

# Reduction of mammalian ferritin

(iron-storage protein/hydrous ferric oxide/electrochemistry/Mössbauer spectroscopy/electron-proton coupling)

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**ABSTRACT** Mammalian ferritin from horse spleen undergoes an electrochemical or chemical reduction reaction in which each iron atom present is reduced by one electron (2300 electrons per ferritin molecule containing 2300  $\text{Fe}^{3+}$  ions). Midpoint potentials of  $-190$  mV,  $-310$  mV, and  $-416$  mV were determined at pH 7.0, 8.0, and 9.0. This variation of potential with pH indicates that  $\approx 2$   $\text{H}^+$  are transferred to the core for each  $\text{Fe}^{3+}$  reduced to  $\text{Fe}^{2+}$ . Mössbauer measurements of partially reduced ferritin give spectra that consist of a ferric quadrupole doublet with a superposed ferrous quadrupole doublet. The relative intensities of these doublets are consistent with the electrochemically determined degree of reduction.

Ferritin is a much-studied (1–4) multisubunit protein, widely distributed in nature, that sequesters a large amount of iron as hydrous ferric oxide in the protein interior. The diameter of the roughly spherical protein interior is about  $60 \text{ \AA}$  (5, 6) and, when filled to capacity, the crystalline polymeric iron core can accommodate up to 4500 iron atoms (7). The 24 identical subunits comprising the ferritin molecule are so arranged as to isolate the sequestered iron core from the cellular environment except for six channels that allow access by small ions and molecules from the solution environment into the protein interior (5, 6). Formation of the iron core in apoferritin is accomplished by reaction of  $\text{Fe}^{2+}$  with an oxidant, most commonly  $\text{O}_2$  or the buffered artificial oxidation system composed of  $\text{KIO}_3/\text{K}_2\text{S}_2\text{O}_8$  (8, 9). The rate of  $\text{Fe}^{3+}$  deposition to form the core has been studied extensively and a two-phase crystal growth model reasonably accounts for the quantitative and qualitative kinetic features observed (9, 10). Iron loss from the core has been studied and can also be accounted for by the same model except that first the  $\text{Fe}^{3+}$  is converted to  $\text{Fe}^{2+}$ , which is then readily lost in the presence of Fe chelators (11). Redox reactions are thus intimately involved in both the oxidation of iron during the process of iron storage and the reduction of iron during iron release. The nature of these redox reactions has not been studied in detail and consequently their role in the mechanism of iron storage and release remains obscure.

We report here results of electrochemical and spectroscopic measurements on horse spleen ferritin. These results suggest that fractional reduction of  $\text{Fe}^{3+}$  bound in the core readily occurs to form  $\text{Fe}^{2+}$ , which is retained in at least a kinetically, if not thermodynamically, stable state in the protein interior.

## MATERIALS AND METHODS

The horse spleen ferritin used in this study (2300 iron atoms per ferritin molecule) was obtained from Sigma and was passed through a  $1 \times 13$  cm Sephadex G-25 column equilibrated in  $0.025 \text{ M}$   $N$ -[Tris(hydroxymethyl)methyl]aminoeth-

anesulfonic acid) at pH 7.0 prior to use. Total iron was determined by the bathophenanthroline method (12). Naturally occurring  $^{57}\text{Fe}$  levels in ferritin samples ranging from 10 to 40 mg/ml were used for the Mössbauer measurements of both oxidized and partially reduced ferritin. Chemical ( $\text{S}_2\text{O}_4^{2-}$ ) and electrochemical methods (13) under controlled pH conditions were used for ferritin core iron reduction.  $\text{S}_2\text{O}_4^{2-}$  solutions were standardized electrochemically (13) or optically (14) by using  $\epsilon_{315} = 8000 \text{ cm}^{-1}\cdot\text{M}^{-1}$ . Reduction potentials were measured (13) at pH 7.0, 8.0, and 9.0 with methyl and benzyl viologens as mediators and ferritin solutions containing  $0.1 \text{ mM}$  total  $\text{Fe}^{3+}$  ( $\approx 0.02 \text{ mg/ml}$  in ferritin).

## RESULTS AND DISCUSSION

Coulometric reduction (13) of ferritin at pH 8 containing an average of 2300 Fe atoms per molecule at  $-500$  mV vs. the normal hydrogen electrode using methyl viologen as a mediator demonstrates that one electron per iron atom (2300 electrons per molecule of ferritin) is transferred during the electrochemical reaction. More detailed coulometric reduction experiments of ferritin as a function of applied potential indicate that reduction occurs over a narrow, well-defined potential range as shown in Fig. 1. Analysis of these curves for reduction of the core iron atoms in ferritin suggests an apparently reversible electrochemical reduction reaction with an  $E_{1/2}$  value of  $-310$  mV at pH 8 and an  $n$  value of 1. Reduction measurements to extreme negative potentials, where water reduction imposes a limit, showed that no further reduction occurs. Both the  $n$  value and the measured stoichiometry are consistent with a single-electron reduction of each core iron atom.

Fig. 1 also shows that the reduction of the core iron atoms is pH dependent, becoming more difficult with increasing pH. This result is consistent with proton uptake, presumably by proton transfer into the core upon reduction. From the magnitude of the potential change with pH (115 mV between pH 7 and 8; 106 mV between pH 8 and 9), values of 1.92 and 1.78 protons transferred per electron transferred were obtained. This result was confirmed by direct pH measurements during the  $\text{Na}_2\text{S}_2\text{O}_4$  reduction of ferritin, which demonstrated that 2.06  $\text{H}^+$  were transferred to ferritin for each  $\text{Fe}^{3+}$  reduced to  $\text{Fe}^{2+}$  in the ferritin core.

The apparent stability of the  $\text{Fe}^{2+}$  formed by reduction and its retention within the ferritin core suggested by the coulometric results in Fig. 1 were verified by direct longer-term chromatography experiments. Five identical 1-ml samples of ferritin of known iron content were each placed in separate containers in a vacuum atmosphere anaerobic enclosure under argon ( $<0.5 \text{ ppm O}_2$ ) and 0, 0.25, 0.5, 0.75, and 1.0 electron equivalent of  $\text{S}_2\text{O}_4^{2-}$  per Fe atom was added and allowed to react 20 min. Following this reaction interval, each

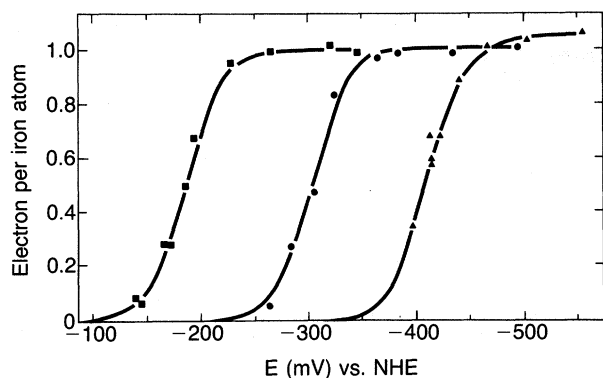


FIG. 1. Coulometric reduction of ferritin at 25°C. Ferritin samples added to 0.01 mM mediator solutions, 0.05 M Tris, and 0.25 M NaCl controlled at the indicated potentials. ■, pH 7.0; ●, pH 8.0; and ▲, pH 9.0. All potentials are relative to the normal hydrogen electrode (NHE).

ferritin sample was passed through an anaerobic Sephadex G-25 column to remove  $\text{S}_2\text{O}_4^{2-}$  oxidation products and any other small ions or molecules (i.e.,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ) and the Fe and protein concentrations were redetermined on these gel-filtered ferritin samples. Table 1 contains the analytical results obtained. Except for the one electron per  $\text{Fe}^{3+}$  sample, much of the original Fe is retained and, as the coulometric results in column 5 of Table 1 show, the expected amount of unreduced  $\text{Fe}^{3+}$  is present commensurate with the amount of added reductant. The one electron per  $\text{Fe}^{3+}$  result has been repeated several times and variable results have been obtained, ranging from little Fe loss to as much as 25% Fe loss. Such factors as length of reduction time, amount and quality of  $\text{S}_2\text{O}_4^{2-}$  used as reductant ( $\text{SO}_3^{2-}$ ,  $\text{S}^{2-}$ , and other products of  $\text{S}_2\text{O}_4^{2-}$  reaction or decomposition may serve as  $\text{Fe}^{2+}$  chelators), and the origin and previous history of ferritin samples (aging, hydration, damaged ferritin molecules) tend to influence the rate and amount of  $\text{Fe}^{2+}$  loss after reduction. Where an iron decrease is noted in the protein emerging from the column, the missing iron is found trailing behind the protein band in the free  $\text{Fe}^{2+}$  form. It thus appears that, as reduction of ferritin approaches one electron per Fe present in the absence of  $\text{Fe}^{2+}$  chelators, some iron is unstable with respect to retention by the core but that, at values less than 1:1, nearly complete retention of  $\text{Fe}^{2+}$  by the core occurs.

Evidence for the reduction of  $\text{Fe}^{3+}$  and retention as  $\text{Fe}^{2+}$  in ferritin was obtained by Mössbauer spectroscopy. Samples were prepared by freezing the ferritin molecules in air-tight plastic containers immediately following reduction and passage through a Sephadex column in an inert atmosphere to remove nonferritin-bound  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  ions. Mössbauer spectra of fully oxidized ferritin, 50% reduced ferritin (0.5 electron per iron atom), and 75% reduced ferritin (0.75

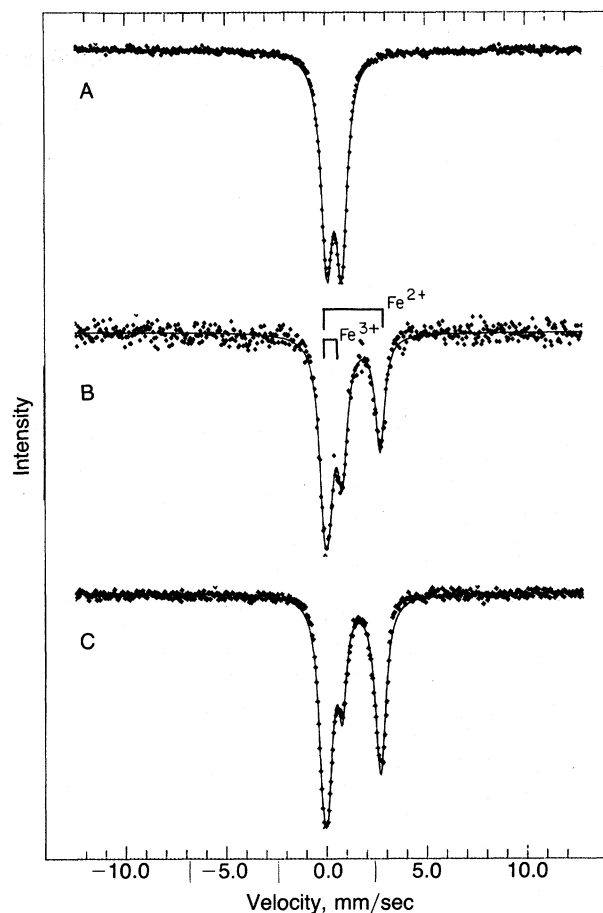


FIG. 2. Mössbauer spectra at 100 K. (A) Fully oxidized ferritin. (B) Fifty percent reduced ferritin. (C) Seventy-five percent reduced ferritin. The solid lines are theoretical fits assuming a ferric doublet in A and ferric and ferrous doublets in B and C.

electron per iron atom) at 100 K are shown in Fig. 2. Whereas the spectrum of oxidized ferritin is a broadened quadrupole doublet with parameters corresponding to ferric iron, the spectra of the reduced samples each consist of the ferric quadrupole doublet with a superposed ferrous quadrupole doublet. Both ferric and ferrous doublets have broadened lines. The relative intensities of the ferric and ferrous quadrupole doublets in each case are consistent with the electrochemically determined degree of reduction considering the uncertainties in the relative recoilless fractions (Table 2). Exposure of the reduced samples to air at room temperature resulted in the Mössbauer spectra of oxidized ferritin.

The 4.2 K spectrum of oxidized ferritin is a magnetic-hyperfine-split sextet resulting from magnetic ordering of the

Table 1. Fractional reduction of ferritin

Sample	Original Fe per mol	Electron per Fe added	After-column Fe per mol	Electron per Fe observed	Electron per Fe expected
1	2300	0	2346	0.92	1.00
2	2300	0.25	2285	0.70	0.75
3	2300	0.50	2115	0.47	0.50
4	2300	0.75	1900	0.31	0.25
5	2300	1.0	1850	0.08	0.00

Reduction of ferritin in 0.025 M *N*-[Tris(hydroxymethyl)methyl]aminoethanesulfonic acid at pH 7.5. Five separate samples of ferritin of known Fe content (2300 Fe per mol of ferritin) were fractionally reduced with  $\text{S}_2\text{O}_4^{2-}$  (electron per Fe added) and allowed to react 20 min. The ferritin samples were then passed through anaerobic Sephadex G-25 columns and each emerging sample was analyzed for protein and Fe content and reduced coulometrically. The last two columns on the right indicate the measured extent of reduction compared to that expected.

Table 2. Mössbauer parameters at 100 K

Electron per iron atom	Isomer shift,* mm/sec	Quadrupole splitting, mm/sec	Relative intensity, %	
			Fe <sup>3+</sup>	Fe <sup>2+</sup>
0	0.45	0.72	100	
0.5	0.47	0.67	48	
	1.25	2.88		52
0.75	0.47	0.63	31	
	1.28	2.82		69

\*Relative to iron metal at 298 K.

hydrous ferric oxide core (Fig. 3). At higher temperatures, transitions of the sublattice magnetization between energetically equivalent easy magnetic axes are activated by thermal energy (15, 16). When the sublattice relaxation time is equal to or shorter than the Larmor precession time of the nuclear moment of the 14.4 keV state of <sup>57</sup>Fe in the magnetic hyperfine field, the Mössbauer spectrum collapses to the quadrupole doublet. This phenomenon also occurs in the ferric subspectra of the partially reduced ferritin samples. The 4.2 K magnetic hyperfine fields are the same as in fully oxidized ferritin, which indicates that the ferric material in the partially reduced samples is the same hydrous ferric oxide as in the fully oxidized ferritin. Moreover, the ferric sextet flanking the ferric doublet is observable in the 60 K spectrum of all three samples. Also, the ferric doublet persists at lower temperatures in the oxidized ferritin than in either reduced

sample. Since at any temperature the ferric doublet and sextet correspond to smaller and larger particles, respectively, this observation suggests that those molecules with fewer iron atoms are preferentially reduced, perhaps on an "all-or-nothing" basis. All-or-nothing accumulation of iron in ferritin has been noted under conditions of rapid oxidation (9, 10).

The ferrous spectra of the partially reduced samples have different temperature dependencies than the ferric spectra. The 4.2 K ferrous spectra are somewhat obscured by the ferric lines, but in each case they appear to consist of a low-intensity quadrupole doublet superimposed on a broad, magnetically split background, which appears as an asymmetry in the overall spectrum. The intensity of the quadrupole doublet increases with increasing temperature, reaching full intensity between 10 and 20 K. These results suggest that the ferrous material might be noncrystalline with a range of magnetic exchange interactions between Fe<sup>2+</sup> ions resulting in a nonunique magnetic hyperfine splitting at 4.2 K. Certainly the ferric and ferrous materials in the partially reduced samples behave independently of each other, suggesting that certain ferritin molecules are preferentially reduced or that reduction occurs in a localized region within or on the hydrous ferric oxide cores.

To summarize, the hydrous ferric oxide cores of horse spleen ferritin can be reduced by one electron per iron atom with an E<sub>1/2</sub> value of -310 mV at pH 8.0. Reduction is accompanied by an uptake of two protons per electron from the surrounding medium and the Fe<sup>2+</sup> produced by reduction is retained in the cores. The reduction potential for mammalian ferritin is ≈100 mV more positive than that reported (17) for the corresponding bacterial ferritin and the latter appears to retain Fe<sup>2+</sup> much more strongly. This result implies that Fe<sup>2+</sup> retention is related to reduction potential. These characteristics raise the questions whether (i) the cores of these two proteins are somewhat different, (ii) the protein shells in these two proteins are influencing their redox potentials, or (iii) some other undisclosed features of these proteins causes variation of the reduction properties. Reduction measurements using ferritins from other sources would be valuable in providing additional information leading to a better understanding of the reduction step of ferritins that precedes Fe<sup>2+</sup> release.

The uptake of two protons, which occurs upon reduction, implies that the oxidation of Fe<sup>2+</sup> to Fe<sup>3+</sup> in ferritin and deposition of the hydrous ferric oxide core involves export of protons. Perhaps phosphate, which is also a constituent of the ferritin cores, is involved in buffering the pH changes within the cores resulting from iron redox reactions. It is intriguing to consider that ferritin in cells could function as a system in which redox reactions involving iron are coupled with the translocation of protons in the cell.

The Francis Bitter National Magnet Laboratory is supported by the National Science Foundation. Research at the Battelle-C.F. Kettering Laboratory was supported by Project PCM-8207775 from the National Science Foundation. R.B.F. was partially supported by the Office of Naval Research. This paper is Battelle-C.F. Kettering Research Laboratory contribution no. 860.

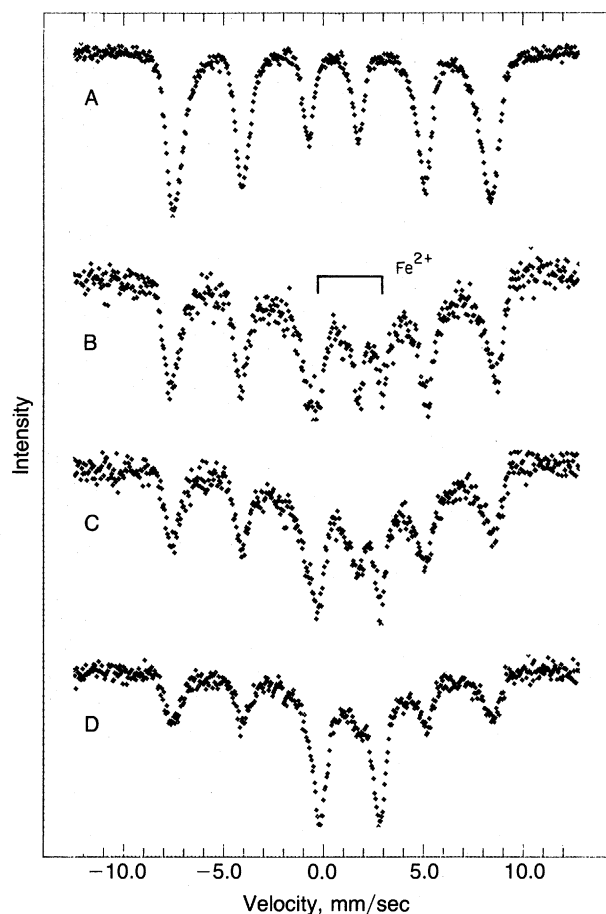


FIG. 3. Mössbauer spectra at 4.2 K. (A) Fully oxidized ferritin. (B) Fifty percent reduced ferritin. (C) Seventy-five percent reduced ferritin. The spectrum in D is 75% reduced ferritin at 10 K. The brackets indicate the position of the ferrous quadrupole doublet.

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