Evidence That a Salt Bridge in the Light Chain Contributes to the Physical Stability Difference between Heavy and Light Human Ferritins*

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Human ferritin, a multimeric iron storage protein, is composed by various proportions of two subunit types: the H- and L-chains. The biological functions of these two genic products have not been clarified, although differences in reactivity with iron have been shown. Starting from the hypothesis that the high stability typical of ferritin is an important property which may be relevant for its iron storage function, we studied ferritin homopolymers of H- and L-chains in different denaturing conditions. In addition we analyzed 13 H-chain variants with alterations in regions conserved within mammalian H-chains. In all the denaturation experiments H-chain ferritin showed lower stability than L-chain ferritin. The difference was greater in guanidine HCl denaturation experiments, where the end products are fully unfolded peptides, than in acidic denaturation experiments, where the end products are peptides with properties analogous to “molten globule.”

The study on H-chain variants showed: (i) ferritin stability was not affected by alterations of regions exposed to the inner or outer surface of the shell and not involved in intra- or inter-chain interactions; (ii) stability was reduced by alterations of sequences involved in inter-subunit interactions such as the deletion of the N-terminal extension or substitutions along the hydrophobic and hydrophilic channels; (iii) stability was increased by the substitution of C-terminal amino acids inside the four-helix bundle with those of the homologous L-chain. One of the residues is involved in a salt bridge in the L-chain, and we concluded that the stability difference between H- and L-ferritins is to a large extent due to the stabilizing effect of this salt bridge on the L-subunit fold.

Ferritin is an ubiquitous iron-containing protein composed of 24 subunits (1–4). The major subunit structural motif is a four-helix bundle, in addition to which there are a short helix, a short non-helical extensions at the N and C termini, and a loop connecting helices B and C at the opposite ends of the bundle (1, 3). The subunits assemble into a protein shell with 4-3-2 symmetry, leaving inter-subunit channels along the 3- and 4-fold axes (1, 3).

Human ferritin is composed of two subunit types, H- and L-chains, with 55% sequence identity, which are encoded by different genes on different chromosomes (5, 6). The different proportions of the two subunits in natural ferritins are under strict genetic control leading to cell-dependent variation (1–4, 7, 8). In order to clarify the functional roles of H- and L-chains they have been overexpressed in Escherichia coli, thus producing ferritin homopolymers in which the specific structural and functional properties of the two chains are more evident (9, 10).

Preliminary x-ray analyses of ferritins containing L-chains (1, 11, 12), H-chains (11, 13), and variant H-chains (13) show homologous subunit conformations and subunit arrangements; the H-chain and variants all had a surface residue converted to that of the L subunit in horse spleen ferritin model to allow an intermolecular salt bridge important for crystallization. Studies on human homopolymers revealed functional distinctions between the two chains: (i) in vitro H-ferritin oxidizes and incorporates iron at faster rates than the L-ferritin, especially at early times after Fe(II) addition to apoferritin (9, 10), a difference probably due to a ferroxidase center on the H-, absent in the L-chain (9, 13); (ii) H-ferritin inhibits iron-induced lipoperoxidation with higher efficiency than L-ferritin (14); (iii) it has been reported that H-ferritin suppresses myeloid and lymphoid cell proliferation (15), with a mechanism which probably involves the interaction with a specific binding site on cell membranes (16) and interference with cellular iron uptake (17). All these properties are lost in H-chain variants carrying the substitution of Glu62 and His65, involved in the ferroxidase center identified in H-chains (13), with Lys and Gly, respectively, residues which are found in the L-chain (17, 18). X-ray structural analyses in progress of the H variant A222 (see Table I), which carries the substitutions Glu62 → Lys and Glu65 → Ala.

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The abbreviations used are: H, heavy; L, light; SDS, sodium dodecyl sulfate; ANS, 8-anilino-1-naphthalenesulfonic acid; GdnHCl, guanidine hydrochloride; rHF, recombinant human H-chain ferritin; rLF, recombinant human L-chain ferritin with a substitution of the first two N-terminal amino acids from Ser-Ser to Asp-Pro.
yses suggest that the greater stability of L-chain ferritins is proteins named "molten globule." H-ferritin was found to be channels also seem to be involved in maintaining shell stabil-

appears to be the presence of a salt bridge situated inside the behaves as a partially folded subunit monomer with properties related to a number of factors, of which an important one results obtained with H-chain variants and structural anal-

Four-helix bundle and cavity

Here we report a study on the denaturation of H- and L-

Electrophoresis—The purified proteins (0.5 mg/ml) were dialed into 50 mM phosphate buffer, in the pH range 2.5–7.4, and in presence or absence of 8 M urea (Merck). After addition of equal volume of 0.25 M Tris-HCl buffer, pH 6.8, containing 20% glycerol and 0.2% SDS to raise the pH and solubilize the peptides, the samples were run on two-layer SDS-polyacrylamide gels with 15% acrylamide. All the variants were purified essentially as in Refs. 9, 10, 13, 17, and 18; briefly, the cellular homogenates were heated at 75 °C for 5 min, clarified, precipitated with ammonium sulfate (80% saturation), and loaded on a Sepharose 6B column. The final protein concentrations were 25–100 μg/ml for far-UV circular dichroism, and 1 mg/ml for near-UV circular dichroism.

**TABLE I**

| N terminal | Alterations | Ref. |
|------------|-------------|-----|
| 115        | Deletion of the N-terminal extension T1–H13 | 9, 17 |
| 119        | Deletion of P88 | 21 |
| 9Cd'       | Duplication of the sequence P88–L106 with the substitutions D91N+D92V | 17, 21 |
| 3-fold channel | Substitution of the sequence 121–125 with the homologous one of L-chain | 21, 22 |
| 175        | D131H,E134H on K86Q | 21, 22 |
| 206        | D131A,E134A on K86Q | 21, 22 |
| C terminus | Deletion of the C-terminal extension G176amber | 21, 23 |
| 105'       | Substitution the last 10 residues with the equivalent residues of L-chain | 21 |
| 152'       | H173L,G176K,D177H,S178D,D179amber | 21 |
| R2'        | L169R | 17, 21, 23 |
| Four-helix bundle and cavity | | |
| A22'       | E61A,E64A,E67A | 17 |
| 222       | E62K,H65G,K86Q | 13, 17, 18 |
| A222      | Substitution A2 + 222 E61A,E62K,E64A,H65G,E67A,K86Q | |

Sequence present in all mammalian ferritins.
Amino acids conserved in all mammalian ferritins.
Made to enable crystallization.
The L-sequence is conserved in all mammalian L-chains.
E62 and H65 are conserved in all H-chains, and K62 is conserved in all mammalian L-chains.

*H*60 → Gly, confirm the formation of a salt bridge between *L*60 and G107.2

Chemical stability studies of ferritin (mainly done on the L-rich protein from horse spleen) showed that it denatures only under extreme conditions: above 80 °C, below pH 3, in boiling 1% SDS, or in high urea or guanidine hydrochloride concentrations at acidic pH (4, 19, 20). Preliminary studies on recombinant human homopolymers indicate that L- and H-ferritin and most of the H-chain variants created by genetic engineering maintain this high stability, e.g. resistance to heat treatment up to 80 °C or to 1% SDS in the cold (9, 10, 22); they also showed that some alterations along the 2-fold axis and at the C terminus decreased stability (9, 21, 23), but they did not explain the molecular bases of high resistance to denaturation typical of ferritin. This property could be important for the major function of ferritin as an iron storage protein and may be related to its slow in vivo turnover with a half-life of 1.5–5.5 days (24, 25).

Here we report a study on the denaturation of H- and L-ferritins with guanidine HCl and at acidic pH values, and of 13 H-chain variants altered in various conserved regions of the molecule. The results show that, at pH below 3, ferritin behaves as a partially folded subunit monomer with properties analogous to the folding intermediates described for other proteins named "molten globule." H-ferritin was found to be less stable than L-ferritin in all denaturation studies. The results obtained with H-chain variants and structural analyses suggest that the greater stability of L-chain ferritins is related to a number of factors, of which an important one appears to be the presence of a salt bridge situated inside the four-helix bundle that is absent from H-chain ferritins. Interactions around the N-terminal residues and the 3- and 4-fold channels also seem to be involved in maintaining shell stabil-

The biological effects of the L-ferritin higher stability are discussed in relation to its interaction with iron.

**MATERIALS AND METHODS**

Ferritins and Variants—Human recombinant H- and L-ferritins (rHF and rLF) were over-expressed in *E. coli* and purified as previously described (9, 10). Variants were obtained by oligonucleotide-directed mutagenesis (26) of the plasmid pEMBLex2HFT (27) by inserting amber codons at the proper position or by substituting or deleting codons. Some of their properties have been described previously (9, 10, 13, 17, 18, 22). All the variants were purified essentially as in Refs. 9, 10, 13, 17, and 18; briefly, the cellular homogenates were heated at 75 °C for 5 min, clarified, precipitated with ammonium sulfate (80% saturation), and loaded on a Sepharose 6B column. All the proteins were the purified proteins by incubation for 18 h in 1% thioglycolic acid, 0.1 M sodium acetate at pH 5.5 in a stoppered test tube; an excess of 2,2'-bipyridine was then added to chelate the ferrous iron, and the sample was dialyzed extensively against 20 mM Tris-HCl buffer, pH 7.4 (28). Protein concentrations were determined by using the BCA assay (Pierce Chemical Co.) calibrated with bovine serum albumin.

Electrophoresis—The purified proteins (0.5 mg/ml) were dialyzed in 50 mM phosphate buffer, in the pH range 2.5–7.4, and in presence or absence of 8 M urea (Merck). After addition of equal volume of 0.25 M Tris-HCl buffer, pH 6.8, containing 20% glycerol and 0.2% SDS to raise the pH and solubilize the peptides, the samples were run on two-layer SDS-polyacrylamide gels with 15% acrylamide at the bottom and 7.5% acrylamide at the top, in order to visualize the native protein and its dissociated subunits on the same gel (23). The gels were stained with Coomassie Brilliant Blue R-250, and, after destaining, densitometry was performed on a 2202 Ultrascan Laser Densitometer (LKB). In other experiments the proteins were equilibrated in 0.1 M phosphate buffer in the pH range 2–4, then run on 6% polyacrylamide gels equilibrated in the same buffer. The gels were stained with Coomassie Blue.

Circular Dichroism—Samples were prepared by diluting the apoproteins in 0.1 M phosphate buffer, in the pH range 2.0–7.4, in the presence or absence of 6 M GdnHCl (Merck), or in 0.1 M phosphate buffer, pH 7.4, in presence of increasing concentrations (0–8 M) of GdnHCl. The final protein concentrations were 25–100 μg/ml for far-UV circular dichroism, and 1 mg/ml for near-UV circular dichroism.

**S. J. Yewdall, P. Hampstead, P. J. Artyomiuk, and P. M. Harrison, unpublished work.**
FIG. 1. Circular dichroism spectra. Far-UV CD spectra of rHF (A) and rLF apoprotein (B) at physiological pH (7.4) and under denaturing conditions (pH 2 and 6 M GdnHCl at pH 3.5). C, near-UV CD spectra of rHF and rLF apoproteins at pH 7.4.

Results

H- and L-ferritins

Circular Dichroism—CD spectra of assembled recombinant H- and L-apoferritins in the 190–250-nm far-UV region were similar to those of horse spleen apoferritin (10, 20), with molar ellipticity values at 222 nm of 19,960 and 22,560, respectively. The far-UV CD spectra indicate that in 6 M GdnHCl, pH 3.5, both proteins are unfolded, while at pH 2 they retain a high proportion of secondary structure with ellipticity values, at 222 nm, of 60–80% of the fully folded proteins (Fig. 1, A and B). The near-UV CD spectra of the two apoproteins were different (Fig. 1C), in agreement with their differences in content and distribution of Phe (6 and 8 in the H- and L-chains, respectively, 4 of which are in conserved positions) and of Tyr (9 and 7 in the H- and L-chains, respectively, 5 of which are in conserved positions). Both ferritins showed a positive peak at 292 nm in the tryptophanyl region, assignable to the single Trp, which is conserved in the two proteins (Fig. 1C). Near-UV CD spectra of the two proteins at pH 2.0 and in 6 M GdnHCl, pH 3.5, were unstructured (not shown).

Guanidine Denaturation—H- and L-apoferritins at pH 7.4 were incubated for at least 18 h with various concentrations of GdnHCl, and the ellipticity values of the samples were measured at 222 nm. The results (Fig. 2A) show that rLF is less stable than rHF (50% unfolding transitions at 4.8 and above 8 M GdnHCl, respectively).

Acidic Denaturation—At pH values below 3, ferritin subunits are dissociated and only partially folded (31–33). We found that in these conditions they bind the hydrophobic probe ANS (30) with fluorescence emission of the L-apofe-
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1. c U T 0 E 0 0.6

\[ \text{FIG. 2. Guanidine HCl denaturation of ferritins and variants.} \]
The ellipticity values at 222 nm of 50 \( \mu \)g/ml apoferritin at \( \text{pH} 7.4 \) and in various GdnHCl concentrations were measured. The data are plotted as fraction of unfolded \( \left( f_U = \frac{\theta_N - \theta}{\theta_D - \theta_D} \right) \), using as references the ellipticity values of the proteins at \( \text{pH} 7.4 \) (native, \( \theta_N \)) and at \( \text{pH} 3.5 \) in 6 M GdnHCl (denatured, \( \theta_D \)). Unfolded, values obtained at \( \text{pH} 3.5 \) in 6 M GdnHCl. A, plots of rHF and rLF; B, plots of the H-chain variants with code names as in Table I.

2. c U T 0 E 0 0.4

\[ \text{FIG. 3. ANS binding.} \]
Fluorescence emission spectra upon excitation at 380 nm of apoferritins (50 \( \mu \)g/ml) at \( \text{pH} 2 \) or at \( \text{pH} 7.4 \) in the presence of 0.1 mM ANS. The spectra of samples in 6 M GdnHCl, \( \text{pH} 3.5 \), are analogous to those of native apoferritins. ANS did not bind to native or unfolded ferritins, and unfolding of the proteins at \( \text{pH} 2 \) by the addition of 3 M GdnHCl abolished ANS fluorescence (not shown). ANS was added to the apoferritins equilibrated at various \( \text{pH} \) values to study acidic denaturation. The 50% transition points were at \( \text{pH} 3.3 \) and 2.6 for rHF and rLF, respectively (Fig. 4A).

3. c U T 0 E 0 0.2

\[ \text{FIG. 4. Acidic denaturation in absence of SDS.} \]
A, monitored by ANS binding: rHF and rLF apoproteins and the H-variant Glu\(^{6'}\) Lys, His\(^{6''}\) + Gly (222) at 50 \( \mu \)g/ml were incubated at various \( \text{pH} \), added of 0.1 mM ANS, excited at 380 nm, and the fluorescence emission at 480 nm was monitored. The plots are expressed as fractions of native ferritin \( (f_N = \frac{F_D - F}{F_0 - F_S}) \) using as references the fluorescence emissions of the samples at \( \text{pH} 7.4 \) (native, \( F_N \)) and at \( \text{pH} 1.5 \) (denatured, \( F_D \)). B, denaturation monitored by gel electrophoresis. rHF (H) and rLF (L) incubated at the \( \text{pH} \) indicated were run anodically on 6% polyacrylamide gels at the same \( \text{pH} \) values (5 \( \mu \)g/lane) and stained with Coomassie Blue. Densitometry of the stained protein bands (Fig. 5A, solid lines), showed that massive dissociation occurs in the \( \text{pH} \) range 3.5–2.5 or slightly higher than in the experiments described above (Fig. 4, A and B), probably due to the dissociating effect of SDS in the gel. The 50% transitions were at \( \text{pH} 3.0 \) and 3.4 for rLF and rHF, respectively (Fig. 5A, solid lines). In similar electrophoreses performed on samples equilibrated in 8 M urea (Fig. 5A, dashed lines) the difference in stability between the two ferritins increased (50% transitions points at \( \text{pH} 3.8 \) and 5.2 for L and H, respectively) (Fig. 5A, dashed lines).

The rLF ferritin used in this study differs from natural L-chain for having the first two N-terminal residues Ser-Ser substituted with Asp-Pro (10). The correct sequence has been...
ferritin stability (data not shown).

The molecular basis for the stability difference between rHF and rLF was investigated by the use of H-ferritin variants.

**H-ferritin Variants**

A series of 13 H-chain variants, produced by site-directed mutagenesis of the p2HFT plasmid, was analyzed. All of them resisted the 75 °C heating step normally used for ferritin purification; they were expressed and recovered from cell homogenates in yields comparable with those of the rHF wild type. Degradation products were not observed during purification. Because of the rather complicated mutations, the variants carry short code names, as in previous papers (9, 10, 13, 17, 18, 21-23). Table I describes the alterations of the variants grouped on the basis of the region of alteration, and Fig. 6 shows the position of the mutations on the subunit sequence. Briefly, the first 13 residues at the N terminus have been deleted (variant 115); the loop connecting B and C helices has been altered by the duplication of an 18-aminoo acid stretch with two mutations (variant M1), by the deletion of Pro near the 2-fold axis (variant 119), and by the substitution Lys→Gln, which has allowed rHF crystallization (variant 9Cd) (13). The region of the hydrophilic channel was altered by substituting the two carboxyl groups lining the channel (Asp Glu) with His (variant 175), or with Ala (variant 206) and by substitution of a 5-amino acid stretch at the exposed mouth of the channel with the homologous L-chain sequence (variant 203). The C terminus was altered by deleting the last 8 residues exposed to the cavity (variant 103), by replacing the last 10 amino acids with the homologous sequence of the L-chain (variant 152), and by substituting the outermost of the leucines lining the hydrophobic channel with Arg (variant R2). The cavity surface was altered by the substitution of three conserved carboxyl groups (Glu Glu, and Glu) with Ala (variant A2), and the inner part of the four-helix bundle was modified by the substitutions of Glu and His with Lys and Gly, which are the residues found in the L-chain (variant 152), and by substituting the outermost of the leucines lining the hydrophobic channel with Arg (variant R2). The cavity surface was altered by the substitution of three conserved carboxyl groups (Glu, Glu, and Glu) with Ala (variant A2), and the inner part of the four-helix bundle was modified by the substitutions of Glu and His with Lys and Gly, which are the residues found in the L-chain (variant 152), and by substituting the outermost of the leucines lining the hydrophobic channel with Arg (variant R2). 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The CD spectra and the ANS fluorescence of the apo-
variants in the assembled (pH 7.4), unfolded (6 M guanidine, pH 3.5), and acidic (pH 2) conditions were analogous to those of the H wild type (not shown). The variants were studied by the methods that most readily distinguished between rHF and rLF stability: guanidine denaturation at pH 7.4 (Fig. 2B) and acidic denaturation in 8 M urea (Fig. 5, B and C). From the denaturation plots we calculated the 50% transition points (Table II). The two methods gave consistent indications of the relative stability of the variants and allowed three groups to be distinguished: (i) variants with stability analogous to H wild type, which included alterations on regions exposed to the cavity (variants A2, 152, 103) and to the outer surface (variants 9Cd, 119, M1, 203) not involved in intra- or inter-subunit interactions; (ii) variants less stable than H wild type, which included the deletion of the N terminus (variant 115) and non-conservative substitutions of the amino acids lining the hydrophilic (variants 175 and 206) and hydrophobic (variant R2) channels; (iii) variants more stable than H wild type, all carrying the substitutions Glu62 → Lys, His65 → Gly (variants 222 and A222) located inside the four-helix bundle. The relative increase of stability of the variants 222 and A222 was confirmed by acidic denaturation experiments with ANS (Fig. 4A, Table II).

DISCUSSION

H- and L-ferritin Stability—Ferritin is an iron storage molecule which exerts its function by incorporating and protecting iron. The high stability in vitro typical of ferritin and slow turnover in vivo may be related properties that have a biological significance. Here we show that human rLF is notably more stable than rHF, as exemplified by the finding that at neutral pH in 6 M GdnHCl rLF is in a fully folded and assembled state, while rHF is 80% denatured (Fig. 2A). Similar large differences in stability (1.4 pH unit difference in 50% transition points) were observed in the acidic denaturation experiments in presence of 8 M urea (Fig. 5A). In contrast in the denaturation induced by pH alone (Figs. 4, A and B, and 5A) the stability difference between the two ferritins was less apparent (about 0.5 pH unit).

Analysis of the end products confirmed that acidic and guanidine denaturation follow different patterns; high GdnHCl concentrations unfold the ferritins completely, while low pH values (below 3) dissociate ferritin in subunit monomers (19, 33) or dimers (34) with a high degree of secondary structure (Fig. 1) (19, 32) which expose hydrophobic surfaces available to ANS binding, absent in the native and unfolded ferritins (Fig. 3). These properties may be attributed to dissociated and folded subunits, which expose the hydrophobic patches responsible for ferritin assembly (1). Alternatively they may be attributed to folding intermediates named molten globules described for various simple globular proteins at acidic pH (29, 30, 35); they have properties analogous to...
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...ferritin at pH 2, they bind ANS, and they have native-like far-UV CD spectra and denatured-like aromatic CD spectra (38). The finding that the H-chain alterations expected to stabilize the helix-bundle fold (see below) increased stability to acidic denaturation (Fig. 4A) may suggest that at pH 2 ferritin is partially unfolded, i.e. in a conformation close to a molten globule state. In addition the absence of detectable intermediates in acidic denaturation (Fig. 4D) (19) suggest that the change in subunit conformation may be the event determining ferritin shell disassembly.

H-variants—In order to obtain indications on the molecular basis for rHF and rLF stability differences we studied 13 H-chain variants and evaluated the results by analogy to the horse L-chain (1) and human H-chain ferritin structures (13) and by determination of the crystal structures of some H-variants. We found that all alterations of residues exposed to solvent and not involved in intra- or inter-chain interactions (and in most cases not conserved in other H-ferritins) had no effect on ferritin stability, whereas alterations of regions involved in intra- and inter-chain interactions (e.g. N-terminal shown in Fig. 7A and hydrophobic and hydrophilic channels), strongly reduced ferritin stability (Figs. 2B and 5, B and C). These channel alterations were found to have only minor effects on ferritin iron uptake (9, 21, 22); thus we conclude that the hydrophobic and hydrophilic channels have only a small effect on iron uptake, but a bigger influence on stability.

The substitution Glu62 to Lys, His65 to Gly had a profound effect on H-ferritin stability and functionality; it abolished the ferroxidase activity typical of HF and consequently reduced the rate of iron core formation to the low rates typical of L-ferritins (18) and increased the stability toward that of L-ferritin has been recently obtained (crystal coordinates de-

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...rich isoferritins may be more resistant to denaturation and degradation within the lysosomes. Data on differences in the metabolism of H and L subunits and of H- and L-rich ferritins are unusual (25, 38–40); the roles of turnover, compartmentalization, and post-translational modifications have yet to be determined.