Evidence that the specificity of iron incorporation into homopolymers of human ferritin L- and H-chains is conferred by the nucleation and ferroxidase centres

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Mammalian ferritins are iron-storage proteins made of 24 subunits of two types: the H- and L-chains. L-chains, in contrast with H-chains, lack detectable ferroxidase activity. When ferritins were subjected to iron loading *in vitro* with increments near the saturation limit of 4000 Fe atoms per molecule, the homopolymers of human H-chains formed insoluble aggregates, caused by non-specific iron hydrolysis, whereas the homopolymers of L-chains remained soluble and incorporated most of the available iron. To analyse the molecular reasons for the difference, Glu-57 and Glu-60, which are conserved and exposed on the cavity of L-chains, were substituted with His, as in H-chains. The double substitution made the L-homopolymers as sensitive as the H-homopolymers to the iron-induced aggregation, whereas the opposite substitution in the H-chain

increased homopolymer resistance to the aggregation only marginally. Millimolar concentrations of citrate and phosphate increased iron incorporation in H-homopolymers by reducing non-specific iron hydrolysis, but inhibited that in L-homopolymers by sequestering available iron. The data indicate that the specific iron incorporation into L-homopolymers is mainly due to the iron-nucleation capacity of Glu-57, Glu-60 and other carboxyl groups exposed on the cavity; in contrast, the specificity of iron incorporation into H-homopolymers is related to its ferroxidase activity, which determines rapid Fe(III) accumulation inside the cavity. The finding that ferroxidase centres are essential for the incorporation of iron in the presence of likely candidates of cellular iron transport, such as phosphate and citrate, confirms their importance in ferritin function *in vivo*.

INTRODUCTION

Ferritins are a class of proteins widely diffused in nature, characterized by a unique tridimensional structure (a hollow protein shell made of 24 helical subunits) and by the capacity to remove Fe(II) from solution in the presence of oxygen [1,2]. They are present in animals, plants and prokaryotes where they play important roles in controlling iron homeostasis, by sequestering the metal in a non-toxic and available form [3]. Plant and bacterial ferritins are homopolymers, whereas mammalian ferritins are composed of two subunit types, the H- and L-chains, which coassemble in different proportions in a tissue-specific manner [3,4]. Human H- and L-chains are of similar size (182 and 174 residues respectively) and share 55% amino acid sequence identity [5].

In vitro, ferritins interact aerobically with ferrous iron in a complex set of reactions that lead to the specific deposition of ferrihydrite iron cores in the cavity, similar to those found in natural ferritins [6]. The reactions involve the selective transport of iron through molecular channels, site-specific oxidation of ferrous iron and the nucleation of ferric oxide in the cavity. After the identification of a specific ferroxidase centre inside the H-chain fold [5,7], various studies analysed the relevance of the centre in the mechanism of ferritin iron uptake. The five metal ligand residues of the centre (Glu-27, Glu-61, Glu-62, His-65 and Glu-107) are conserved in all ferritins (but *Escherichia coli* ferritins have Asp-61), except mammalian L-chains, where Glu-27, Glu-62 and His-65 are substituted [4]. Consequently all ferritins except L-homopolymers have ferroxidase activity [8–12]. The centres act by inducing rapid iron oxidation, with the formation of early oxo-bridged Fe(III) dimer intermediates which

dissociate within about an hour of their formation, and are partially replaced by mononuclear Fe(III) and later by Fe(III) clusters [13–15]. The reaction consumes a half dioxygen molecule per Fe(II) oxidized, with the formation of H₂O₂ [16,17]. The involvement of a transient Fe-tyrosyl radical has been indicated in the very early phase of the reaction, but its significance in iron uptake remains unclear [18,19]. In keeping with the proposed mechanism, alteration of outer-surface residues and of the hydrophobic 4-fold channels, where iron is not expected to be transported, had little effect on the rate and specificity of the reaction [8,20], whereas modifications of the carboxyl groups lining the hydrophilic 3-fold channel, which form metal-binding sites, reduced the rate of iron uptake about 2-fold [20,21]. In addition, substitution of Ala for the conserved Glu-61, Glu-64 and Glu-67, which form a cluster of carboxyl groups exposed on the cavity surface (the putative nucleation centre), decreased the rate of iron oxidation [6,22]. Human L-ferritin has no evident ferroxidase activity, because of the substitutions Glu-62 → Lys and His-65 → Gly which eliminate metal ligands from the ferroxidase centre [5,9]. Activity can be produced by replacing these and other neighbouring residues with those of the H-chain [23]. Nevertheless, wild-type L-ferritin (rLF) incorporates iron *in vitro* with a specificity and efficiency that, under some conditions, is even higher than that of H-ferritin (rHF) [24]. For example, rLF incorporates some of the iron oxidized by rHF at acidic pH [22], and, more importantly, under conditions of high iron loading (more than 2000 Fe atoms per molecule), it prevents the non-specific iron hydrolysis that induces the ferritin aggregation that occurs with rHF [24]. H-chain variants with inactivated ferroxidase centres showed rates of iron oxidation marginally lower than those of rLF, as expected, but did not exhibit the high

Abbreviations used: rHF, recombinant H-chain human ferritin; rLF, recombinant L-chain human ferritin.

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efficiency and specificity of iron incorporation typical of rLF, under the conditions indicated [24]. The substitution of Ala for Glu-61, Glu-64 and Glu-67 in the H-chain variant with inactivated ferroxidase centres virtually abolished specific iron incorporation [6,22], suggesting that carboxyl groups exposed on the cavity participate in iron incorporation by binding Fe(III) and promoting ferrihydrite nucleation. L-ferritins have exposed on the cavity a higher number of carboxylates than the H-chains [4], among which Glu-57 and Glu-60 are of particular interest, as they are conserved in L-chains, are replaced by conserved His in H-chains, are located next to the putative nucleation centre and protrude inside the cavity. They have been suggested to play a role in the higher mineralization efficiency of rLF and in the cooperativity with H-chains in heteropolymers [22–25].

We here describe variants of human H- and L-chains in which residues 57 and 60 were cross-substituted. The results show that the substitution of His for Glu-57 and Glu-60 abolished the efficiency of iron incorporation typical of rLF, whereas the opposite substitution increased iron incorporation into rHF only to a limited extent. In addition, they show that only ferritins with active ferroxidase centres incorporate iron in the presence of millimolar concentrations of phosphate and citrate. The data indicate that the specificity of iron incorporation under conditions of high iron loading is determined by the ferroxidase centres when they are active and by the nucleation capacity of the cavity carboxylates when they are inactivated.

MATERIALS AND METHODS

Ferritins and variants

Human ferritin H- and L-chain variants were obtained by oligonucleotide-directed mutagenesis of the plasmids pEMBLEx2LFT and pEMBLEx2HFT as described previously [8,9]. Homopolymers of human H- and L-chains and of the variants were expressed as soluble proteins by transformed *E. coli* strains and were purified as described previously [9,22]. Briefly, expression was induced by heat shock at 42 °C; cells were disrupted by sonication; the soluble homogenates were heated at 75 °C for 10 min, precipitated with $(\text{NH}_4)_2\text{SO}_4$ (520 g/l) and treated with DNase and RNase. The final purification steps consisted of gel filtration on a Sepharose 6B or Sephacryl S-200 column, for the H- and L-homopolymers respectively, followed by anion-exchange column chromatography. All ferritins were electrophoretically pure. Iron was removed by incubation with 1 % thioglycolic acid, pH 5.5, and 2,2-bipyridine followed by dialysis against 0.1 M Hepes, pH 7.0. The apoferritins had an A_{280}/A_{260} ratio higher than 1.9. Protein concentration was determined with bicinchoninic acid reagent (Pierce), with BSA as standard.

Iron incorporation

Apoferritins (0.1 μM ; 50 $\mu\text{g}/\text{ml}$) in 0.1 M Hepes buffer, pH 7.0, were added to freshly made 0.1 mM ferrous ammonium sulphate. Iron oxidation was monitored by measuring A_{310} [8,9]. When iron incorporation was to be monitored by gel electrophoresis, apoferritins (1 μM ; 0.5 mg/ml) in 0.1 M Hepes buffer, pH 7.0, were incubated for 2 h at room temperature with 0.5–4.0 mM ferrous ammonium sulphate [8,9]. The samples were electrophoresed on non-denaturing 6 % or 7.5 % polyacrylamide gels and stained for protein (Coomassie Blue) or iron (Prussian Blue) [22]. After destaining, densitometry was performed using a Computing Densitometer (Molecular Dynamics).

RESULTS

The ferritins analysed in this study are listed in Table 1. They include the two new doubly substituted variants and rHF(E62K + H65G) in which the ferroxidase centres had been inactivated [7]. The proteins were expressed in *E. coli* as stable assembled proteins which could be purified by normal procedures involving heating at 75 °C. All ferritins had iron removed before analysis. The study of the rates of iron oxidation (Table 1) showed that the activities of rHF and rHF(H57E + H60E) are essentially the same, whereas that of rLF is severalfold lower, but still higher than that of rLF(E57H + E60H) and rHF(E62K + H65G).

The relative efficiency of iron incorporation was analysed by competition experiments in which the mutants were mixed with equimolar amounts (1 μM) of rLF or rHF, incubated at pH 7.0 for 2 h with 1 mM Fe(II) and separated by electrophoresis. The mutant rHF(H57E + H60E) incorporated about 3 times as much iron as rHF (Figure 1B), whereas rLF(E57H + E60H) and rHF(E62K + H65G) incorporated little if any iron in the presence of rLF (Figure 1A). On the other hand, rLF did not take up any iron in the presence of rHF or rHF(H57E + H60E) (Figures 1A and 1B). Thus, under these conditions, the apparent efficiency of iron incorporation was in the order rHF(H57E + H60E) > rHF $\gg$ rLF > rHF(E62K + H65G) = rLF(E57H + E60H). The ratio between the Prussian Blue and Coomassie Blue stains

Table 1 Rates of iron oxidation of ferritin variants

All the mutants carried the substitution K86Q on the outer surface to facilitate crystallization. It had no effect on ferritin function. The reaction conditions were: 0.1 μM apoferritins in 0.1 M Hepes, pH 7.0, 25 °C, 0.1 mM ferrous ammonium sulphate. Rates were calculated as the change in A_{310} during the first minute of the reaction. Data are expressed as means $\pm$ S.D. from at least three independent experiments.

Variant	Site of substitution	Rate ($\Delta A_{310}/\text{min}$)
rHF	—	104 $\pm$ 13
rHF(H57E + H60E)	Cavity	101 $\pm$ 2
rHF(E62K + H65G)	Ferroxidase centre	3.8 $\pm$ 0.8
rLF	—	8.0 $\pm$ 0.4
rLF(E57H + E60H)	Cavity	2.6 $\pm$ 0.2

Figure 1 Competition experiments between rHF, rLF and variants for iron incorporation at pH 7.0

Apoferritins (1 μM ; 0.5 mg/ml) alone or mixed with equimolar amounts of a competitor ferritin were added to 1 mM ferrous ammonium sulphate, incubated for 2 h at 20 °C, and loaded on to 6 % polyacrylamide gels. After an electrophoretic run, the gels were stained with Prussian Blue. The competing ferritins are indicated above the lanes. The arrows mark the mobility of the reference ferritins. (A) Competition experiments with rLF; (B) competition experiments with rHF.

Figure 2 Iron incorporation at various iron increments

Non-denaturing 7.5% PAGE was performed on apoferritins incubated with various iron increments, and gels were stained with Prussian Blue. Conditions were as in Figure 1. Top, the concentrations of added Fe(II) are indicated above the lanes. The arrows mark the mobility of the ferritin monomers. Bottom, the iron incorporated into the ferritin monomers was evaluated by densitometry of the Prussian Blue-stained gels. The area of the bands is expressed in arbitrary units and plotted against the iron increments.

of samples subjected to iron loading provided an indication of the saturation of the ferritin monomers. The results, mainly from experiments with increments of 1000 Fe atoms per molecule, consistently indicated that the rHF monomer incorporated less iron than rLF (ratios 0.22 and 0.32 respectively) and that it behaved very similarly to the three variants (ratios 0.21–0.22).

In another approach to the study of the efficiency of iron incorporation, the ferritins were incubated for 2 h at pH 7.0 with various iron increments up to the saturation limit of 4000 Fe atoms per molecule. The proteins were then separated by electrophoresis, stained with Prussian Blue and iron taken up by the monomers quantified by gel densitometry. Iron incorporation into rLF increased almost linearly with the increase in iron loading (Figure 2); Coomassie Blue staining of the gels revealed no signs of protein aggregation (not shown). In contrast, in all other ferritins including rLF(E57H + E60H), apparent iron incorporation was much lower, reaching a maximum at increments

Figure 3 Effect of BSA on ferritin iron incorporation

Conditions were as in Figure 1, with 4.0 mM Fe(II) increments and in the presence of variable concentrations of BSA. Top, Prussian Blue-stained 7.5% polyacrylamide gels of rHF and rLF to which was added 0, 5, 2, 1, 0.5 and 0.1 mg/ml of BSA. Middle, plots of the areas of the monomer band against BSA concentrations. Bottom, Prussian Blue-stained gels of the various ferritins in the presence (+) or absence (–) of 5 mg/ml BSA.

of about 2000 Fe atoms per molecule (Figure 2, bottom). The apparent reduced capacity of these ferritins to incorporate iron was associated with the formation of slow moving oligomers, evident mainly in the H-ferritins (Figure 2, top) and insoluble aggregates that did not enter the gels (not shown). The mutant rHF(H57E + H60E) appeared to incorporate about twice as much iron as rHF and aggregated less at high iron increments (Figure 2, top).

Other experiments were performed with 4 mM Fe(II) increments (4000 atoms per molecule), conditions that most

Figure 4 Effect of phosphate and citrate on ferritin iron incorporation

Conditions were as in Figure 1 with 4.0 mM Fe(II) increments in the presence of variable concentrations of phosphate and citrate. Top, Prussian Blue-stained 7.5% polyacrylamide gels of ferritins to which was added 0 (control lane) or 0.4, 1, 4 or 16 mM sodium phosphate or sodium citrate. Middle, densitometry of the ferritin monomer bands plotted against the molar phosphate/iron ratio. Bottom, densitometry of the ferritin monomer bands plotted against the molar citrate/iron ratio. □, rHF(H57E+H60E); ○, rHF; ●, rLF; ■, rLF(E57H+E60H).

clearly differentiate the apparent incorporation efficiency of rLF. The addition of BSA to the reaction mixture had no effect on rLF, whereas it increased the apparent iron incorporation in rHF in a dose-dependent manner (Figure 3, top and middle). At the

highest BSA concentration (5 mg/ml) the iron associated with the rHF monomer was more than 20-fold higher than in the control in the absence of BSA, and almost equal to that of rLF (Figure 3, top and middle). Similarly, mutant ferritins appeared to incorporate much more iron in the presence than in the absence of 5 mg/ml BSA, but the total amount differed, being 100, 63 and 17% of that of rHF for rHF(H57E+H60E), rLF(E57H+E60H) and rHF(E62K+H65G) respectively (Figure 3, bottom). Coomassie Blue staining revealed that, in the presence of BSA, iron-induced ferritin aggregation was greatly reduced or eliminated (not shown).

We next analysed the effect of phosphate and citrate on iron incorporation in the various ferritins. Experiments with increments of 1000 Fe atoms (not shown) and 4000 Fe atoms (Figure 4, top and middle) revealed that the addition of either at concentrations above a 1:1 molar ratio with Fe fully inhibited iron uptake in rLF and rLF(E57H+E60H), whereas it increased by severalfold the apparent iron incorporation in rHF and rHF(H57E+H60E). Both compounds prevented iron-induced ferritin aggregation, even with an increment of 4000 Fe(II), as detected by Coomassie Blue staining of the gels (not shown), and the iron not incorporated into the ferritins formed insoluble aggregates with phosphate and fast moving soluble complexes with citrate (not shown). Low concentrations of citrate (0.4–1.0 mM) increased the Prussian Blue staining of the iron taken up by all ferritins except rLF(E57H+E60H), an effect that may be due to the abolition of ferritin aggregation or to the formation of iron cores that are more accessible to ferrocyanide. Densitometry of Prussian Blue- and Coomassie Blue-stained gels from experiments with 1000 Fe atom increments indicated that 1 mM phosphate had little effect on the saturation of rHF monomers, whereas citrate decreased it by about 30% (not shown).

DISCUSSION

The aim of this work was to study the molecular reasons for the higher mineralization efficiency of rLF compared with rHF, revealed by its resistance to aggregation caused by large iron increments. The present data partially confirm that the difference is due to the presence of two carboxyl groups exposed on the L-chain cavity (Glu-57 and Glu-60) which are replaced by His in H-chains [24]. The replacement of Glu-57 and Glu-60 with His made rLF as sensitive to iron aggregation as rHF, eliminating its typically high mineralization efficiency (Figure 2). However, the opposite substitution (His-57 → Glu and His-60 → Glu) had a less dramatic effect on rHF, increasing to some extent its mineralization efficiency, but not to the levels of rLF. This is probably due to the different mechanisms of iron uptake in the presence and absence of active ferroxidase centres.

The variants rLF(E57H+E60H) and rHF(E62K+H65G) both have inactive ferroxidase centres and His in positions 57 and 60 and exhibited very similar functional properties; they had the same rates of iron uptake, they were easily aggregated by high iron increments (Figure 2), they did not take up iron in the presence of rLF or rHF (Figure 1) and they were the least efficient at incorporating iron in the presence of 5 mg/ml BSA (Figure 3, bottom). This finding implies that the ferroxidase centre of H-chains and Glu-57 and Glu-60 of L-chains are the major sites that differentiate between the functions of the two chains. Glu-57 and Glu-60 are next to the putative conserved nucleation centre (Glu-61, Glu-64 and Glu-67), and the substitution of Ala in this centre has been shown to abolish specific iron incorporation in an H-chain variant with inactivated ferroxidase activity [6]. It is concluded that, in the absence of

active ferroxidase centres, the specificity and capacity of ferritins to incorporate iron is related to the number and distribution of carboxyl groups around the nucleation centre. Glu-57 and Glu-60 protrude into the cavity in such a way as to increase the capacity of the site to nucleate ferrihydrite. In the absence of ferroxidase activity the competing reactions leading to either iron-core formation or non-specific iron hydrolysis in bulk solution appear to be chemically similar: both are slow and have sigmoid progression plots [9], occur at detectable rates above pH 6.0 [22] and oxidize one dioxygen molecule per 4 Fe(II) atoms [16,17]. Thus the specificity of iron incorporation *in vitro* is determined by efficient nucleation sites, together with mechanisms that facilitate iron entry into the cavity, without the need for sites that promote iron oxidation. This hypothesis is supported by the finding that, in these ferritins, iron uptake is inhibited by phosphate and citrate, molecules that, when not in large excess, accelerate the rate of Fe(II) oxidation by dioxygen by virtue of their capacity to bind and stabilize Fe(III) and to promote its hydrolysis [26]. Thus, in the absence of active ferroxidase centres, the capacity to oxidize/hydrolyse iron is in the order phosphate or citrate > rLF nucleation centres > rHF nucleation centres > bulk solution.

The mechanism of iron uptake in ferritins with active ferroxidase centres is characterized by much faster rates of Fe(II) oxidation, a different stoichiometry (2 Fe(II) per O₂ [17,27]) and the mobility of Fe(III), which first forms dimers in the ferroxidase centres, then splits into monomers and moves into clusters [13–15]. This complex pathway implies the involvement of various Fe-binding sites, one of which, probably the final one, is where iron is nucleated and hydrolysed. Substitutions of cavity residues, e.g. Glu-61, Glu-64 and Glu-67 to Ala [6,22] or His-57 and His-60 to Glu (Figure 2), affected iron incorporation, but only to a limited extent, which could be detected only by direct competition experiments (Figure 1). They did not modify the specificity of iron incorporation and had little effect on the rate of iron oxidation; in fact, the low ferroxidase activity of the mutant rHF(E61A + E64A + E67A) is mainly due to the substitution of Glu-61 which is part of the ferroxidase centre [22]. Thus nucleation centres seem to play a minor role in this mechanism, a finding that may explain the high variability of cavity surfaces in plant and bacterial ferritins, all of which are homopolymers with active ferroxidase centres. The specificity of iron incorporation is simply explained by a fast and local accumulation of Fe(III) in the cavity caused by the ferroxidase centres; iron is then nucleated spontaneously without the need of specific sites. Under conditions of high iron loading, the specificity is apparently lost and ferritins aggregate (Figure 2), but experiments in the presence of BSA, phosphate or citrate, which prevent protein aggregation, showed that iron incorporation in rHF is the same as or even higher than that in rLF. Thus aggregation appears to be caused by a minor proportion of Fe(III) escaping from the cavity, in keeping with the high mobility of the iron oxidized at the ferroxidase centre [14,22]. The minor, but significant, reduction in rHF(H57E + H60E) aggregation suggests that its more efficient nucleation centres retain more iron inside the cavity by reducing its mobility. A similar effect explains the higher iron-incorporation efficiency of H/L-heteropolymers over the homopolymers: the efficient L-chain nucleation together with a lower proportion of H-chains reduce movements of Fe(III) outside the cavity and non-specific iron hydrolysis [25].

Phosphate is a natural constituent of the iron core, being a major component of bacterial and plant ferritins and a minor one of vertebrates [28]. We found that phosphate did not modify the rate of iron oxidation of rHF or rHF(H57E + H60E) at

concentrations as high as 1 mM (monitored by measuring A_{310} ; results not shown), nor did it form major aggregates with Fe, and more importantly, it did not inhibit iron incorporation (Figure 4). In contrast, phosphate inhibited iron incorporation into rLF and variants with inactive ferroxidase centres with the formation of insoluble Fe-phosphate complexes. These results are in partial agreement with previous reports showing that addition of phosphate to horse spleen ferritin (which has 5–10 % H-chain) increased the rate of iron oxidation when monitored by Mossbauer spectroscopy [29] but not by oxygen consumption [30], and induced iron aggregation outside ferritin cores. Thus phosphate seems to have minor effects on the mechanism of iron uptake mediated by ferroxidase activity, although it modifies the structure of the mineral core [31]. Experiments in the presence of citrate have not been reported previously. We show that it behaves similarly to phosphate, except for a stimulatory effect on the incorporation at low concentrations, due to its ability to eliminate non-specific iron hydrolysis which causes ferritin aggregation. At high concentrations, exceeding a 1:1 molar ratio with iron, it partially competes for Fe binding with H-ferritins.

In conclusion we have shown that the specificity of ferritin iron incorporation *in vitro* is determined by either cavity nucleation efficiency or the ferroxidase centres. The centres seem to be biologically important as they allow incorporation to occur in the presence of physiological concentrations of phosphate or citrate, two likely candidates for involvement in intracellular iron transport. This goes some way to explaining why ferroxidase centres are present in virtually all natural ferritins, both homo- and hetero-polymers.

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