RESEARCH COMMUNICATION

Ferritin does not accumulate iron oxidized by caeruloplasmin

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Ferritin is an iron-storage protein ubiquitous in mammals, plants and bacteria. It can be reconstituted, *in vitro*, from the apoprotein and Fe(II) salts in the presence of dissolved oxygen. Recently it has been reported that caeruloplasmin can facilitate apoferritin reconstitution and that iron oxidized by caeruloplasmin is sequestered within the ferritin shell. Here we show that the primary effect of adding caeruloplasmin to horse spleen ferritin

during reconstitution is the competition between the two molecules for the iron. This competition results in overall increased rates of iron oxidation and a mixture of products, namely iron-containing ferritin and iron hydroxy polymers attached to caeruloplasmin. Iron oxidized by caeruloplasmin is not incorporated, to any significant extent, into horse spleen ferritin.

INTRODUCTION

Iron is stored in animals, plants and bacteria as ferritin, a molecule (M_r 500 000) with a large (8 nm) iron-storage cavity within a roughly spherical protein shell [1,2]. This shell (2.5 nm in thickness) is composed of 24 subunits. Experiments carried out *in vitro* have led to the following picture of iron loading by ferritin. All native ferritins so far examined have the ability to catalyse Fe(II) oxidation. This ability resides in a conserved ferroxidase centre situated within the polypeptide chain [2–5]. Ferritins from plants and bacteria contain 24 such chains [2]. However, mammalian ferritins are heteropolymers of two types of chain known as ‘H’ and ‘L’, and only H-chains exhibit significant catalytic activity [6,7]. Although L-chains (in recombinant L-homopolymers) do promote Fe(II) oxidation, they do so at rates that are only slightly greater than those shown by protein-free controls. Fe(III) produced on H-chains migrates into the iron-storage cavity, where it is deposited as an inorganic (hydrous iron oxide) mineral [1]. Iron oxidized on one ferritin molecule can also be transferred to another ferritin molecule and bound by its iron core [7]. L-chain homopolymers can act as acceptors, and L-chains are thought to promote iron-core nucleation more effectively than H-chains [7,8].

Relatively little is known about cellular iron metabolism. Given the conservation of ferroxidase centres in ferritin, it has generally been assumed that the Fe(II) oxidative mechanism of iron storage found *in vitro* also applies *in vivo*. However, not all research workers accept this picture [9]. One problem is that the initial oxidation of Fe(II) by apoferritin, *in vitro*, is accompanied by the formation of H_2O_2 [10], and the Fenton-chemistry-derived damage to cellular constituents is well known. Modification of ferritin’s amino acids has been reported [9], as well as the production of radical species [11]. Yet ferritin isolated from mammalian tissues does not show oxidative damage. To explain this, Boyer and Schori [12] and Aust and colleagues [9] have proposed that, *in vivo*, the iron that ferritin sequesters has not been oxidized within its own shell, but is donated by other proteins or that other proteins may provide the oxidizing equivalents to Fe(II) inside the ferritin cavity. They studied the oxidation of iron by caeruloplasmin (Cp) (which does not give

rise to H_2O_2 production) and concluded that, in the presence of ferritin, this iron was sequestered within the ferritin shell. They also found no oxidation of ferritin’s amino acids under these conditions. However, the experiments on which these conclusions were based used an increase in absorbance at 310 or 380 nm to monitor incorporation of iron into ferritin. It was also assumed that iron, which was not removed by incubation with EDTA, or by passage through Sephadex G-25 or Chelex-100, had been incorporated into ferritin. Here we show that this conclusion is unsafe, and that ferritin does not accumulate iron oxidized by Cp.

EXPERIMENTAL

Horse spleen ferritin (HoSF) was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). Its iron was removed by reduction with sodium dithionite [4]. The concentration of apoferritin was calculated from its absorbance at 280 nm ($A_{280}^{1\text{ mg/ml}} = 0.9$). Human Cp was obtained from Sigma and used without further purification. Its concentration was calculated from its absorbance at 610 nm ($A_{610}^{1\text{ mM}} = 10.9$) [13]. Apo-Cp was prepared as described in [14].

Reconstitution of iron cores was carried out either in 0.1 M Mes buffer, pH 5.5, or 0.1 M Mops buffer, pH 7.0, in the presence or absence of Cp. Conditions used, which were comparable with those of [9,12], were 0.25–0.5 μM apoferritin, 0.4–0.5 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$ and 0.05–3 μM Cp. In some experiments the Fe(II) solution was labelled with ^{59}Fe and catalase (10 units/ml) was included in the reaction mixture.

Rates of iron oxidation were measured either by the disappearance of Fe(II), using a discontinuous ferrozine assay [15], or by monitoring the increase in absorbance at 310 or 380 nm due to the formation of hydrous ferric oxide [12,9]. The products of the reconstitution were analysed by native PAGE (6 or 7% gel) after staining with potassium ferrocyanide or Coomassie Brilliant Blue. The Prussian Blue colour associated with ferritin bands indicates the extent of iron-core reconstitution. When ^{59}Fe was used, the location of Fe bands was detected by autoradiography.

Abbreviations used: HoSF, horse spleen ferritin; Cp, caeruloplasmin; rHuHF, recombinant human H-chain ferritin; rHuLF, recombinant human L-chain ferritin.

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Figure 1 Iron oxidation by apo-HoSF or Cp

Iron oxidation was monitored as an increase in A_{310} . The reaction mixture contained $0.5 \mu\text{M}$ Cp (0.15 mg/ml), $0.2 \mu\text{M}$ apo-HoSF (0.1 mg/ml), 0.1 mM Fe and 0.1 M Mes buffer, pH 5.5.

RESULTS AND DISCUSSION

Iron oxidation by Cp or apo-HoSF

Iron oxidation by either HoSF or Cp alone results in an increase in u.v. absorbance, as measured at 310 nm, due to an accumulation of oxo-iron species (Figure 1). Similar results (not shown) were obtained at 380 nm. The iron oxidized by Cp alone remained soluble, and was not removed from the solution by treatment with Fe(III) chelators or Chelex-100, or by passage through Sephadex G-25 after incubation with EDTA. Thus none of these assays can distinguish between iron oxidation by Cp and iron oxidized by, and incorporated into, ferritin. The fact that, in contrast with iron oxidized in buffer solutions, iron oxidized by Cp (or in the presence of BSA [16]) is soluble, indicates that the iron must be bound to the protein.

Iron incorporation into ferritin in the presence or absence of caeruloplasmin

Iron was oxidized in mixtures of HoSF and Cp and the extent of iron incorporation into HoSF was determined by electrophoretic separation of the products and compared, semi-quantitatively, with the rates of Fe(II) oxidation by Cp or HoSF alone (Figure 2a). In mixtures of HoSF and Cp, the amount of iron deposited in ferritin was found to correspond to the relative rates of oxidation by the two proteins. As the concentration of Cp increased, thus increasing the rate of Cp-promoted Fe(II) oxidation, the incorporation of iron into ferritin decreased (Figures 2b and 2c). When radiolabelled iron was used, a fast-moving Fe species was detected at the solvent front in addition to the iron incorporated into ferritin (Figure 3). In the absence of Cp, most of the iron was incorporated into the ferritin (Figure 3, lane 1). The addition of Cp decreased iron accumulation by ferritin and increased the amount of the fast-moving species in a dose-dependent manner. In the presence of Cp alone, this species was the sole product of iron oxidation (Figure 3, lane 6). It is probably an oxyhydroxy polymer of Fe(III) [17], which, when formed, is associated with the protein to form a soluble complex, but which may be dissociated from the protein during electrophoresis. This species was not detected in the iron-stained gels,

Figure 2 Comparison of rates of iron oxidation by Cp or apo-HoSF with iron sequestration in HoSF

The reaction mixture contained 0.5 mM Fe(II) and 0.1 M Mes buffer, pH 5.5. (a) Iron oxidation was monitored as the rate of Fe(II) disappearance using the discontinuous ferrozine assay [15]. (b) and (c), native PAGE analysis (iron stain) of ferritin reconstituted either (b) using the same conditions as in (a) or in 0.1 M Mops buffer, pH 7.0 (c). Samples were left overnight before electrophoresis. Lane 1, $0.25 \mu\text{M}$ HoSF + 0.5 mM Fe(II); lane 2, as lane 1 + $0.5 \mu\text{M}$ Cp; lane 3, as lane 1 + $1.5 \mu\text{M}$ Cp; lane 4, as lane 1 + $3.0 \mu\text{M}$ Cp.

presumably because it was washed out of the gels during the acid staining process.

These results indicate that, while iron oxidation by Cp and ferritin was additive, each protein promoted the formation of its own typical product, as reflected in the correlation between the Cp/ferritin ratio and the ratio of fast-moving iron to ferritin iron products. The addition of apo-Cp to ferritin did not increase the rate of iron oxidation (results not shown), did not change the amount of iron incorporated into HoSF and did not produce a fast-moving iron species (Figure 4).

When ferritin reconstitution was carried out in the absence of Cp, a third product was observed at the top of the gel (Figures 2b and 2c). This product stained for both protein and iron, and was also detected by autoradiography when ^{59}Fe was used (Figure 3). It indicates that some large iron-ferritin aggregates are formed during the reconstitution. More aggregates were formed at pH 7.0 than at pH 5.5, and the aggregation was greatest at high iron concentrations and high iron/protein ratios. Aggregation could not have been due to protein cross-linking by radicals derived from Fenton reactions, since their formation

Figure 3 Effect of Cp on the products of ferritin reconstitution

Ferritin was reconstituted in 0.1 M Mops buffer, pH 7.0, 10 units/ml catalase, 0.4 mM ^{59}Fe , 0.5 μM apo-HoSF and Cp as stated. Native gel electrophoresis was carried out a few hours after reconstitution and developed by autoradiography. Lane 1, 0.5 μM apo-HoSF + 0.4 mM ^{59}Fe ; lane 2, as lane 1 + 0.05 μM Cp; lane 3, as lane 1 + 0.1 μM Cp; lane 4, as lane 1 + 0.5 μM Cp; lane 5, as lane 1 + 1.5 μM Cp; lane 6, 1.5 μM Cp + 0.4 mM ^{59}Fe .

Figure 4 Effect of apo-Cp on the products of ferritin reconstitution

Ferritin was reconstituted as described for Figure 3. Lane 1, 0.5 μM apo-HoSF + 0.4 mM ^{59}Fe ; lane 2, as lane 1 + 1.5 μM Cp; lane 3, 1.5 μM Cp + 0.4 mM ^{59}Fe ; lane 4, as lane 1 + 1.5 μM apo-Cp; lane 5, 1.5 μM apo-Cp + 0.4 mM ^{59}Fe ; lane 6, 0.4 mM ^{59}Fe and no protein (except catalase)

was not inhibited by the presence of catalase (Figures 3 and 4). In contrast, the presence of Cp considerably decreased the formation of aggregated ferritin (Figures 2b, 2c and 3). Apo-Cp also protected against aggregate formation (Figure 4), indicating that this effect of Cp is independent of its ferroxidase activity. The protection by Cp and apo-Cp is not specific for this protein, since ferritin aggregation is also decreased by BSA (results not shown). Thus, although when not overloaded the structure of ferritin facilitates the sequestration of iron as hydroxy polymers within its shell, Cp or albumin are also capable of binding those polymers. In so doing these proteins can prevent the aggregation of ferritin molecules caused by interaction with iron polymers that they have failed to sequester.

A protective effect has also been observed in mixtures of recombinant human H- (rHuHF) and L- (rHuLF) homopolymers

[8]. When relatively large amounts of Fe(II) were added to H-chain apoferritin, precipitation of the protein occurred and this could be prevented by the addition of L-chain homopolymer. The explanation proposed in this case was that, under the experimental conditions used, the capacity of rHuHF to sequester iron was exceeded, and rHuLF, although unable itself to oxidize a significant amount of iron, was able to incorporate iron oxidized by rHuHF [8]. In the absence of the L-ferritin, the Fe(III) released from rHuHF caused its aggregation.

It has been suggested that Cp assists the incorporation of iron into ferritin [9,12]. Our data do not support this hypothesis and indicate that Cp competes for the iron rather than promoting its utilization by ferritin. Iron oxidized by Cp may eventually migrate into the ferritin core. However, although it has been shown that some ferritin molecules can bind iron oxidized by other ferritin molecules [7,8], there is as yet no evidence that it can accept a significant amount of Fe(III) from different protein donors. In summary, the primary effect of adding Cp to ferritin during reconstitution is the competition between the two molecules for the iron. This competition results in an overall increased rate of iron oxidation and a mixture of products, namely iron-containing ferritin and iron hydroxy polymers attached to Cp. The presence of Cp or apo-Cp prevents the formation of a mixed iron-ferritin aggregate.

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