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Rapid reduction of iron in horse spleen ferritin by thioglycolic acid measured by dispersive X-ray absorption spectroscopy

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Summary. The release of iron from ferritin is important in the formation of iron proteins and for the management of diseases in both animals and plants associated with abnormal accumulations of ferritin iron. Much more iron can be released experimentally by reduction of the ferric hydrous oxide core than by chelation of Fe^{3+} which has led to the notion that reduction is also the major aspect of iron release in vivo. Variations in the kinetics of reduction of the mineral core of ferritin have been attributed to the redox potential of the reductant, redox properties of the iron core, the structure of the protein coat, the analytical method used to detect Fe^{2+} and reactions at the surface of the mineral. Direct measurements of the oxidation state of the iron during reduction has never been used to analyze the kinetics of reduction, although Mössbauer spectroscopy has been used to confirm the extent of reduction after electrochemical reduction using dispersive X-ray absorption spectroscopy (DXAS). We show that the near edge of X-ray absorption spectra (XANES) can be used to quantify the relative amounts of Fe^{2+} and Fe^{3+} in mixtures of the hydrated ions. Since the nearest neighbors of iron in the ferritin iron core do not change during reduction, XANES can be used to monitor directly the reduction of the ferritin iron core. Previous studies of iron core reduction which measured by Fe^{2+}·bipyridyl formation, or coulometric reduction with different mediators, suggested that rates depended mainly on the redox potential of the electron donor. When DXAS was used to measure the rate of reduction directly, the initial rate was faster than previously measured. Thus, previously measured differences in reduction rates appear to be influenced by the accessibility of Fe^{2+} to the complexing reagent or by the electrochemical mediator. In the later stages of ferritin iron core dissolution, reduction rates drop dramatically whether measured by DXAS or formation of Fe^{2+} complexes. Such results emphasize the heterogeneity of ferritin core structure.

Key words: Ferritin - Iron core - Iron reduction - X-ray absorption spectroscopy - Thioglycolic acid

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Introduction

Iron is stored in ferritin reversibly and is used for proteins important for respiration, cell division, and photosynthesis (reviewed in Theil 1990; Harrison et al. 1989). An inorganic core of hydrous ferric oxide forms inside the protein coat of ferritin which varies in size (up to 4500 iron atoms with the most common range 1000–2000), composition (e.g. P/Fe can vary over 1.2–8; Watt et al. 1986; Mann et al. 1986) and long-range order (amorphous to microcrystalline, Harrison et al. 1967; Mann et al. 1986, 1987). Variations in ferritin core composition also affect short-range interactions (Rohrer et al. 1990).

Iron core formation, studied in vitro, involves Fe^{2+} which is bound and/or sequestered by the protein and oxidized (Chasteen et al. 1985; Yang et al. 1987; Rohrer et al. 1987, 1989; Bauminger et al. 1989). Earlier studies which indirectly indicated the participation of Fe^{2+} during ferritin iron core formation include Macara et al. (1972), Chasteen and Theil (1982) and Treffry; Harrison (1984) and Chasteen et al. (1985). Polynuclear Fe^{3+} complexes form by hydrolysis of coordinate H_2O; proton loss averages $\approx 2.5\ H^+/Fe^{3+}$ during ferritin core formation (Spiro et al. 1967). Such observations suggest that a form of Fe^{2+} is also used in vivo for ferritin iron core formation, followed by hydrolysis and polynuclear complex formation.

Iron is released from ferritin core in vivo in response to intracellular or organismal iron deficits but the signal is unknown. The dissolution of iron from the core of ferritin experimentally requires reduction of Fe^{3+}, suggesting that conversion of Fe^{3+} to Fe^{2+} is required for iron release in vivo; only small numbers of Fe^{3+} atoms are removed from ferritin with Fe^{3+} chelators either in vivo or in vitro (see Crichton et al. 1980;

Theil and Aisen 1989). To reverse the reactions which produce the core in vitro, reduction of iron and rehydration is required. Reduction of the iron in ferritin has been studied using the absorbance of the Fe^{2+}·bipyridyl or 1,10-phenanthroline complex to monitor the reaction with a variety of electron donors such as dithionite, cysteine, $FMNH_2$ and ascorbate as electron donors (e.g. Macara et al. 1972; Hoy et al. 1974; Crichton et al. 1980; Jones et al. 1978; Bienfait and van den Briel 1980). Apparent rates of reduction varied depending upon the conditions used and the source of the ferritin, but the dilemma of separating the contribution of reduction form complex formation remained unresolved.

Recently, reduction of the iron in the core of ferritin has been studied electrochemically using mediators such as flavins, dithionite, and substituted viologens (Watt et al. 1985, 1986; Jacobs et al. 1989) which eliminates the contribution of Fe^{2+} complex formation and measures rates of reduction directly, assuming that the effect of the mediator is restricted only to the redox potential. The appearance of Fe^{2+} was measured by Mössbauer spectrometry at selected times (Watt et al. 1985, 1986). Rates of reduction were determined by microcoulometry and changes in the visible absorbance spectrum (Jacobs et al. 1989). The reactions were carried out anaerobically and in the absence of any chelator; essentially all the Fe^{2+} remained associated with the protein (Watt et al. 1985, 1986). A lag in Fe^{2+} formation was observed whether or not bipyridyl was present (Jones et al. 1978; Jacobs et al. 1989) which was interpreted as indicating the lag was due to reduction of the core itself rather than to iron release (Jacobs et al. 1989). Since the oxidation state of the iron in the ferritin core has only been deduced from indirect measurements, except for Mössbauer spectroscopy at fixed times, the contribution of Fe^{2+} complex formation or electrochemical mediators could not be assessed.

The near-edge region of X-ray absorption spectra (XAS) can be used to determine the oxidation of iron in ferritin, since the ligand geometry of the enar-neighbor environment is unchanged by reduction and release; octahedral coordination to oxygen is preserved. Collection of X-ray absorption spectra in the dispersive mode is rapid (scan times of 30 s), continuous and can be carried out under a variety of temperatures and reactions conditions (Tourillon et al. 1986). In this report we show that the near edge of the XAS (X-ray absorption near-edge structure, XANES) can be used to quantify the relative amounts of Fe^{2+} and Fe^{3+} in mixtures and use XANES to measure the iron in horse spleen ferritin. The results show a very rapid initial rate of reduction, even in the presence of air, followed by a sharp decrease in the rate of reduction.

Materials and methods

Materials. Horse spleen ferritin, twice crystallized and cadmium-free, was obtained from Boehringer and used without further purification. Hepes was obtained from the same source, and Tris (Trizma) from Sigma. All other chemicals were reagent grade and obtained from Fisher. Sample measurements were made in a plastic cuvette designed to have the same surface/volume ratio as a typical 1-ml cuvette with a 1-cm path length.

Collection of X-ray absorption spectra. The cuvette was mounted and data collection begun immediately (20 s) after adding buffered thioglycolate (final concentration 140 mM) to ferritin (−20 mM) at pH = 7 in 0.05 M Hepes·Na sodium acetate or Tris. Standards for Fe^{2+} or Fe^{3+} were 20 mM $FeSO_4$ and $Fe(NO_3)_3$ in 0.1 M H_2SO_4. The dispersive X-ray absorption beam line used at the Laboratoire des Utilisation Rayonnement Electromagnetique has been described previously (Tourillon et al. 1986). Spectra were collected, at room temperature, at intervals of 30 s, for 2 h.

Mathematical analysis of XANES for the relative amounts of Fe^{2+} and Fe^{3+}. The near edges (−10 to 30 eV) of normalized X-ray absorption spectra (XANES) were analyzed for Fe^{2+}, Fe^{3+} and 2:1, 1:1 and 1:2 mixtures measured under the same conditions as the protein. The spectra were analyzed using a least-squares fitting program and the assumption that the spectra of mixtures were linear combinations of the spectra of pure samples, i.e. $Z_i(E) = aX_i(E) + bY_i(E)$ where Z is the spectrum of the mixture, X and Y the spectra of pure Fe^{2+} or Fe^{3+} and a and b are the relative concentrations of the two oxidation states.

Results

Use of linear, least-squares fit of mathematically added X-ray absorption near-edge spectra (XANES) of aquo Fe^{2+} and aquo Fe^{3+} to quantify the relative amount of Fe^{2+} and Fe^{3+} in a mixture

The X-ray absorption near-edge spectrum (XANES) is sensitive to several factors including the oxidation state of the absorbing atom. When the ligands of a metal ion in two different oxidation states are essentially the same, the XANES of a mixture of iron in the two oxidation states should be that of the XANES for each ion weighted by the relative amount of each in the mixture. To test the validity of such an assumption, spectra of acidic solutions of Fe^{2+} and Fe^{3+} were used to fit the spectra of 2:1, 1:1 and 1:2 mixtures of the solutions of Fe^{2+} and Fe^{3+}. The data in Fig. 1 and Table 1 show that the mathematical models fit the data to within 10%.

Iron atoms in the ferritin iron core are octahedrally coordinated to oxygen ligands whether or not the iron is Fe^{2+} or Fe^{3+} (Theil 1990; Harrison et al. 1989). Moreover, the X-ray absorption spectrum for the Fe nearest neighbors in ferritin are well modelled by Fe^{3+} in 0.1 M HNO_3 (Yang et al. 1987). Moreover, analysis of the Fe^{3+} content of native horse spleen ferritin using XANES and fitting using the XANES of aquo Fe^{3+} and Fe^{2+}, as shown in Fig. 1, gave the correct value for the Fe^{3+} content. The XANES analysis of several samples and spectra showed the Fe^{3+} content of horse spleen ferritin to be 89–98% (Table 1) compared to 85% determined as the 1,10-phenanthroline complex after acid digestion and reduction with hydroxylamine (Rohrer et al. 1989).

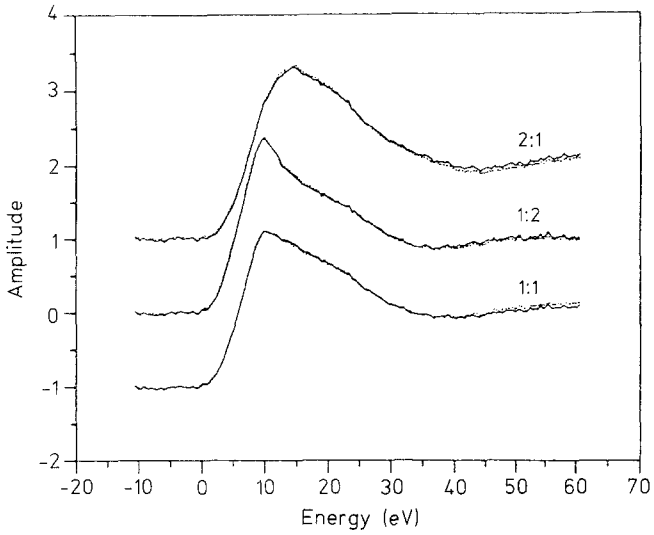

Fig. 1. Comparison of experimental and fitted XANES for mixtures of aquo Fe(III) and Fe(II). X-ray absorption near-edge spectra (XANES) of solutions of $Fe(NO_3)_3$ and $FeSO_4$ in 0.1 M H_2SO_4 were obtained for each solution as Fe^{3+} and Fe^{2+} standards, respectively, and for 1:2, 1:1 and 2:1 mixtures. The XANES (solid line) are plotted with the model (dotted line) produced using a linear fitting program and the assumption that the spectra were a linear combination of the pure samples, i.e. that $Z_i(e) = aX_i(E) + bY_i(E)$, where E is the spectrum of the mixture, X and Y are the spectra of the Fe^{2+} or Fe^{3+} standards, and a and b are the relative concentrations of the two oxidation states. (— Exper.; ... Theor.)

Table 1. Accuracy of numerical fitting of X-ray absorption spectra near edges (XANES) for mixtures of Fe(II) and Fe(III)

Actual mixture [Fe(II):Fe(III)]	Calculated mixture [%Fe(II):Fe(III)]	Regression error $(R) \times 10^{-3}$
1:2	30:70	1.3
1:1	55:45	0.8
2:1	73:27	0.9
0:100 (horse spleen ferritin)	8:92	1.8

The near edges (−10 to 30 eV) of normalized X-ray absorption spectra (XANES) were obtained for mixtures of Fe(II) and Fe(III) in 0.1 M NHO_3; XANES were fitted as described in Materials and Methods (see also Fig. 1)

Reduction of Fe^{3+} in horse spleen ferritin by thioglycolic acid with various buffer ions

Thioglycolic acid effectively removes iron from ferritin and has been widely used to prepare iron-free protein coats (e.g. Bryce and Crichton 1973; Chasteen and Theil 1982; Treffry and Harrison 1984). The burgundy color of the dialyzable complex of iron with dimeric thioglycolate at neutral values of pH (Leussing and Kolthoff 1953) appears immediately when thioglycolate is added to ferritin at neutral values of pH in air. However, several days of dialysis are required before all the iron is removed. The rapid appearance of the $Fe(SCH_2COO)_2^{2-}$ suggests rapid reduction/solubilization of the iron in the ferritin core.

The immediate and rapid reduction of iron in ferritin by thioglycolic acid is confirmed by the data in Figs. 2 and 3. Thioglycolic acid is present at a sevenfold excess over iron (the concentration of iron is 20 mM and that of thioglycolic acid 140 mM, pH = 7). After 40 s,

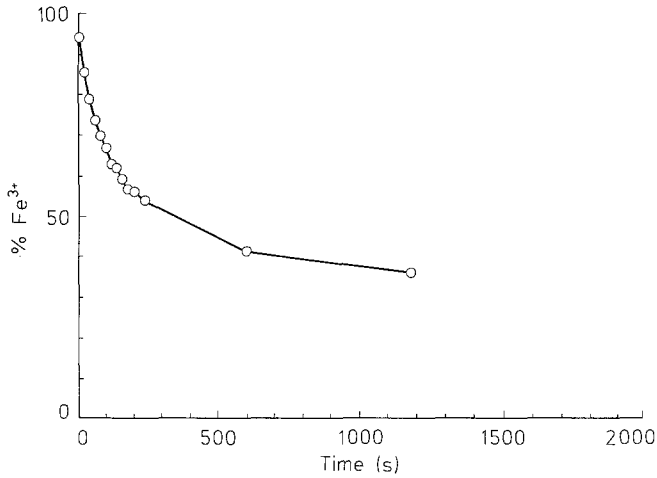

Fig. 2. Reduction of iron in the core of horse spleen ferritin by thioglycolic acid. Horse spleen ferritin (Fe = 20 mM) was mixed with buffered thioglycolic acid (final concentration: 1.40 mM in 0.05 M Hepes·Na pH = 7). X-ray absorption spectra were collected every 30 s, in the dispersive mode, beginning 20 s after mixing. The decrease in Fe^{3+} is plotted vs time. The data are representative or two independent experiments

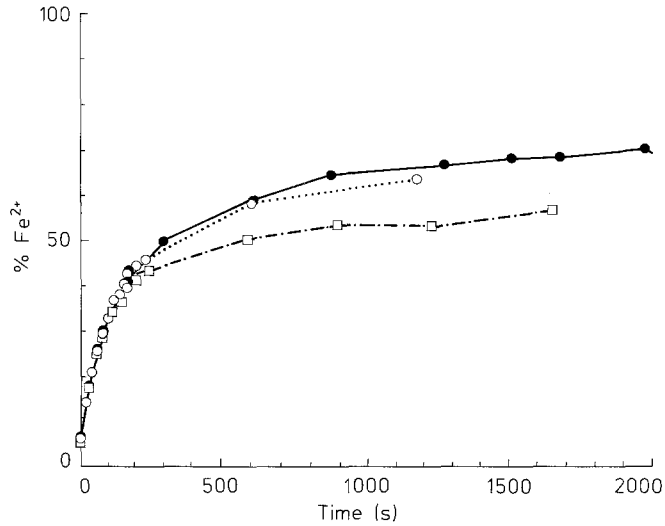

Fig. 3. The effect of solvent ions on the rate of reduction of ferritin iron cores by thioglycolic acid. Thioglycolic acid, buffered with Hepes (●—●), sodium acetate (□ · — · □) or Tris (○ · · · · ○), was added to a solution of horse spleen ferritin as described in Fig. 2. The increase in Fe^{2+} is plotted vs time. Note the similarity of the initial reduction rate for each condition, but the significantly earlier change of the slow rate of reduction when acetate is present. The data are representative of two independent sets of experiments

Table 2. The effect of solvent ions on reduction rates of ferritin iron cores

Time (s)	Fe(II) (%) with		
	Tris	Hepes	acetate
0	94	95	94
20	86	82	82
60	79	74	74
10	41	40	49
1200	36	31	47

XAS were collected within 20 s of adding thioglycolic acid (to 140 mM) ot the horse spleen ferritin (20 mM Fe) at pH = 7 in air. Spectra were normalized and fitted as described in Fig. 1. The deviation between the fitted and the actual XANES data ranged over 0.5–0.8%. The results are representative of two experiments with each type of solution

21% of the 2000 iron atoms were converted to Fe^{2+}, but the rate decreased continuously (Fig. 2). After 200 s, the rate became extremely slow so that only 64% of the iron had been reduced after 23 min (Table 2).

The possibility that the reduction rate was affected by ions in solution was tested by comparing the rates of reduction in Tris, Hepes · Na and sodium acetate. While varying the buffer ions had no affect on the initial rate of reduction, the dramatic decrease in the rate of reduction occurred several seconds earlier with acetate (Fig. 3). The addition of freshly prepared thioglycolic acid to the reaction mixture after the decrease in the initial rate had little affect on the slow rate of reduction indicating that consumption of the reductant was not the cause of the rate change.

Discussion

Iron is released or reduced in ferritin at different rates depending upon the buffer, source of ferritin, iron content and source of electrons (e.g. Hoy et al. 1974; Jones et al. 1978; Bienfait and van den Briel 1980; Crichton et al. 1980; Mertz and Theil 1983; Funk et al. 1985; Watt et al. 1985, 1986; Jacobs et al. 1989). If dissolution of the iron core of ferritin is the reverse of core formation, then proton consumption, hydration, and reduction are required. When the problem of dissolving a lump of rust or iron oxide under physiological conditions is considered outside the protein coat of ferritin, interactions at the interface of the mineral with H_2O and protons as well as with the electron source become paramount in understanding rates of reaction. Ferritin is a deceptively simple 'solution' of the mineral. A few discussions of reduction of ferritin iron cores have considered the structure of the core itself, e.g. Hoy et al. (1974) and Jones et al. (1978) who used a model of homogeneous core structure of varying size, Watt et al. (1986) who noted differences that are related to core composition; and Funk et al. (1985) who suggested that reduction rates are a composite of electron transfer and interactions at the mineral surface with the reductant. However, many interpretations of reduction rates focus on redox potentials or protein structure because of the indirect methods used to monitor reduction of iron. Direct measurement of the oxidation state of the iron in ferritin during reduction could simplify the interpretation of variations in the rate of reduction. Note that the oxidation state of the iron in ferritin during reduction has never been monitored directly before except by Mössbauer spectroscopy where continuous measurements have not been possible and where the time after adding electrons was not clear (Watt et al. 1985, 1986).

The oxidation state of iron in mixtures with the same near-neighbor geometry can be measured accurately (Fig. 1, Table 1) and directly when monitored by quantitative analysis of the X-ray absorption near-edge spectra. Using thioglycolic acid as the electron donor and the XANES collected in the dispersive mode with rapid scan times (30 s; Tourillon et al. 1986) to monitor reduction, iron in ferritin was reduced rapidly and immediately at pH = 7; no lag was detected (Figs. 2 and 3). A dramatic decrease in the reduction rate occurred when approximately 50% of the iron was reduced.

Earlier studies had suggested that the rates of reduction of ferritin depended mainly on the redox potential of the electron donor. However, the initial rate of reduction of iron in the ferritin core, by thioglycolate, measured by DXAS (Figs. 2 and 3) was comparable to that for $FMNH_2$ measured using bipyridyl (Funk et al. 1985). (Note that in both studies the same supplier of horse spleen ferritin was used which minimizes the possibility of difference due to sample variability.) Moreover, the rate of reduction by thioglycolate measured by DXAS (Fig. 3) was about sixfold higher than the rate measured with bipyridyl (Funk et al. 1985). Formation of the Fe^{2+} · bipyridyl complex thus appears to have been rate-limiting. Why Fe^{2+} formed during iron release was relatively inaccessible to chelators of Fe^{2+} is not clear, but sequestration of Fe^{2+} inacessible to chelators has recently been observed during ferritin core formation in air when large amounts of iron are added (Rohrer et al. 1987, 1989).

A slow initial rate in reduction of the iron core of ferritin were observed when measured electrochemically (Jacobs et al. 1989) or by measuring the Fe^{2+} · bipyridal complex (Jones et al. 1978; Funk et al. 1985). No lag was observed when the oxidation state of iron was monitored during the same time period using DXAS (Figs. 2 and 3). Thus, both chelators and electrochemical mediators appear to influence the reaction.

The later stages of reduction of iron in the ferritin core are slow and involve hours or days (e.g. Figs. 2 and 3; Hoy et al. 1974; Bienfait and van den Briel 1980; Funk et al. 1985). After about half the iron was reduced, the rate of reduction dropped dramatically (Figs. 2 and 3). Variables such as the method of measurement or the electron donor seem relatively unimportant at this stage of core dissolution. The consumption of reductant, or accumulation of reaction products inside the protein coat, do not explain the phenomenon, since neither the addition of fresh reductant, nor the re-

moval of reaction products by dialysis or ultrafiltration, increased the rate of reduction and/or the removal of iron from ferritin. Even the removal of oxygen had little effect on the rate (Chasteen and Theil 1982). Thus it is in the later stages of dissolution of the ferritin iron core that properties of the core itself become rate-limiting. Since the surface/volume ratio should increase as core dissolution proceeds, accessibility of the mineral surface alone is not a likely cause of the decreased rate. Differences in core structure could involve dehydration and crystallinity particularly if hydrated or imperfect regions of the mineral were preferentially reduced and dissolved. Anions appear to influence the rate of core reduction in the later stages (Fig. 3). Thioglycolic acid interactions with iron are also well documented and described (Leussing and Kolthoff 1953; Funk et al. 1985). Cores formed in the presence of anions such as phosphate, which has been associated with disruptions in the iron-oxy complex (Mann et al. 1986, 1987; Rohrer et al. 1990), or sulfate which has been associated with greater dehydration (Yang et al. 1986) might alter the fraction of ferritin iron cores which is readily reduced and dissolved. The future systematic exploration of the structural features of the ferritin iron core which affect later stages of ferritin core reduction, using cores altered by various anions for example, will be important for understanding mechanisms of biomineralization, for evaluating the role of the ferritin protein coat and the environment in core structure, and ultimately for understanding diseases of iron overload and iron mobilization.

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