Evidence that residues exposed on the three-fold channels have active roles in the mechanism of ferritin iron incorporation

Sonia LEVI*‡, Paolo SANTAMBROGIO*, Barbara CORSI*, Anna COZZI* and Paolo AROSIO†

*DIBIT Department of Biological and Technological Research, H. San Raffaele Scientific Institute, Via Olgettina 58, Milano, Italy, and †Cattedra di Chimica Applicata alle Scienze Biomediche, University of Brescia, Brescia, Italy

Iron is thought to enter the ferritin cavity via the three-fold channel, which is lined in its narrowest part by the residues Asp-131 and Glu-134. We describe here variants of human ferritins with active and inactive ferroxidase centres having Asp-131 and Glu-134 substituted with Ala and Ala or with Ile and Phe respectively. The two types of substitution had similar effects on ferritin functionality: (i) they decreased the amount of iron incorporated from Fe(II) solutions and decreased ferroxidase activity by about 50%; (ii) they inhibited iron incorporation from Fe(III) citrate in the presence of ascorbate; (iii) they resulted in loss of Fe and Tb binding sites; and (iv) they resulted in a marked decrease in the inhibition of iron oxidation by Tb (but not by Zn). In addition, it was found that substitution with Ala of Cys-130 and His-118, both of which face the three-fold channel, decreased the capacity of H-ferritin to bind terbium and to incorporate iron from Fe(III) citrate in the presence of ascorbate. The results indicate that: (i) the three-fold channels are the major sites of iron transfer into the cavity of H- and L-ferritins; (ii) at least two metal binding sites are located on the channels which play an active role in capturing and transferring iron into the cavity; and (iii) the permeability of the channel is apparently not affected by the hydrophilicity of its narrowest part. In addition, it is proposed that iron incorporation from Fe(III) citrate complexes in the presence of ascorbate is a reliable, and possibly more physiological, approach to the study of ferritin functionality.

INTRODUCTION

Ferritins from bacteria, plants and animals have similar three-dimensional structures; they all are symmetrical protein shells composed of 24 subunits folded into a four-α-helix bundle [1]. All ferritins have the capacity to remove Fe(II) ions from solution in the presence of oxygen, and to deposit iron into the protein interior in a mineral form [2,3]. The formation of the mineral core is probably a multistep process mediated by the protein, involving Fe(II) binding to the protein, its oxidation by oxygen, and hydrolysis of the resulting Fe(III) followed by nucleation/mineralization in the cavity [4,5]. The process is thought to be conserved in ferritins from different organisms, although the resulting iron cores are structurally different: mainly ferrihydrite in animal ferritins and ferric phosphate in bacterial ferritins [6].

Bacterial ferritins are composed of a single subunit type, although two types of ferritins have been recognized so far [7,8], while animal [9], and possibly plant, ferritins [10] are composed of two or more subunit types which co-assemble in variable compositions. The two main subunit types found in mammals have been named H- and L-chains. The ferroxidase centres in the four-helical bundles of the ferritin fold are highly conserved in all the vertebrate H-chains, as well as in bacterial and plant ferritins [1,11]. Accordingly, all these ferritins have ferroxidase activity, i.e. they induce the aerobic oxidation of iron at rates several-fold faster than that of iron auto-oxidation [1,8,12,13]. Some residues in the centres are altered in mammalian L-chains, and L-ferritins oxidize iron at low rates, similar to those of iron auto-oxidation and of H-ferritins with artificially inactivated ferroxidase centres [14]. In heteropolymers, such as natural ferritins, one or a few H-chains are sufficient to confer ferroxidase activity on the protein and to promote rapid iron incorporation [15,16]. Details of the stoichiometry of the reactions catalysed by the ferroxidase centre have been recently reported [17]. The reaction occurs with the transfer of one or two electrons from dioxygen to Fe(II) and with the production of hydrogen peroxide [18,19]. Mossbauer studies have shown that ferritin iron incorporation occurs via the formation of oxo-bridged Fe(III) dimers followed by the formation of mononuclear Fe(III) and core deposition [5,20]. The Fe(III) dimers have been localized to the ferroxidase centres and the mononuclear Fe(III) to the hydrophilic channels, with Asp-131 and Glu-134 acting as ligands [21].

Iron incorporation by ferritin *in vitro* can also occur in the absence of active ferroxidase centres (e.g. by L-chain homopolymers) via a slow reaction that is probably driven by the formation of iron nuclei inside the cavity which provide surface sites for Fe(II) binding and oxidation [22]. This mechanism of iron incorporation differs from those driven by the ferroxidase centre in both stoichiometry [one O_2 consumed for four Fe(II) oxidized] and pathway [the oxidized iron is incorporated directly into the iron core without formation of transient di- or mononuclear Fe(III) species] [16,23]. Most data indicate that iron enters the ferritin cavity via hydrophilic three-fold channels that are lined in their narrowest part by Asp-131 and Glu-134, residues that are conserved in all mammalian ferritins [21,24,25]. However, evidence that some of the iron newly oxidized by H-ferritin can be transferred to other macromolecules, including other L-ferritins or transferrin [23,26], suggests that part of the iron oxidized by the ferroxidase centres lies on or near the protein outer surface. Crystallographic data have shown the presence of an H-chain-specific 'one-fold' intrachain channel linking the ferroxidase centres with the protein outer surface [11] which might allow iron oxidation to occur near the surface. However, the functionality of these channels has not been established.

The complex mechanism of iron incorporation involves the

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

‡ To whom correspondence should be addressed.

presence of various iron binding sites which may be available to other metals. The interaction of apoferritins with metal ions has been extensively studied. Crystallization studies identified a number of heavy atom binding sites [including those for Tb(III) and Cd(II)] [27], and a Cd(II) binding site on the protein surface was engineered to allow crystallization [11]. Up to five binding sites per subunit were identified for Zn(II) and Tb(III) with both high and low affinities [28,29]. Cd(II) binding, possibly to the three-fold channel, was found to compete with that of iron [30]. Werdeska et al. [31] found that a variety of metal probes bound to ferritin with a stoichiometry of 0.3 or 0.7 per subunit, compatible with binding of one or two ions per three-fold channel. They also showed that Mn(II) and Fe(II) competed for the same site. Chasteen and Theil [32] showed that vanadyl competed with Fe(II), Fe(III), Tb(III) and, to a lesser extent, Zn(II) for binding to ferritin. Detailed physical characterization indicated that vanadyl binding sites involved nitrogen atoms, probably from histidine, suggesting that the site is not in the narrowest part of the three-fold channels, where only carboxy groups are present [33,34]. Under anaerobic conditions apoferritin was shown to bind 8 ± 0.5 Fe(II) atoms per molecule, consistent with the presence of one binding site per three-fold channel [35]. More recently, three types of Fe–nitrosyl complexes have been identified on H-ferritin, and two of these have been localized by mutational mapping around the three-fold channels and involve Cys-130, His-128 and probably also His-118, but not Asp-131 and Glu-134 [36]. The substitution of Asp-131 and Glu-134 with Ala or His greatly decreased the inhibition of H-ferritin iron uptake by Tb(III), but had no effect on inhibition by Zn(II) [21]. Experiments using capillary zone electrophoresis and gel chromatography showed that ferritins bind a large variety of metals, mostly on the protein surface, but Cd(II) appeared to compete with Fe for sites in the cavity [37,38].

In order to study further the functional role of the three-fold channels, we have produced variants of H- and L-ferritin mutants in which the two residues lining the narrowest part of the three-fold channels (Asp-131 and Glu-134) were substituted with Ala or with Ile and Phe respectively to modify the hydrophilicity of the area. In addition, other residues facing the channels were substituted in the H-chain. The proteins were analysed for their capacity to incorporate iron from Fe(II) solutions and from Fe(III) citrate complexes in the presence of ascorbate, and for Tb(III) binding and Tb(III) inhibition of ferroxidase activity.

MATERIALS AND METHODS

Ferritins and variants

Human ferritin L- and H-chains and their variants were obtained by oligonucleotide-directed mutagenesis of the plasmids pEMBLex2LFT and pEMBLex2HFT respectively [12,22]. All ferritins were expressed as soluble proteins by transformed *Escherichia coli* strains and were purified using the procedures already described [12,22]. Briefly, expression of the ferritins was induced by heat shock at 42 °C, cells were disrupted by sonication, and the soluble homogenates were heated at 75 °C for 10 min, precipitated with ammonium sulphate (520 g/l) and treated with DNase and RNase. The final purification steps consisted of gel filtration on a Sepharose 6B or a Sephacryl S-200 column, for the H- and L-homopolymers respectively, followed by anion-exchange column chromatography. All ferritins were electrophoretically pure, and iron was removed by incubation with 1% thioglycollic acid, pH 5.5, and 2,2-bipyridine followed by dialysis against 0.1 M Hepes, pH 7.0. Proteins concentrations were determined using the BCA reagent (Pierce), with BSA as standard.

Iron incorporation

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 1 mM ferrous ammonium sulphate with or without 1 mM sodium phosphate, pH 7.0. In other experiments apoferritins (1 μM in 0.5 M Tris/HCl, pH 8.4) were incubated for 2 h with 0.5–5.0 mM Fe(III) ammonium citrate in the presence of 1–10 mM ascorbic acid. The samples were run on non-denaturing 7.5% polyacrylamide gels and stained for iron (Prussian Blue) [22]. After destaining, densitometry was performed using a Computing Densitometer (Molecular Dynamics). ^{59}Fe(III) citrate complexes were prepared as follows: 100 μl of 1 mM $^{59}FeCl_3$ (Amersham) was added to 10 μl of 10 mM sodium citrate, 60 μl of 1 M Tris/HCl, pH 8.8, and 30 μl of 1 M NaOH. Aliquots of 0.5–5.0 μl of the solution were added to 5 μl of 1.5 μM apoferritin, the volume was corrected to 10 μl with 0.1 M Tris/HCl, pH 8.4, and the solution was incubated for 2 h at room temperature. After electrophoresis the incorporated iron was detected by autoradiography and quantified by densitometry.

Iron oxidation

Apoferritins (0.1 μM; 50 μg/ml; 0.5 ml) in 0.1 M Hepes buffer, pH 7.0, at 25 °C were added to 5 μl of 10 mM ferrous ammonium sulphate freshly dissolved in water to a final Fe(II) concentration of 0.1 mM. The rate of iron oxidation was monitored by the change in absorbance at 310 nm [26]. In some experiments the proteins were incubated with 0–15 μM $TbCl_3$ or $ZnSO_4$ for at least 5 min at 25 °C before measuring iron oxidation. Iron uptake was monitored by the variation in absorbance at 260 nm in experiments in 0.1 mM Hepes buffer, pH 7.0, containing 0.2 μM apoferritin, 10 μM ferrous ammonium sulphate and 0–10 μM $TbCl_3$.

Terbium binding

Apoferritins at 0.2 μM or 1 μM in 0.1 M Hepes buffer, pH 7.0, were added to sequential increments of 1.2 μM $TbCl_3$ and incubated for 10 min at room temperature, and the fluorescence emission spectra upon excitation at 295 nm were collected using an SFM 25 spectrofluorimeter (Kontron). The variation in fluorescence was calculated by the difference in the emissions at 545 and 560 nm.

RESULTS

H- and L-ferritin variants

The ferritin variants used in the study are listed in Table 1. For simplicity, the H-ferritins in which the ferroxidase activity has been inactivated by the substitutions Glu-62 → Lys and His-65 → Gly or by Glu-62 → His are indicated as rHF-Δf (where rHF is recombinant human H-chain ferritin). The three-fold channel variants of rHF, rHF-Δf and rLF (recombinant human L-chain ferritin) have the conserved residues Asp-131 and Glu-134 substituted by Ala and Ala or by Ile and Phe respectively. The rHF variants around the three-fold axes have the conserved residues His-118, His-128 and Cys-130 (which face the channels) substituted with Ala [36]. The rHF variants of the cavity surface have the conserved residues Glu-61, Glu-64 and Glu-67 (forming the putative nucleation centre) substituted with Ala [39], and the non-conserved residues His-57 and His-60 substituted with Glu, like in rLF. The rLF variant of the cavity surface has the opposite substitutions (Glu-57 → His; Glu-60 → His) [40]. The ferritins and variants were expressed by *E. coli* as soluble,

Table 1 Properties of the ferritin variants

Iron incorporation was determined by densitometry of Prussian Blue-stained gels of iron-loaded ferritins, as described in the legend to Figure 1, in the presence (+P) or absence (−P) of 1 mM sodium phosphate. The amount of iron associated with the ferritin monomer band is expressed as a percentage of that associated with rLF in the same electrophoretic run. Values are means of at least two independent experiments. Iron oxidation is given as the change in absorbance (in milli-absorbance units; mA) at 310 nm of the reaction of apoferritins (0.1 μM in 0.1 M Hepes, pH 7.0, 25 °C) with 0.1 mM Fe(II) ammonium sulphate. The values were calculated from the first 1 min of the reaction. Values are means of at least three independent measurements. Terbium/subunit is the molar ratio of Tb(III)/ferritin subunits at which the fluorescent emission at 545 nm reached a plateau. Conditions were as described in the legend to Figure 4. Data were obtained from at least two independent measurements.

Code	Substitutions	Chain type	Iron incorporation (%)		Iron oxidation (ΔmA/min)	Terbium/subunit
			−P	+P		
Ferritin wild types						
rLF	–	L	100	7	<	4
rHF	–	H	33	64	104	5
222	rHF(E62K + H65G)	rHF-Δf	34	21	<	4
Substitutions at the three-fold channel						
204	rHF(E134A)	H	25	–	81	3
Cr11	rLF(D131A + E134A)	L	13	4	<	2
206	rHF(D131A + E134A)	H	10	18	45	3
F100	rHF(E62K + H65G + D131A + E134A)	rHF-Δf	15	10	<	2
S14	rHF(D131I + E134F)	H	9	18.2	40	3
S15	rHF(E62H + D131I + E134F)	rHF-Δf	8	<	<	2
Substitutions around the three-fold channel						
S9	rHF(H118A)	H	44	65	71	4
S10	rHF(H128A)	H	38	71	110	5
S5	rHF(C90E + C102A + C130A)	H	42	53	95	4
Substitutions on the cavity surface						
A2	rHF(E61A + E64A + E67A)	H	7	18	19	3
S8	rHF(H57E + H60E)	H	70	–	103	5
Cr12	rLF(E57H + E60H)	L	40	8	<	4

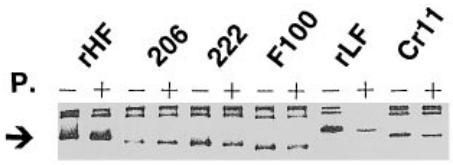

Figure 1 Fe(II) incorporation by ferritin in the presence or absence of phosphate

Apoferritins (1 μM in 0.1 M Hepes, pH 7.0) were added to 1 mM Fe(II) ammonium sulphate in the presence (+) or absence (−) of 1 mM sodium phosphate (P.). After a 2 h incubation the samples were separated by 7.5% polyacrylamide-gel electrophoresis, stained with Prussian Blue, and the gel pictures digitalized. The code names of the variants (see Table 1) are indicated above the lanes. The arrow indicates ferritin monomers. Each ferritin has a different electrophoretic mobility, caused by the amino acid substitutions.

assembled proteins with similar yields. The H-ferritins with active ferroxidase centres were purified as yellow iron-containing molecules, whereas the others were colourless, indicating that they contained little or no iron.

Iron incorporation

The apoferritins were exposed to increments of 1000 Fe(II) atoms per molecule at pH 7.0 in the presence or absence of 1 mM sodium phosphate, non-denaturing gel electrophoresis was carried out, and the iron incorporated into the ferritin monomers was quantified by densitometry of Prussian Blue-stained gels. The results are shown in Figure 1 and summarized in Table 1. In the absence of phosphate, rLF took up most of the available iron without signs of aggregation [40], and thus was used as a reference to evaluate the relative iron incorporation of the other ferritins. Under the same conditions rHF, rHF-Δf and the H-chain variants around the three-fold axes incorporated about one-third of the iron taken up by rLF, with minor differences. More importantly, the channel mutants of rHF, rLF and rHF-Δf having Ala/Ala or Ile/Phe in positions 131/134 all incorporated only about one-tenth of the iron taken up by rLF (Table 1; Figure 1). To verify that the differences in iron incorporation by the ferritin monomers were due to their capacity to incorporate iron, rather than to aggregation caused by iron, we performed experiments in the presence of 1 mM sodium phosphate, which was shown to decrease non-specific iron hydrolysis and ferritin precipitation [40]. Under these conditions iron incorporation into ferritins with active ferroxidase centres increased about two-fold, as expected, and the differences between rHF and its channel variants [e.g. rHF(D131A + E134A)] were maintained (Table 1). Phosphate decreased iron incorporation into rHF-Δf and to a greater extent into rLF (Figure 1), consistent with the absence of ferroxidase activity [40]. The results indicate that, in all the three ferritin types, substitution of Asp-131 and Glu-134 with Ala decreased the capacity of the protein to incorporate iron; the effect was greater with rLF than with rHF. The introduction of Ala or of the more bulky and hydrophobic residues Ile and Phe into positions 131 and 134 had similar effects on iron incorporation.

It has been shown that natural ferritins from plants and horse spleen take up iron supplied as Fe(III) citrate at pH values above 8.4 in the presence of ascorbate [41]. We performed experiments by incubating apo-rHF with 0.5–5.0 mM Fe(III) ammonium citrate at pH 8.4 and in the presence of 1–10 mM ascorbate. The amount of iron incorporated, detected by Prussian Blue staining, varied in a complex manner with the Fe and ascorbate concentrations (Figure 2, upper panel). In the presence of 10 mM ascorbate, iron incorporation into rHF increased with iron

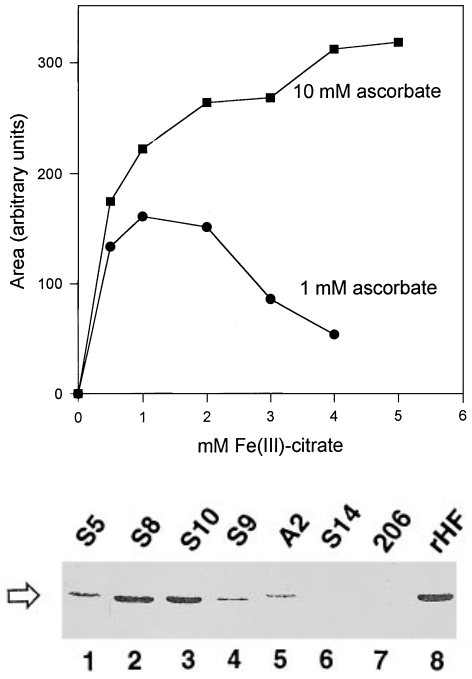

Figure 2 Iron incorporation from Fe(III) ammonium citrate in the presence of ascorbate

Apoferritins (1 μM) were incubated for 2 h with 0.5–5.0 mM Fe(III) ammonium citrate in the presence of 1 or 10 mM ascorbic acid in 0.5 M Tris/HCl, pH 8.4. The samples were electrophoresed, stained and the picture digitalized as in Figure 1. Upper panel: densitometry of the monomeric bands of rHF after incubation with 1 or 10 mM ascorbate and the indicated concentrations of Fe(III). Lower panel: iron loading from 1 mM Fe(III) in the presence of 10 mM ascorbate of the variants (indicated by code names; see Table 1). The arrow indicates ferritin monomers.

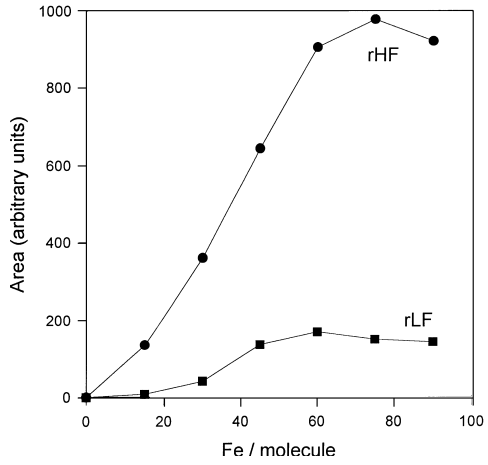

Figure 3 Iron uptake from ^{59}Fe(III) citrate

Apoferritins and rHF or rLF (1.5 μM) in 0.1 M Tris/HCl, pH 8.4, were added to various amounts of ^{59}Fe(III)/citrate (1:1) and 10 mM sodium ascorbate and incubated for 2 h at room temperature. Then 1 mM batophenanthroline was added and the sample was loaded on to 7.5%-PAGE. The gels were exposed to autoradiography and the radioactivity associated with the ferritin monomer bands was evaluated by densitometry.

concentration to reach a plateau at about 4 mM Fe(III) (Figure 2) while, under these conditions, rHF and rHF-Δf variants having Asp-131 and Glu-134 substituted with Ala or with Ile and Phe respectively (Figure 2, lower panel, lanes 6 and 7), and rLF (results not shown), did not take up detectable iron. All the other rHF variants incorporated iron, although the ones with His-118 or Cys-130 substituted with Ala only incorporated minor amounts (Figure 2, lower panel, lanes 1 and 4).

Experiments with ^{59}Fe(III) citrate at pH 8.4 confirmed that iron incorporation into rHF increased with increasing iron concentration, and also showed that rLF was able to bind low, but significant, amounts of iron (Figure 3), calculated to be about 10–20 Fe atoms per molecule, which could not be extracted by a further 1 h incubation with 1 mM desferrioxamine or with 1 mM batophenanthroline (results not shown). ^{59}Fe binding was totally absent in the rLF and rHF variants having Asp-131 and Glu-134 substituted with Ala or with Ile and Phe respectively (results not shown), while all the other variants, including rHF altered at the putative nucleation centre or at the ferroxidase centre, bound iron in amounts comparable to that bound by rLF (results not shown). The results indicate that the residues on and around the three-fold channels, on the ferroxidase centre and on the putative nucleation centre play active roles in iron incorporation under these conditions.

Terbium binding

Terbium fluorescence increases on binding to proteins and, accordingly, the fluorescence emission at 545 nm (excitation at 295 nm) of apoferritins increased with terbium addition to reach a saturation point (Figure 4, top panel). This point, which is related to maximum number of Tb atoms bound to the protein, was reached after the addition of about five and four Tb atoms per subunit for rHF and rLF respectively (Figure 4, middle and bottom panels). The results obtained with various mutants (Table 1) show that the saturation points were reached at about three, two and two Tb atoms per subunit in rHF(D131A + E134A), rHF(E62K + H65G + D131A + E134A) and rLF(D131A + E134A) respectively (Figure 4). The other substitutions that decreased Tb binding were Glu-134 → Ala, His-118 → Ala and Cys-130 → Ala (but not Hys-128 → Ala) near the three-fold channels, Glu-61 → Ala + Glu-64 → Ala + Glu-67 → Ala forming the putative nucleation centre, and Glu-62 → Lys in the ferroxidase centre. The five Tb binding sites on rHF could be localized with some precision: one site on the ferroxidase centres, involving Glu-62 and absent from L-ferritin, two sites on and near the hydrophilic channels, involving the conserved residues Asp-131, Glu-134, His-118 and Cys-130, and other two sites on the cavity surface, involving the conserved residues Glu-61, Glu-64 and Glu-67 which form the putative nucleation centres.

Ferroxidase activity

The initial ferritin iron oxidation rates were evaluated by measuring the variation in absorbance at 310 nm after the addition of 1000 Fe(II) atoms per molecule. The results, summarized in Table 1, confirmed that rLF, rHF-Δf and their variants had no detectable ferroxidase activity. The substitutions around the three-fold axes, as well as His-57 → Glu and His-60 → Glu in the cavity, did not modify rHF activity to a significant extent, whereas the substitution of Glu-61, Glu-64 and Glu-67 with Ala greatly decreased the iron oxidation rate, in keeping with the observation that Glu-61 is part of the ferroxidase centre [11]. More important was the finding that the rHF mutants having Ala/Ala or Ile/Phe in positions 131/134 on the hydrophilic channels showed an activity about half that of rHF, and comparable with that reported for rHF(D131H + E134H) [42].

rHF variants with different abilities to bind Tb were studied for the inhibitory effects of Tb(III) and Zn(II) on iron oxidation.

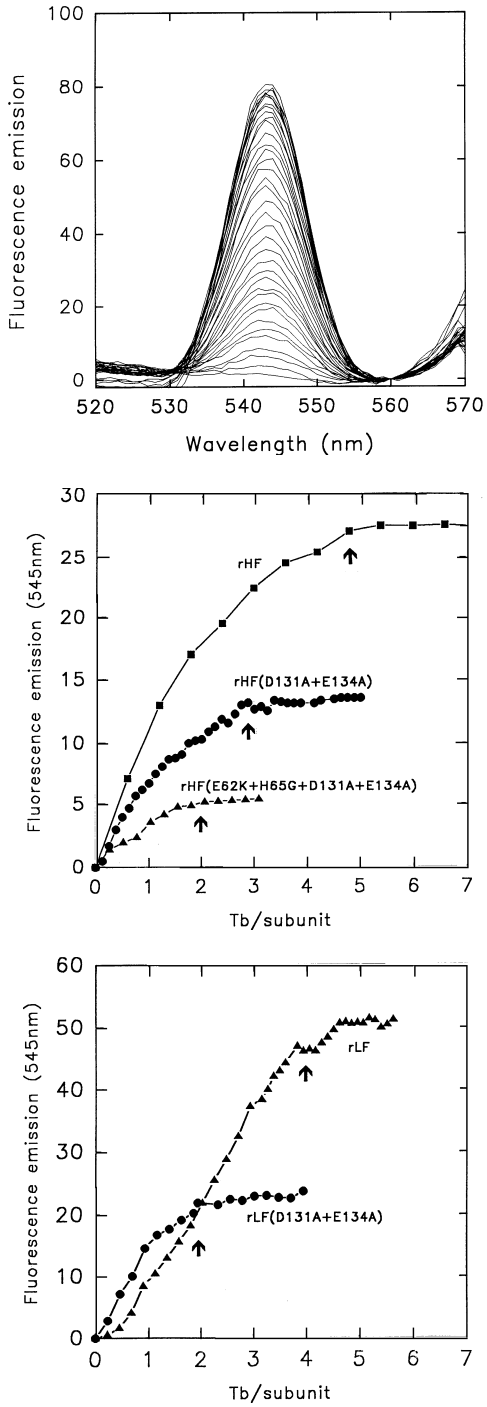

Figure 4 Terbium titration experiments

Top panel: Tb at increments of 1.2 μM was added to apo-rHF (0.2 μM), and the emission spectra (excitation at 295 nm) were recorded. Middle and bottom panels: fluorescence emission values at 545 nm plotted against the molar Tb/subunit ratio for rHF and its variants at 0.2 μM (middle), and rLF and its variants at 1 μM (bottom). The points at which the plateaus were reached are indicated by arrows.

The two metals inhibited rHF ferro-oxidation in similar concentration ranges, up to a maximum of 70–80 % at 10 μM (i.e. 100 metal ions per molecule) (Figure 5). Almost complete inhibition of activity was achieved when the two metals (each at 10 μM) were added together (Table 2). Zn and Tb (10 μM) inhibited to a similar extent all the rHF variants analysed, except for those carrying substitutions of Asp-131 and Glu-134, including rHF(E134A), which were much less sensitive to inhibition by Tb (Table 2).

Finally, we studied the effect of Tb on a reaction system in which ferritins were added of 50 Fe atoms increments; the reaction was monitored at 260 nm, the wavelength at which the absorption difference between apo- and holo-ferritin is greatest. The results showed that Tb strongly inhibited the reaction catalysed by rHF, whereas it had a minor effect on that catalysed by rHF(D131A + E134A) (Figure 6). This finding implies that binding of Tb to Asp-131 and Glu-134 inhibits iron oxidation occurring at the ferroxidase centres, but not that occurring on the surface of the iron core, which, under these conditions, is minimized.

DISCUSSION

The evaluation of the amount of iron incorporated from an Fe(II) solution provides a simple way to compare the functionality of ferritins with active and inactive ferroxidase centres. Here we show that, in this respect, the functionality of rLF, rHF and rHF-Δf is greatly decreased by the double substitution Asp-131 → Ala/Glu-134 → Ala, implying that the two carboxy groups located in the narrowest part of the three-fold channels participate in the mechanisms of iron incorporation of H- and L-chain homopolymers.

The double substitution Asp-131 → Ile/Glu-134 → Phe in rHF was designed to decrease the size of the narrowest part of the three-fold channels and to make the channels strongly hydrophobic without interfering with protein assembly. The rHF mutant so obtained assembled correctly and showed functional properties (i.e. rates of iron oxidation and amount of incorporated iron) similar to the rHF variants in which the two residues were substituted with Ala or His [21,42]. To evaluate whether the residual activity of the mutants (which is 30–50 % of that of rHF wild type) was due to iron transiting through the described 'one-fold' intrasubunit channel connecting the ferroxidase centre with the outer surface [11], the same Asp-131 → Ile/Glu-134 → Phe double substitution was made to an rHF-Δf variant in which this type of channel could not be functional. The variant so constructed incorporated similar amounts of iron as the corresponding one with Ala in positions 131 and 134. This implies either that iron can enter the cavity through alternative and unknown pathways, or that the three-fold channels lined by positively charged histidines, small alanines or bulky and hydrophobic Ile and Phe residues are similarly permeable to iron. This is not unlikely on the basis of recent evidence that ferritin allows the entrance of molecules much larger than the channel size [43], even bulky haem molecules [44], implying a large flexibility of the protein shell. These substitutions on the three-fold channels did not inhibit iron access to the cavity, but iron incorporation was much more efficient in wild-type ferritins having Asp and Glu in positions 131 and 134 respectively, suggesting that these residues are actively involved in iron transfer.

The data from Tb binding and inhibition studies support the importance of Asp-131 and Glu-134 for iron uptake. At least three regions involved in Tb binding were identified in rHF: around the ferroxidase centres, around the putative nucleation centres and around the three-fold channels. Tb inhibition of rHF ferroxidase activity was decreased to a large extent by the substitution of Asp-131 and Glu-134 (or of Glu-134 alone), whereas it was not affected by the other substitutions. This supports previous evidence that inhibition by Tb is caused by specific binding at the three-fold channels, which limits iron

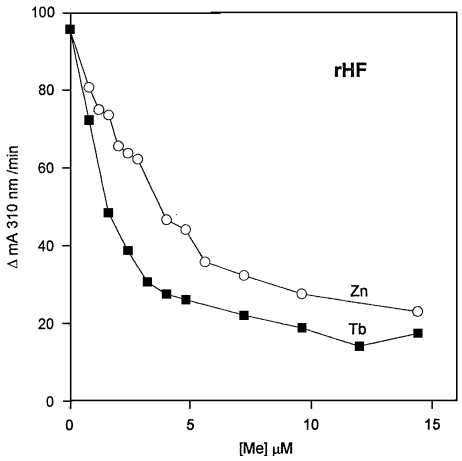

Figure 5 Inhibition of ferroxidase activity by terbium and zinc

Rates of iron oxidation by rHF were monitored by the change in absorbance (mA = milli-absorbance units) at 310 nm in the presence of various concentrations of $TbCl_3$ and $ZnCl_2$ ([Me] = metal ion concentration). Conditions: 0.1 μM apoferritin, 0.1 mM Fe(II), 0.1 M Hepes, pH 7.0, at 25 °C.

access to the cavity [21]. This is also indirect evidence that the ferroxidase centres are accessible to iron only from the cavity side and that the proposed 'one-fold' channels are not functional. Zinc, which showed inhibition plots similar to those of Tb, decreased the ferroxidase activity in all of the rHF variants analysed, indicating that it acts via a different mechanism, probably by binding to, or near, the ferroxidase centres.

Further evidence for an active role for Asp-131 and Glu-134 in iron incorporation came from studies with Fe(III) citrate in the presence of ascorbate. Under controlled conditions of pH and Fe(III)/ascorbate ratios, rHF was able to build up large iron cores, while rHF(D131A + E134A) and rHF(D131I + E134F) were inactive (Figure 2). In addition, rLF was unable to form iron cores, but it bound 10–20 atoms of iron per molecule, probably in a mono- or oligo-nuclear form, whereas the variants rHF and rLF(D131A + E134A) did not bind any detectable iron (results not shown). These findings demonstrate that Asp-131

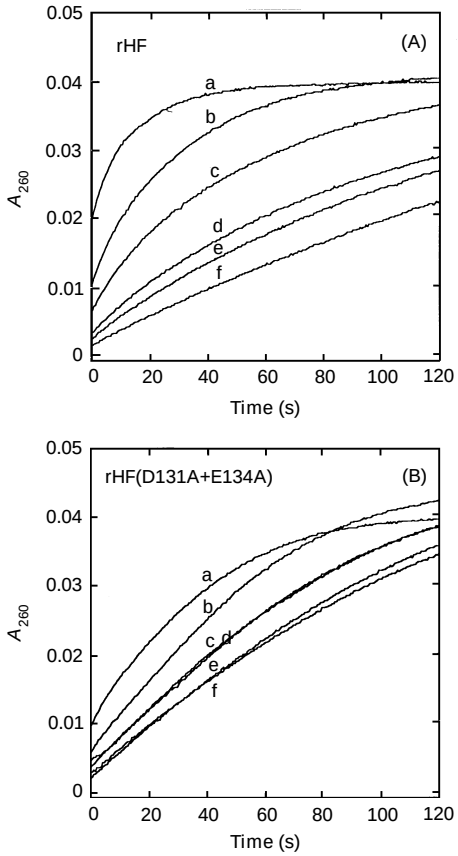

Figure 6 Effect of Tb on reactions with small increments of Fe(II) atoms

Progression plots of the reaction of apoferritins with 50 Fe(II) atoms per molecule monitored at 260 nm, in the presence of various Tb(III) concentrations. (**A**) rHF; (**B**) variant rHF(D131A + E134A). Plots a–f, Tb(III) concentrations of 0, 1.6, 2.4, 3.2, 4.8 and 6.4 μM respectively. Conditions: 0.2 μM apoferritin, 10 μM Fe(II), 0.1 M Hepes, pH 7.0, at 25 °C.

and Glu-134 form an iron binding site which is essential for iron incorporation under some conditions. The variants rHF(C130A) and rHF(H118A) were less efficient in iron incorporation than

Table 2 Effect of Tb(III) and Zn(II) on the rate of iron oxidation by rHF variants

Initial rates of iron oxidation (ΔA_{310}/min) are expressed as a percentage of that of the rHF wild type in the absence of Zn(II) or Tb(III). Experiments were performed at 25 °C, pH 7.0, with 0.1 μM apoferritin and 0.1 mM Fe(II), after a 5 min preincubation with 10 μM $TbCl_3$, 10 μM $ZnCl_2$ or both.

Variant	Relative initial rate of iron oxidation (%) in the presence of:			
	–	10 μM Zn	10 μM Tb	Zn + Tb
rHF	100	17.1	16.0	2.2
Substitutions at the three-fold channel				
D131A + E134A	43.3	4.5	36.5	5.7
D131I + E134F	38.5	9.4	21.8	5.7
E134A	77.9	11.7	65.3	7.1
Substitutions around the three-fold channel				
H118A	68.3	12.6	14.1	3.2
C90E + C102A + C130A	91.3	14.6	7.2	1.7
H128A	110.8	13.6	–	–
Substitutions on the cavity surface				
E61A	16.3	4.3	4.0	2.2
E61A + E64A + E67A	18.9	3.1	2.9	1.3
H57E + H60E	99	16.1	8.0	1.6

rHF, suggesting that Cys-130 and His-118 also take part in the reaction.

Iron uptake by ferritin is a complex mechanism that involves various metal binding sites, the importance of which varies under different conditions. When the rate of spontaneous iron hydrolysis is high, e.g. in neutral/alkaline Fe(II) solutions, efficient nucleation centres in the ferritin cavities appear to be sufficient to determine iron mineralization without the need for ferroxidase centres and, under these conditions, rLF is even more efficient than rHF in building up large iron cores [22]. When the spontaneous iron hydrolysis is slow, e.g. in acidic Fe(II) solutions, the nucleation centres are no longer sufficient to induce iron mineralization, and the activity of the ferroxidase centres becomes essential to promote accumulation/hydrolysis of Fe(III) inside the cavity [12]. In Fe(III) citrate solutions with ascorbate, spontaneous iron hydrolysis is slow and is inhibited by the presence of low concentrations of citrate [45]; thus, in order to mineralize iron, ferritin requires functional sites involving Asp-131 and Glu-134, in addition to ferroxidase centres. These residues form metal binding sites that are active in the sequestration of Fe(III) bound to citrate and in its transfer inside the cavity. In addition, Cys-130 and His-118, which are ligands of Tb (Table 1) and of Fe–nitrosyl complexes [36], appear to have a role in this reaction, as shown by the finding that substitution of these two residues with Ala decreases iron incorporation from Fe(III) citrate (Figure 2).

Our results suggest that the three-fold channel binds iron before it is oxidized and mineralized. The substitutions Asp-131 → Ala and Glu-134 → Ala were shown to abolish the transient formation of mononuclear Fe(III) during iron oxidation in rHF, implying that the two carboxy groups co-ordinate Fe(III) as well as Fe(II) [21,23]. This is consistent with earlier reports showing that Fe(II) and Fe(III) compete for binding to the same site and that both are displaced by other metals [30–32]. The present and previous data do not support the hypothesis that iron is oxidized on the three-fold channels [24,25], but indicate that the channels are active in the mechanism of iron incorporation and probably in the transfer of newly oxidized iron to other macromolecules, including ferritins [26].

In conclusion, the present findings indicate that the three-fold channel is the major, and probably the only, site of iron entry into the ferritin cavity, and support previous indications that Asp-131 and Glu-134 in the narrowest part of the three-fold channels (along with Cys-130 and His-118) form metal binding sites that are active in the mechanism of iron incorporation. Indeed, these residues appear to be essential for iron incorporation under some conditions, such as those with Fe(III) citrate in the presence of ascorbate and dioxygen, which may be closer to the physiological situation than those with Fe(II) salts and dioxygen, where the functionality of these residues is not obvious. It should be noted that the four residues are conserved in all vertebrate ferritins for which sequences are known [1].

REFERENCES

1 Andrews, S. C., Arosio, P., Bottke, W., Briat, J. F., von Darl, M., Harrison, P. M., Laulhere, J. P., Levi., S., Lobreaux, S. and Yewdall, S. J. (1992) J. Inorg. Biochem. **47**, 161–174
2 Theil, E. C. (1987) Annu. Rev. Biochem. **56**, 289–316
3 Harrison, P. M., Andrews, S. C., Artymiuk, P. J., Ford, G. C., Guest, J. R., Hirzmann, J., Lawson, D. M., Livingstone, J. C., Smith, J. M. A., Treffry, A. and Yewdall, S. J. (1991) Adv. Inorg. Chem. **36**, 449–486
4 Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L. and Yariv, J. (1984) Philos. Trans. R. Soc. London B **304**, 551–565
5 Bauminger, E. R., Harrison, P. M., Hechel, D., Hodson, N. W., Nowik, I., Treffry, A. and Yewdall, S. J. (1993) Biochem. J. **296**, 709–719
6 St. Pierre, T. G., Kim, K. S., Webb, J., Mann, S. and Dickson, D. P. E. (1990) Inorg. Chem. **29**, 1870–1874
7 Yariv, J., Kalb, A. J., Sperling, R., Bauminger, E. R., Cohen, S. G. and Ofer, S. (1981) Biochem. J. **197**, 171–175
8 Cheesman, M. R., Le Brun, N. E., Kadir, F. H. A., Thomson, A. J., Moore, G. R., Andrews, S. C., Guest, J. R., Harrison, P. M., Smith, J. M. A. and Yewdall, S. J. (1993) Biochem. J. **292**, 47–56
9 Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) J. Biol. Chem. **253**, 4451–4458
10 Wicks, R. E. and Entsch, B. (1993) Biochem. Biophys. Res. Commun. **192**, 813–819
11 Lawson, D. M., Artymiuk, P. J., Yewdall, S. J., Livingstone, J. C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C. D., Shaw, W. and Harrison, P. M. (1991) Nature (London) **349**, 541–544
12 Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. and Arosio, P. (1988) J. Biol. Chem. **263**, 18086–18092
13 van Wuytswinkel, O., Savino, G. and Briat, J. F. (1995) Biochem. J. **305**, 253–261
14 Lawson, D. M., Treffry, A., Artymiuk, P. J., Harrison, P. M., Yewdall, S. J., Luzzago, A., Cesareni, G., Levi, S. and Arosio, P. (1989) FEBS Lett. **254**, 207–210
15 Santambrogio, P., Levi, S., Cozzi, A., Rovida, E., Albertini, A. and Arosio, P. (1993) J. Biol. Chem. **268**, 12744–12748
16 Sun, S., Arosio, P., Levi, S. and Chasteen, N. D. (1993) Biochemistry **32**, 9362–9369
17 Chen-Barrett, Y., Harrison, P. M., Treffry, A., Quail, M. A., Arosio, P., Santambrogio, P. and Chasteen, N. D. (1995) Biochemistry **34**, 7847–7853
18 Sun, S. and Chasteen, M. D. (1992) J. Biol. Chem. **267**, 25160–25166
19 Xu, B. and Chasteen, N. D. (1991) J. Biol. Chem. **266**, 19965–19970
20 Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. and Treffry, A. (1989) Biochemistry **25**, 5486–5493
21 Treffry, A., Bauminger, E. R., Hechel, D., Hodson, N. W., Nowik, I., Yewdall, S. J. and Harrison, P. M. (1993) Biochem. J. **296**, 721–728
22 Levi. S., Santambrogio, P., Cozzi, A., Rovida, E., Corsi, B., Tamborini, E., Spada, S., Albertini, A. and Arosio, P. (1994) J. Mol. Biol. **238**, 649–654
23 Bauminger, E. R., Treffry, A., Hudson, A. J., Hechel, D., Hodson, N. W., Andrews, S. C., Levi, S., Nowik, I., Arosio, P., Guest, J. R. and Harrison, P. M. (1994) Biochem. J. **302**, 813–820
24 Desideri, A., Stefanini, S., Polizio, F., Perruzzelli, R. and Chiancone, E. (1991) FEBS Lett. **287**, 10–14
25 Yablonsky, M. J. and Theil, E. C. (1992) Biochemistry **31**, 9680–9684
26 Levi, S., Santambrogio, P., Cozzi, A., Rovida, E., Albertini, A., Yewdall, S. J., Harrison, P. M. and Arosio, P. (1992) Biochem. J. **288**, 591–596
27 Artymiuk, P. J., Bauminger, E. R., Harrison, P. M., Lawson, D. M., Nowik, I., Treffry, A and Yewdall, S. J. (1991) in Iron Biominerals (Frankel, R. B. and Blakemore, R. P., eds.), pp. 269–294, Plenum, New York
28 Treffry, A. and Harrison, P. M. (1984) J. Inorg. Biochem. **21**, 9–20
29 Harrison, P. M. and Lilley, T. H. (1989) Phys. Bioinorg. Chem. Ser. **5**, 123–238
30 Stefanini, S., Desideri, A., Vecchini, P., Drakenberg, T. and Chiancone, E. (1989) Biochemistry **28**, 378–382
31 Werdeska, J. G., Viglione, B. and Chasteen, N. D. (1986) J. Biol. Chem. **262**, 6677–6683
32 Chasteen, N. D. and Theil, E. C. (1982) J. Biol. Chem. **257**, 7672–7677
33 Hanna, P. M., Chasteen, N. D., Rottman, G. A. and Aisen, P. (1991) Biochemistry **30**, 9210–9216
34 Gerfen, G. J., Hanna, P. M., Chasteen, N. D. and Singil, D. J. (1991) J. Am. Chem. Soc. **113**, 9513–9519
35 Jacobs, D., Watt, G. D., Frankel, R. B. and Papaefthymiou, G. C. (1989) Biochemistry **28**, 9216–9221
36 Lee, M., Arosio, P., Cozzi, A. and Chasteen, N. D. (1994) Biochemistry **33**, 3679–3687
37 Zhao, Z., Malik, A., Lee, M. L. and Watt, G. D. (1994) Anal. Biochem. **218**, 47–54
38 Pead, S., Durrant, E., Webb, B., Larsen, C., Heaton, D., Johnson, J. and Watt, G. D.(1995) J. Inorg. Biochem. **59**, 15–27
39 Wade, V. J., Levi, S., Arosio, P., Treffry, A., Harrison, P. M. and Mann, S. (1991) J. Mol. Biol. **221**, 1443–1452
40 Santambrogio, P., Levi, S., Cozzi, A., Corsi, B. and Arosio, P. (1996) Biochem. J. **314**, 139–144
41 Laulhere, J. P. and Briat, J. F. (1993) Biochem. J. **290**, 693–699
42 Levi, S., Luzzago, A., Franceschinelli, F., Santambrogio, P., Cesareni, G. and Arosio, P. (1989) Biochem. J. **264**, 381–388
43 Yang, D. and Nagayama, K. (1995) Biochem. J. **307**, 253–256
44 Precigoux, G., Yariv, J., Gallois, B., Dautant, A., Courseille, C. and Langlis d'Estaintot, B. (1994) Acta Crystallogr. **D50**, 739–743
45 Harris, D. C. and Aisen, P. (1973) Biochim. Biophys. Acta **329**, 156–158

Received 2 January 1996/28 February 1996; accepted 21 March 1996