecule) that the possibility of protein binding of all of the individual Fe(II) atoms was unlikely; the protein coat is saturated when 8–12 Fe(II) atoms bind,[10] while the remainder appear to fill the hollow center of the protein, where they become inaccessible to *o*-phenanthroline. At first, the results seemed to be inconsistent with previous studies of ferritin core formation in which the complete oxidation of Fe(II) in the presence of the protein appeared to be rapid (less than 1 h).[7,8] However, the earlier studies measured the oxidation state of the iron indirectly and were biased toward Fe accessible to chelators or toward Fe(III) in optically detectable complexes; the properties of the complexes were incompletely known in terms of stoichiometry. Apparent differences between the several sets of data could be due to quantitative effects in the analyses used: XAS measured contributions from all the iron atoms, while the other analyses measured iron atoms in particular environments. A model consistent with all the data requires that when large numbers of Fe(II) atoms are present, the protein provides an environment in which most of the iron is initially sequestered as Fe(II), with a small number oxidizing rapidly to Fe(III); eventually all the iron would be oxidized. An analogous model during dissolution of ferritin iron cores was indicated by previous observations of retention of Fe(II) and Fe(III) by the protein after partial reduction of the core.[9]

We now report that the Fe(II), which was inaccessible to *o*-phenanthroline in the native protein, is sequestered and can be recovered after denaturing the protein. In addition, using $^{57}Fe(II)$ to reconstitute ferritin and monitoring oxidation with Mössbauer spectroscopy, we show that a small amount of iron is oxidized relatively quickly but much of the Fe remains in the ferrous state for many hours. Moreover, quantitative analyses of Fe(II):Fe(III) from *X*-ray absorption and Möbauer spectroscopy show a good correlation and support studies on the retention of Fe(II) by ferritin after partial reduction of the cores.[9,13] Thus, all the available observations are consistent with the model, which provides new insight into the formation of ferritin iron cores and a new perspective about the possible role and evolutionary age of ferritin.

Experimental Section

Materials. Horse spleen ferritin was obtained from Boehringer–Mannheim and the iron removed as previously described.[10] All materials were of reagent grade and were obtained from Boehringer-Mannheim or Fisher Scientific.

Methods. To test the accessibility of Fe(II) to *o*-phenanthroline, solutions of protein coats, prepared from horse spleen ferritin[10] (<0.2 Fe atom/molecule; 0.0417 mM protein; in 0.15 M HepesNa, pH 7.0), and fresh, aqueous solutions of $FeSO_4$ were mixed (final volume 0.5 mL; 480 Fe atoms/molecule; final concentration of Fe 20 mM) and incubated at room temperature in a spectrophotometer cuvette (10 × 4 × 14 mm) of the dimensions used previously.[6] With ambient air as used, the ratio of iron to dioxygen was comparable to that estimated for liver.[6] Previous experiments showed that the aeration of the protein solution had no detectable effect on the oxidation of Fe(II) under these conditions.[6] Denatured protein was produced to determine the effect on accessibility of Fe(II) to *o*-phenanthroline, by removing an aliquot of the Fe(II)/protein mixture and heating in 1 N HCl at 100 °C for 30 min before adding the *o*-phenanthroline.

Solutions of $^{57}Fe(II)$ for Mössbauer spectroscopy were prepared by using wire enriched in the isotope to 93.3% (obtained from NEN division of Du Pont) and H_2SO_4 incubated at 50 °C;[12] additions to solutions of the protein were volumes sufficiently small so that the pH was not changed detectably. Mixtures were prepared as described in ref 6 and as described for measurement of accessibility to *o*-phenanthroline. Samples were removed at the time indicated, frozen in liquid nitrogen, and stored at −80 °C until measured. Measurements were made as described in ref 9 and 13.

Results and Discussion

Recovery of Fe(II) inaccessible to chelators was possible after denaturing the protein by heating in dilute acid (Table I). For example, after 15 min 94.5% of the added Fe(II) was recovered as the Fe(II)–*o*-phenanthroline complex after denaturing the protein, but only 52% was available to react with the chelator in the presence of native protein. After 2 h some oxidation of Fe(II) had occurred, since only 77% of the added Fe formed the Fe(II)–*o*-phenanthroline complex after denaturation of the protein; the Fe(II) added remained inaccessible to the chelator before denaturation of the protein. Thus, protein coats of ferritin can incorporate large amounts of Fe(II) in a form that is not readily accessible to small chelators but only slowly oxidized, confirming the earlier XAS results.[6]

To extend the results of XAS and to analyze the properties of the small amount of oxidized Fe in the presence of bulk Fe(II), Mössbauer spectra were collected for ferritin protein coats incubated with $^{57}Fe(II)$ for various periods of time (Figure 1). The Mössbauer spectra confirm the XAS data on the stabilization of Fe(II) by ferritin protein coats;[6] 12 h after mixing, 38% of the Fe is still Fe(II) by Mössbauer analysis and 33% by XAS (Figure 1 and Tables I, II). The Mössbauer parameters of both oxidation states of the iron in the protein are presented in Table II. It is not possible to distinguish between monoatomic and polynuclear forms of Fe(II) on the basis of published Mössbauer parameters.[14] However, in the case of the oxidized Fe, some of the Fe(III) is polynuclear even at 45 min; possibly Fe(III) in such an environment gives rise to the amber color that was used in the past to measure iron core formation (e.g. ref 7, 8, 11). Note that XAS analysis of the oxidation rate in MOPS, compared to HEPES at pH 7, produced qualitatively similar results.

Clearly, Fe(II) added to the protein coats of ferritin can follow at least two paths: rapid oxidation of a small number of Fe(II) atoms or sequestration by the protein and slow oxidation of large numbers of Fe(II) atoms, possibly inside the hollow center of the protein coat, where an insoluble ferrous precipitate could form. (Note that 480 Fe atoms in a volume the size of the hollow of the ferritin protein coat are equivalent to a concentration of 0.3 M in an isolated system; 0.3 M exceeds the solubility of Fe(II) at pH 7.0.)[15] Oxidation would then occur slowly at the buffer/ferrous oxide hydroxide interface. Once partial oxidation

(13) Frankel, R. B.; Papaefthymiou, G. C.; Watt, G. D. *Hyperfine Interact.* **1987**, *33*, 233–240.
(14) Pritchard, A. M.; Mould, B. J. *Corros. Sci.* **1971**, *11*, 1–9.
(15) Leussing, D. L.; Kolthoff, I. M. *J. Am. Chem. Soc.* **1953**, *75*, 2476–2479.

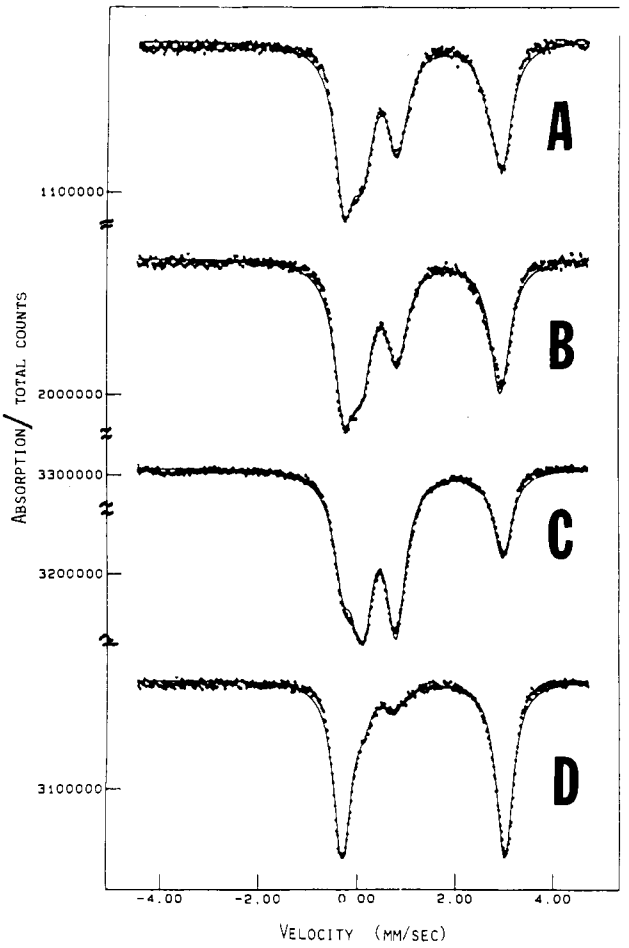

Figure 1. Mössbauer spectra of ^{57}Fe(II) mixed with protein coats from spleen ferritin. Spectra were obtained at 80 K as previously described: (A–C) protein after mixing with Fe(II) for 0.75, 2, or 12 h (see Experimental Section); (D) Fe(III) sample added to deaerated water, transferred to a sample vial, and frozen immediately after addition of ^{57}Fe(II) to the protein sample was completed.

occurred, the surface iron(III) oxide hydroxide might render the remaining Fe(II) inaccessible to *o*-phenanthroline. Such a model also explains the increased availability of Fe(II) in ferritin to chelators when Fe(II) is added in the absence of dioxygen.[9,13,16] An apparently analogous phenomenon occurs when Fe(II) is added to the buffer: ferro/ferri oxide hydroxides (blue-green) precipitate that oxidize (rust) first at the surface; when the protein is present, the iron complexes are maintained in solution.

Two possible ramifications of the sequestration of Fe(II) by protein coats of ferritin when large amounts of iron are added concern the bioenergetics of storing Fe in ferritin and the role of ferritin in evolution. The energetics of cellular iron storage in ferritin predicts a large electron flux for oxidation of iron during ferritin formation and reduction of iron during ferritin core dissolution. The flux may be less than predicted for large amounts of iron when iron turnover is rapid, in macrophages for example, if some of the iron remains as Fe(II) during short recycling times.[5] The possible evolutionary age of ferritin has been related to he appearance of dioxygen in the atmosphere, which produced the need for a variety of proteins to control or detoxify the results of iron/dioxygen interactions in living cells. However, even the metabolic role of Fe(II) is complex.[17–19] Thus, the ability of the protein coats of ferritin to sequester Fe(II) when it is available in large amounts and the high concentrations of Fe(II) used by bacteria thought to represent features of primitive life in the absence of dioxygen, e.g. sulfate-reducing bacteria, suggest that ferritin or ferritin-like proteins may be among the oldest of proteins.

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Registry No. Fe, 7439-89-6.

(16) Watt, G. D.; Jacobs, D.; Frankel, R. B. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7457–7461.
(17) Postgate, J. R. *The Sulfate-reducing Bacteria*, 2nd ed.; Cambridge University Press: Cambridge, England, 1984; pp 98, 99, 103–105.
(18) Bagg, A.; Neilands, J. B. *Biochemistry* **1987**, *26*, 5471–5477.
(19) Williams, R. J. P. *FEBS Lett.* **1982**, *140*, 3–10.

Contribution from the Institute of Molecular Physics, Polish Academy of Sciences, Smoluchowskiego 17, PL-60179 Poznań, Poland, and Department of Chemistry, University of Florence, I-50144 Florence, Italy

Temperature and Pressure Effects on the EPR Zero-Field Splitting of $Cu_2Cl_6^{2-}$ Dimers in $[(C_6H_5)_4Sb]_2Cu_2Cl_6$ Crystals

D. Gatteschi,[†] J. Goslar,[‡] W. Hilczer,[‡] S. K. Hoffmann,*[,‡] and C. Zanchini[†]

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The zero-field-splitting parameter D of EPR spectra is commonly dependent on temperature and pressure for multielectron paramagnetic ions with $S > {}^1/_2$. It is related to changes in the crystal field gradient at an ion site as a result of a rearrangement of the effective ligand charges due to thermal lattice contraction and/or changes in the amplitude of atomic vibrations.

These mechanisms seem to not be effective in binuclear complexes of ions with $S = {}^1/_2$ since the **D** tensor is not "quadrupolar" in origin there and is, rather, determined by dipolar and exchange coupling. Thus, the **D** tensor is an intrinsic property of a dimeric unit and is related to the geometrical structure, which is stable over a large temperature range, as is proved by magnetic susceptibility χ data. In fact, in the vast majority of copper(II) dimers the $\chi(T)$ dependence can be described by the temperature-independent singlet–triplet splitting J, which is known to be very sensitive to the dimer geometry.[1] Thus, a **D** tensor for binuclear $S_1 = S_2 = {}^1/_2$ complexes is not expected to be affected by temperature.

We have found, however, that in the chlorocuprates $[(C_6H_5)_4A]_2Cu_2Cl_6$ (A = P, Sb, As) the D values increase on cooling.[2] The dipolar contribution to the **D** tensor is overdominated by anisotropic exchange splitting in these crystals; thus the $D(T)$ dependence seems to be related to the temperature dependence of the anisotropic exchange coupling between Cu(II) ions within Cu_2Cl_6 units. Since generally an increase in pressure affects the spectra in the same way as a decrease in temperature, we decided to perform EPR experiments over a large temperature range under hydrostatic pressure up to 400 MPa on powdered samples of $[(C_6H_5)_4Sb]_2Cu_2Cl_6$ with the aim of having a closer insight into the factors determining the behavior of D.

The compound was prepared as described in ref 2. X-Band EPR spectra were recorded with a Radiopan SE/X-2542 spectrometer equipped with a hydrostatic pressure apparatus.[3,4] A cylindrical TE_{112} corundum resonator and 80-Hz modulation were used. Powder spectra were recorded as depending on temperature under different pressures and as depending on hydrostatic pressure at different temperatures.

EPR spectra under atmospheric pressure are presented in Figure 1. The following three effects appear with a lowering of temperature as is clearly seen in Figure 1: (a) an increase in the splitting of the principal **D**-tensor components D_i ($i = x, y, z$), as marked by arrows, (b) an increase in the intensity of the

[†] University of Florence.
[‡] Polish Academy of Sciences.

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