Evidence that H- and L-chains have co-operative roles in the iron-uptake mechanism of human ferritin

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The ability to incorporate iron *in vitro* was studied in homopolymers of human ferritin L-chain, human ferritin H-chain and its variants and in homopolymer mixtures. The H-chain variants carried amino acid substitutions in the ferroxidase centre and/or in carboxy residues on the cavity surface. Iron incorporation was examined by gel electrophoresis of the reaction products by staining for iron and protein. It was found that inactivation of the ferroxidase centre combined with the substitution of four carboxy groups on the cavity abolished the ability of H-chain ferritin to incorporate iron. Competition experiments with limited amounts of iron showed that, at neutral pH, L-chain ferritin is more efficient in forming iron cores than the H-chain variants altered at the ferroxidase activity or in the cavity. Competition experiments at pH 5.5 demonstrated that L-chain apoferritin is able to incorporate iron only when in the presence of H-chain variants with ferroxidase activity. The results indicate that L-chain apoferritin has a higher capacity than the H-chain apoferritin to induce iron-core nucleation, whereas H-chain ferritin is superior in promoting Fe(II) oxidation. The finding of co-operative roles of the H- and L-chains in ferritin iron uptake provides a clue to understanding the biological function of isoferritins.

INTRODUCTION

The iron-storage protein, ferritin, is a spherical shell composed of 24 subunits with a large cavity where iron is deposited as the mineral ferrihydrite (Ford *et al.* 1984). Each subunit is a bundle of four long helices with a fifth short helix, a short non-helical extension at the N- and C-termini and a long loop connecting helices B and C at opposite ends of the bundle (Ford *et al.*, 1984; Harrison *et al.*, 1987, 1991). The major function of this molecule is the sequestration of iron by formation of an inorganic core of hydrous Fe(III) oxide or ferrihydrite. The steps by which the core is laid down are not completely specified, but it is clear that iron uptake *in vitro* involves Fe(II) oxidation and nucleation of ferrihydrite on the cavity surface (Harrison *et al.*, 1991) and that carboxy groups are implicated (Wetz & Crichton, 1976; Harrison *et al.*, 1986; Wardeska *et al.*, 1986; Yang *et al.*, 1987). Natural ferritins are composed of various proportions of two subunit types, named H and L (Arosio *et al.*, 1978), which are genetically distinct but share 55 % amino acid sequence identity (Boyd *et al.*, 1985). The study of recombinant H (rHF; Levi *et al.*, 1987, 1988) and L homopolymers (rLF; Levi *et al.*, 1989a) of human ferritin provides insights into functional differences between H- and L-chains. Both homopolymers can incorporate iron, but rHF does so at rates several times those of rLF (Levi *et al.*, 1988, 1989a). Lawson *et al.* (1989, 1991) identified a ferroxidase centre within the four-helix bundle of the H-chain. It has been established that oxo-bridged Fe(III) dimers that are early intermediates of core formation are formed at this centre (Bauminger *et al.*, 1991b). Residues Glu-27, Glu-61, Glu-62, His-65 and Glu-107 act as metal ion ligands with Glu-61 also adopting a second position when it binds metal on the cavity surface (Lawson *et al.*, 1991). Site-directed modification of Glu-62→Lys and His-65→Gly (H- to L-subunit changes, variant previously named 222) results in the loss of ferroxidase activity, in the ability to form Fe(III) dimers (Bauminger *et al.*, 1991b) and in a decrease in the rate of

iron incorporation (Lawson *et al.*, 1989; Broxmeyer *et al.*, 1991). Engineering of rHF at various sites, including the hydrophobic fourfold and hydrophilic threefold intersubunit channels, the N- and C-terminal extensions, or the loop connecting B–C helices, has little influence on iron uptake (Levi *et al.*, 1989b; Treffry *et al.*, 1989), and the substitution of three carboxy groups exposed to the cavity (E61, 64, 67) caused a 5–6-fold decrease in the rate of iron uptake (Levi *et al.*, 1990; Wade *et al.*, 1991). Alteration of the ferroxidase site, together with the substitution of these three cavity carboxy groups, results in a ferritin molecule apparently unable to incorporate iron (Wade *et al.*, 1991). These findings indicate that specific iron incorporation in ferritin involves both residues required for ferroxidase activity and the carboxylate ligands in the cavity, which promote nucleation of ferrihydrite (Wade *et al.*, 1991). However, functional differences between rLF and the rHF variant E62K–H65G–K86Q (Glu-62→Lys, His65→Gly) with inactivated ferroxidase activity, have not so far been demonstrated, thus the significance of the large number of genetically controlled amino acid substitutions between these two proteins remains unclear.

The present study aims to elucidate the functional difference between rLF and rHF, including the relative importance of the ferroxidase and putative nucleation centres. rLF, rHF and rHF variants with substitutions in the ferroxidase centre and/or in carboxy groups on the cavity surface were compared in conditions in which iron is allowed to oxidize in the presence of two competing ferritins. The results show that, at pH 7.0, rLF facilitates iron-core formation to a greater extent than the above rHF variants (including E62K–H65G–K86Q) and confirm that cavity carboxy groups play an important role in core formation. It is suggested that the higher efficiency of rLF in core formation is related to a higher concentration of negative charges in its cavity. Another finding of considerable significance is that the ability of rLF to incorporate iron, which is lost under acidic conditions, is restored by the presence of rHF.

Abbreviations used: rHF, recombinant human H-chain ferritin; rLF, recombinant human L-chain ferritin; FeNTA, ferric nitrilotriacetate complex.
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Table 1. List of rHF variants

Code	Site	Alteration
E61A-K86Q	Cavity*	Glu-61 → Ala + Lys-86 → Gln
E61A-E64A-E67A	Cavity	Glu-61, -64, -67 → Ala
E62K-H65G-K86Q	Ferroxidase*	Glu-62 → Lys + His-65 → Gly + Lys-86 → Gln
A222	Ferroxidase and cavity*	Glu-62 → Lys + His-65 → Gly + Lys-86 → Gln + Glu-61, -64, -67 → Ala
S1	Ferroxidase and cavity*	Glu-62 → Lys + His-65 → Gly + Lys-86 → Gln + Glu-61, -64, -67 → Ala + Asp-42 → Ala

* The modification K-86 → Q on the loop region was introduced to enable crystallization.

MATERIALS AND METHODS

Ferritins and variants

Production of human H-ferritin from *Escherichia coli* strains transformed with plasmid pEMBLex2HFT has been described (Levi *et al.*, 1987, 1988). The H-chain ferritin variant E62K-H65G-K86Q is described in Lawson *et al.* (1989), rHF variants E61A-E64A-E67A and E61A-E64A-E67A-E62K-H65G-K86Q (code A222) were described in Wade *et al.* (1991). The rHF variants E61A-K86Q and E62K-H65G-K86Q-E61A-E64A-E67A-D42A (code S1) were constructed by deoxyoligonucleotide-directed mutagenesis (Sollazzo *et al.*, 1985) on the plasmid pEMBLex2HFT, and the constructs were checked by DNA sequencing. The plasmid for the overexpression of the complete sequence of human L-chain was constructed with a procedure analogous to the one used for pEMBLex2HFT (Levi *et al.*, 1987). Briefly, the L-chain cDNA fragment of 820 bp, containing the entire coding sequence, was obtained by partial digestion of the plasmid pFr36 with *pst*I (Costanzo *et al.*, 1984). This fragment was cloned in the M13 phage derivative mp7, excised by *Sal*I digestion and cloned in the expression vector pEMBLex2 (Sollazzo *et al.*, 1985). A stretch of 155 bp was then deleted by site-directed mutagenesis in order to put the coding region in frame with the translational initiation codon, under the control of the λ P_L promoter. The resulting plasmid encodes the complete and correct sequence of the human ferritin L-chain. It was named pEMBLex2LFT.

Overexpression of H-chain ferritins and variants was performed by the procedure described in Levi *et al.* (1988, 1989b) consisting of heat induction of the transformed *E. coli* strains at 42 °C for 4 h. With this procedure, H-chain ferritin and variants were recovered in the soluble fraction of *E. coli* extracts, whereas most of the L-chain ferritin accumulated in the insoluble fraction. We found that by limiting the heat induction of the cells at 42 °C for 5 min, followed by 2–3 h at 37 °C, L-chain ferritin was expressed as efficiently as H-chain ferritins (5–10% of cell proteins), recovered in high proportion (70–90%) in the soluble fraction, and could be easily purified. In some experiments, 1 mM-ferric nitrilotriacetate (FeNTA) was added to the broth during induction.

The same purification procedure was used for rHF and variants. Briefly, cells were disrupted by sonication, the soluble fraction was heated at 75 °C for 10 min, precipitated with (NH₄)₂SO₄ (520 g/l), treated with DNAase and subjected to gel filtration on a Sepharose 6B column. Expression and recovery of purified protein were similar for rHF and its variants. Purification of recombinant L-chain ferritin was analogous but included chromatography on Sephacryl S200 and DEAE-Sepharose columns instead of Sepharose 6B, as described in Levi *et al.* (1989a). All ferritins were electrophoretically pure. Apoferritins

were prepared by treatment with 1% (v/v) thioglycolic acid, pH 5.5, and 2,2'-bipyridine followed by dialysis against 0.1 M-Hepes, pH 7.0 (Levi *et al.*, 1988). The apoferritins had a 280/260 nm absorbance ratio higher than 1.3. Protein concentration was determined with BCA reagent (Pierce) with BSA as standard.

Iron incorporation

This was carried out in 0.1 M-Hepes buffer, pH 7.0, or in 0.1 M-Mes, pH 5.5, with Fe/ferritin molar ratio of 1000:1. Iron-uptake kinetics were performed as in Levi *et al.* (1988, 1989b). When iron uptake was to be monitored by gel electrophoresis, apoferritins (0.5 mg/ml) were incubated for 2 h at room temperature with 1 mM-ferrous ammonium sulphate (Levi *et al.*, 1988, 1989a,b). The reaction was stopped by addition of 10 mM-4,7-diphenyl-1,10-phenanthroline sulphonate and 5 mM-desferrioxamine. Electrophoresis was performed in 7.5% (w/v) or 6.0% (w/v) polyacrylamide gels. In the experiments at pH 5.5 the stacking gels were made at the same pH. Gels were stained with either Coomassie Blue or Prussian Blue.

RESULTS

Human H- and L-chain ferritins and five rHF variants were studied. The latter are described in Table 1. In the first two variants E61A-K86Q and E61A-E64A-E67A, glutamates 61 or 61, 64 and 67 which lie on the cavity surface (see Fig. 1) are replaced by alanine. Note that Glu-61 can occupy an alternative position as part of the ferroxidase centre or on the inside surface (Lawson *et al.*, 1991). The third variant E62K-H65G-K86Q has inactivated ferroxidase centres owing to the substitution of two residues with those found in L-chains (E62K-H65G) (Lawson *et al.*, 1989; Broxmeyer *et al.*, 1991); the final two variants carried substitution of the ferroxidase centre combined with substitution of the three cavity Glu-61, -64, -67 with Ala (code name A222) and a further substitution of the cavity Asp-42 with Ala (code S1). Ferritins and variants had analogous expression yields and thermostability, and were purified with the same procedure. The ferritins rHF, E61A-E64A-E67A and E61A-K86Q were purified as amber-coloured iron-containing proteins, whereas the other ones were colourless, even when extracted from *E. coli* grown in 1 mM-FeNTA (not shown).

In our previous studies we used a rLF preparation in which the first two N-terminal amino acids Ser-Ser were substituted with Asp-Pro (Levi *et al.*, 1989a). In the present work we cloned the correct sequence of the human L-chain in the same expression vector used for rHF, which facilitates site-directed mutagenesis. The two L-chain ferritins show the same functional properties of iron uptake and incorporation (not shown) and differ only in electrophoretic mobility. In fact, the correct rLF, unlike the N-

Fig. 1. Schematic diagram of the inside surfaces of human H-chain and L-chain homopolymers

The computer plots were drawn with the aid of the program MOLSCRIPT (Kraulis, 1991) and show either a single H (a) or L (b) subunit or four subunit stereopairs of H (c) and L (d) viewed along a twofold axis. Glutamates and aspartates are shown as open circles, and arginines, lysines and histidines as filled circles. The numbering of acidic residues on the single subunits corresponds to the amino acid sequence of human H-chain ferritin. (a) and (c) are based on the three-dimensional structure of human H-chain ferritin as determined by Lawson *et al.* (1991). (b) and (d) show a model of the three-dimensional structure of human L-chain homopolymer based on that of recombinant rat L-chain ferritin (Lawson *et al.*, 1991) with amino acid substitutions where appropriate (85% identity). Note that the greater density of acidic residues in the cavity surface of the L-chain is somewhat misleading because the last six residues at the C-termini of human H-chains (Asp-Ser-Asp-Asn-Glu-Ser) have been omitted, since there is no visible electron density for these residues. It was shown that the substitution of the last seven C-terminal residues of the H-chain by the three C-terminal residues of the L-chain has little effect on iron uptake (Levi *et al.*, 1989b). Note that a cluster of carboxy residues around the twofold axes of L-chain homopolymers is decreased in H-chain homopolymers owing to the replacement by histidines of glutamates 57 and 60 [marked by arrows in (b)]. This cluster comprising Glu-57, -60, -61 and possibly -64 and -67 (although the latter participates in a salt bridge with the twofold intersubunit interface) extends over two subunits and may be important for ferrihydrite nucleation in L-chains; this may account for the greater ability to drive nucleation under conditions in which the supply of Fe(III) is not limiting. The cluster of hydrophilic residues in the centre of the H-subunit in (a), marked by an arrow, represents the ferroxidase centre. In L-subunits (b), several of the ferroxidase-centre residues are changed and activity is lacking.

terminus variant, can be separated by non-denaturing PAGE from rHF and its variants, a property exploited in the present study.

Iron uptake

At pH 6 and below, only rHF, E61A-K86Q and E61A-E64A-E67A showed iron-uptake activity, whereas rLF and the other variants were inactive (not shown). At pH 7.0, rHF, E61A-K86Q and E61A-E64A-E67A exhibited faster

iron uptake than the iron autoxidation control, rLF and E62K-H65G-K86Q showed iron-uptake kinetics slightly faster than the control, whereas both A222 and S1, which also contain alterations in the cavity, exhibited rates that were not significantly different from autoxidation (Fig. 2). Prussian Blue staining of the reaction products confirmed that only A222 and S1 did not incorporate iron in these conditions (not shown). However, as shown in Fig. 3, some iron incorporation into the A222 variant was achieved by increasing fourfold the protein concentration in

Fig. 2. Iron-uptake progress curves of apoferritins

Ferrous ammonium sulphate (0.1 mM) was added to the recombinant apoferritins (0.1 μ M) in 0.1 M-Hepes, pH 7.0, at 30 °C and iron uptake was monitored by measuring absorbance at 310 nm. H, rHF; L, rLF; A5, E61A-K86Q; A2, E61A-E64A-E67A; 222, E62K-H65G-K86Q; A222, E62K-E61A-E64A-E67A-H65G-K86Q; ----, control in the absence of ferritin.

Fig. 3. Iron incorporation into apoferritins at pH 7.0

Apoferritins (1 μ M) were incubated for 2 h at 20 °C in 0.1 M-Hepes pH 7.0 with 1 mM-ferrous ammonium sulphate and then were loaded (10 μ g) on to 7.5% (w/v) non-denaturing polyacrylamide for PAGE. A222 loading was achieved with 4 μ M-ferritin and 4 mM-Fe(II) concentrations. The gels were stained with Prussian and Coomassie Blue. H, rHF; L, rLF; A2, E61A-E64A-E67A; 222, E62K-H65G-K86Q; A222, E62K-E61A-E64A-E67A-H65G-K86Q.

the reaction mixture while keeping the same Fe/protein ratio (other conditions induced massive protein precipitation). All attempts to load detectable iron into S1 failed, suggesting that this protein cannot incorporate iron *in vitro*.

Competition experiments

The ability to separate rLF and rHF or H-chain variants by electrophoresis enabled the results of competition experiments to be analysed. Limited amounts of ferrous iron were added to equimolecular mixtures of rLF and rHF variants. After incubation for 2 h at room temperature, the proteins were separated by electrophoresis and stained with Prussian Blue. The experiments showed that at pH 7.0 in the presence of rLF, rHF

Fig. 4. Competition between rLF and rHF or its variants for iron incorporation at pH 7.0

Ferrous ammonium sulphate (1 mM) was added to the apoferritins (1 μ M) and the samples were incubated for 2 h at 20 °C. They were loaded on to 7.5% (w/v) polyacrylamide gels, and after electrophoresis, the gels were stained with Coomassie Blue or Prussian Blue. The arrows indicate the mobility of ferritin monomers. H, rHF; L, rLF; A5, E61A-K86Q; A2, E61A-E64A-E67A; 222, E62K-H65G-K86Q; A222, E62K-E61A-E64A-E67A-H65G-K86Q; S1, D42A-E62K-E61A-E64A-E67A-H65G-K86Q.

Fig. 5. Competition experiments at pH 5.5

Conditions were the same as in Fig. 4, except that incubations were performed at pH 5.5. (a) Ferritin code names as in Fig. 4. (b) Competition between rHF and variant E62K-H65G-K86Q (222), separated on 6% polyacrylamide gels (lane 2; lanes 1 and 3 are the controls of the two separated proteins). The positions of ferritin monomers are marked by arrows; A, Coomassie Blue stain; B, Prussian Blue stain.

incorporated most of the available iron (Fig. 4). In contrast, the rHF variants E61A-K86Q, E61A-E64A-E67A and E62K-H65G-K86Q incorporated much less iron than rLF (Fig. 4), and variants A222 and S1 with both ferroxidase centre and cavity alterations were unable to compete with rLF for the available iron (Fig. 4).

Similar experiments were performed at pH 5.5, conditions in which rLF and E62K-H65G-K86Q alone do not form iron

Fig. 6. Effects of rHF on iron incorporation into rLF at pH 5.5

Various amounts of rHF were mixed with 10 µg of rLF and Fe(II) was added at 1000:1 molar ratio. After incubation and electrophoretic separation as in Fig. 5, the iron incorporated into the ferritin monomers was measured by densitometry of the Prussian Blue-stained gels. L, iron incorporated in rLF; H+L, total iron incorporated in both rHF and rLF.

cores (Fig. 5a). However, the addition of rHF to rLF enabled it to incorporate iron as did the two rHF variants with ferroxidase activity E61A-E64A-E67A and E61A-K86Q, whereas the E62K-H65G-K86Q variant lacking this activity did not (Fig. 5a). When rHF and E62K-H65G-K86Q were incubated with iron, only rHF was able to form iron cores (Fig. 5b). When the ratio of rHF to rLF was varied at constant rLF and iron concentrations, pH 5.5, ferritin iron incorporation reached a plateau at a molar ratio of rHF/rLF equal to 1:2, and the fraction of iron in rLF was maximum (50%) at this ratio (Fig. 6).

DISCUSSION

The results presented here confirm that the rate of iron uptake of H-chain ferritin is diminished by substitutions of ferroxidase-centre residues (Lawson *et al.*, 1991) or of carboxy groups on the cavity surface (Wade *et al.*, 1991). Replacement of Glu-61 or Glu-61, Glu-64 and Glu-67 by Ala in the variants E61A-K86Q and E61A-E64A-E67A lowers the rate of iron uptake to about the same extent. This could mean that Glu-61 is a ferroxidase-centre ligand that facilitates the migration of iron from oxidation to nucleation centres (Lawson *et al.*, 1991).

All the ferritins with inactive ferroxidase centres have rates of iron uptake similar to the rate of autoxidation in the control (Fig. 2) and it is difficult to assess functional differences between them from progress curves. However, they were found to differ in iron incorporation: the substitution of both cavity carboxy groups and ferroxidase-centre residues in variant A222 greatly decreases (Wade *et al.*, 1991) but does not fully abolish (Fig. 3) the iron-incorporation capacity, and this is completely lost by the additional substitution of Asp-42 in variant S1. Further differences in the ability to form iron cores were demonstrated by the competition experiments with rLF (Fig. 4): under the conditions used the order of efficiency is rHF > rLF > E61A-K86Q = E61A-E64A-E67A > E62K-H65G-K86Q (A222 and S1 are inactive). Thus rLF is superior to the ferroxidase-inactivated H-chain variant E62K-H65G-K86Q in promoting core formation, but also, and surprisingly, it competes successfully for iron with the rHF variants E61A-K86Q and E61A-E64A-E67A in which the ferroxidase centres are intact and which show faster rates of iron uptake when examined separately (Fig. 2). Clearly the ability to form a stable iron core in ferritin is not governed solely by the ferroxidase centres, but also by structural differences between H- and L-chain ferritins. The present findings support recent data obtained by the use of

Mossbauer spectroscopy: when small numbers of Fe(II) atoms (12 or 38 per molecule) were allowed to oxidize at pH 7.0 in the presence of apoferritin, a larger fraction of Fe(III) was found as a cluster when the ferritin was L-chain rich (about 85% L) than in H-chain homopolymers (Bauminger *et al.*, 1991b).

It has been shown previously that only ferritins with active ferroxidase centres incorporate iron at pH below 6 (Levi *et al.*, 1988, 1989a,b). It is again shown that, at pH 5.5, rHF incorporates iron, whereas rLF and E62K-H65G-K86Q are inactive on their own (Fig. 5a). Surprisingly, however, rLF although inactive alone, does incorporate iron when in the presence of rHF or variants E61A-K86Q and E61A-E64A-E67A (Fig. 5a). Variants E62K-H65G-K86Q and A222 are inactive even in the presence of rHF (Fig. 5b). This helper activity of rHF is evident for rHF/rLF ratios as low as 1:10, and reaches a plateau at ratios above 1:2 (Fig. 6). In Figs. 4-6, the two ferritins were mixed before the addition of Fe(II). If rLF was added to rHF after Fe(II), incorporation of iron into rLF occurred during the first 3-5 min, but not after 30 min (results not shown).

The experimental findings can be interpreted as follows. The formation of ferrihydrite mineral in apoferritin is promoted on the one hand by catalysing Fe(II) oxidation, thus accelerating the supply of Fe(III), and on the other hand by increasing the likelihood of ferrihydrite nucleation. These two functions are, to a large extent, segregated within different chains, respectively H and L, and differently affected by pH. Higher pH values promote iron hydrolysis (Schneider, 1988) and favour iron-core formation in ferritins (Macara *et al.*, 1972; Bauminger *et al.*, 1989) including the ones without ferroxidase centres. At pH 5.5-6.0, the reaction is essentially oxidation driven and rHF provides its own cavity with a sufficient concentration of Fe(III) to allow nucleation to proceed. At these pH values, rLF, which lacks the ferroxidase centre, does not produce iron core when alone, but its nucleation efficiency is sufficiently high to sequester part of the iron oxidized by rHF.

The present data indicate that iron-core nucleation is dependent on carboxy groups on the cavity surface. Schematic diagrams of the cavity surface of human H- and L-chain ferritins are shown in Fig. 1. The ferroxidase centre is seen as a cluster of hydrophilic residues within the H-chains with Glu-61 on the cavity side, and the diagrams show that the substitutions in variant S1 remove most of the carboxy groups in the cavity (Glu-61, -64, -67, Asp-42 to Ala). In the L-chain model, the ferroxidase centre is replaced by a salt bridge (Lawson *et al.*, 1991), and Glu-61 is swung into a position facing that cavity, where it may participate in ferrihydrite nucleation (Harrison *et al.*, 1991; Lawson *et al.*, 1991). In addition, it shows that near to Glu-61 in the L-chain, there are Glu-57 and Glu-60 which in the H-chain are His. These two glutamates participating in a cluster of negative charges may be responsible for the greater efficiency of L-chains in iron-core nucleation, and this hypothesis will be tested by site-directed mutagenesis.

Another important finding of the present experiments is that iron can exchange between ferritin molecules. This is in agreement with the results of Mossbauer spectroscopic studies (Bauminger *et al.*, 1991a) which showed that iron bound to one ferritin molecule, but not incorporated into large clusters, could move to the iron core of another molecule. Such iron is also available to transferrin (Bakker & Boyer, 1986; Levi *et al.*, 1988) and it is conceivable that ferritin H-chains could act as general ferroxidases providing Fe(III) for other molecules.

In conclusion, we have shown that H- and L-chains can act co-operatively when in separate molecules. It is likely that within the same molecule they will have even more co-operative roles, and that the different H/L-chain ratios in various isoferritins may preserve a delicate balance between the need for fast iron

oxidation and nucleation. It is of interest that in the iron-storage organs, such as liver and spleen, the ferritins are L-chain-rich and many of them contain large crystalline cores (Mann *et al.*, 1986), possibly emphasizing the importance of L-subunit nucleation-driven reactions. In heart (Arosio *et al.*, 1978) and erythrocytes (Cazzola *et al.*, 1983), the ferritins are H-chain-rich, and here their major role may be in removing toxic iron rather than providing a reserve. The evidence of co-operative roles of H- and L-chains goes some way to explaining why nature prefers its ferritins to be heteropolymers, at least in mammals.

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REFERENCES

- Arosio, P., Adelman, T. G. & Drysdale, J. W. (1978) *J. Biol. Chem.* **253**, 4451–4458
- Bakker, G. R. & Boyer, R. F. (1986) *J. Biol. Chem.* **261**, 13182–13185
- Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. & Treffry, A. (1989) *Biochemistry* **25**, 5486–5493
- Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. & Treffry, A. (1991a) *Proc. R. Soc. London Ser. B* **244**, 211–217
- Bauminger, E. R., Harrison, P. M., Hechel, D., Nowik, I. & Treffry, A. (1991b) *Biochim. Biophys. Acta* **118**, 48–58
- Boyd, D., Vecoli, C., Belcher, D. M., Jain, S. K. & Drysdale, J. J. (1985) *J. Biol. Chem.* **260**, 11755–11761
- Broxmeyer, H. E., Cooper, S., Levi, S. & Arosio, P. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 770–774
- Cazzola, M., Dezza, L., Bergamaschi, G., Barosi, G., Bellotti, V., Caldera, D., Ciriello, M. M., Quaglini, S., Arosio, P. & Ascari, E. (1983) *Blood* **62**, 1078–1087
- Costanzo, F., Santoro, C. & Cortese, R. (1984) in *Ferritins and Isoferritins as Biochemical Markers* (Albertini, A., Arosio, P., Chiancone, E. & Drysdale, J. W., eds.), pp. 79–85, Elsevier, Amsterdam
- Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L. & Yariv, J. (1984) *Philos. Trans. R. Soc. London Ser. B* **304**, 551–565
- Harrison, P. M., Treffry, A. & Lilley, T. H. (1986) *J. Inorg. Biochem.* **27**, 287–293
- Harrison, P. M., Ford, G. C., Rice, D. W., Smith, J. M. A., Treffry, A. & White, J. L. (1987) *Biochem. Soc. Trans.* **15**, 744–748
- 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. & Yewdall, S. J. (1991) *Adv. Inorg. Chem.* **36**, 449–486
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- Lawson, D. M., Treffry, A., Artymiuk, P. J., Harrison, P. M., Yewdall, S. J., Luzzago, A., Cesareni, G., Levi, S. & Arosio, P. (1989) *FEBS Lett.* **254**, 207–210
- 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. & Harrison, P. M. (1991) *Nature (London)* **349**, 541–544
- Levi, S., Cesareni, G., Arosio, P., Lorenzetti, R., Soria, M., Sollazzo, M., Albertini, A. & Cortese, R. (1987) *Gene* **51**, 267–272
- Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. & Arosio, P. (1988) *J. Biol. Chem.* **263**, 18086–18092
- Levi, S., Salfeld, J., Franceschinelli, F., Cozzi, A., Dorner, M. H. & Arosio, P. (1989a) *Biochemistry* **28**, 5179–5184
- Levi, S., Luzzago, A., Franceschinelli, F., Santambrogio, P., Cesareni, G. & Arosio, P. (1989b) *Biochem. J.* **264**, 381–388
- Levi, S., Luzzago, A., Santambrogio, P., Cozzi, A., Cesareni, G. & Arosio, P. (1990) in *Iron Biominerals* (Frankel, R. B. & Blackmore, R. P., eds.), pp. 339–348, Plenum Press, New York
- Macara, I. G., Hoy, G. & Harrison, P. M. (1972) *Biochem. J.* **126**, 151–162
- Mann, S., Bannister, J. V. & Williams, R. J. P. (1986) *J. Mol. Biol.* **188**, 225–232
- Schneider, W. (1988) *Chimia* **42**, 9–20
- Sollazzo, M., Frank, R. & Cesareni, G. (1985) *Gene* **37**, 199–206
- Treffry, A., Harrison, P. M., Luzzago, A. & Cesareni, G. (1989) *FEBS Lett.* **247**, 268–272
- Wade, V. J., Levi, S., Arosio, P., Treffry, A., Harrison, P. M. & Mann, S. (1991) *J. Mol. Biol.* **221**, 1443–1452
- Wardeska, J. G., Viglione, B. & Chasteen, N. D. (1986) *J. Biol. Chem.* **261**, 6677–6683
- Wetz, K. & Crichton, R. R. (1976) *Eur. J. Biochem.* **61**, 545
- Yang, C. Y., Meagher, A., Huynh, B. H., Sayers, D. E. & Theil, E. C. (1987) *Biochemistry* **26**, 497–503

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