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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 2 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 fifth 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-[1] 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 Glu^{62} and His^{65}, 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 $Glu^{62} \rightarrow$ Lys and

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[1] 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.

TABLE I

Human H-ferritin variants studied for stability properties

One-letter amino acid codes are used.

Code names	Alterations	Ref.
N terminus		
115[a]	Deletion of the N-terminal extension T1–H13	9, 17
Loop sequence		
119[b]	Deletion of P88	21
M1	Duplication of the sequence P88-L106 with the substitutions D91N+D92V	17, 21
9Cd[c]	K86Q	13, 21, 22
3-fold channel		
203[d]	Substitution of the sequence 121–125 with the homologous one of L-chain A121G,T122S,D123A,K124R,N125T, K86Q	21, 22
175[b]	D131H,E134H on K86Q	21, 22
206[b]	D131A,E134A on K86Q	21, 22
C terminus		
103[e]	Deletion of the C-terminal extension G176amber	21, 23
152[e]	Substitution the last 10 residues with the 6 equivalent residues of L-chain H173L,G176K,D177H,S178D,D179amber	21
R2[b]	L169R	17, 21, 23
Four-helix bundle and cavity		
A2[b]	E61A,E64A,E67A	17
222[f]	E62K,H65G, K86Q	13, 17, 18
A222	Substitution A2 + 222 E61A,E62K,E64A,H65G,E67A, K86Q	

[a] Sequence present in all mammalian ferritins.
[b] Amino acids conserved in all mammalian ferritins.
[c] Made to enable crystallization.
[d] The sequence ATDKN conserved in mammalian H-chains was substituted with the homologous sequence of the L-chain GSART (GSA*X*T is conserved in mammalian L-chains).
[e] The L-sequence is conserved in all mammalian L-chains.
[f] E62 and H65 are conserved in all H-chains, and K62 is conserved in all L-chains, while G65 is Ala in rabbit L-chain.

$His^{65} \rightarrow Gly$, confirm the formation of a salt bridge between Lys^{62} and Glu^{107}.[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 stability. The biological effects of the L-ferritin higher stability are discussed in relation to its interaction with iron.

[2] S. J. Yewdall, P. Hampstead, P. J. Artymiuk, and P. M. Harrison, unpublished work.

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, 21–23). 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 found to be pure by gel electrophoresis. Iron was removed from the 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 Brillant 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 ug/ml for far-UV circular dichroism, and 1 mg/ml for near-UV circular dichroism

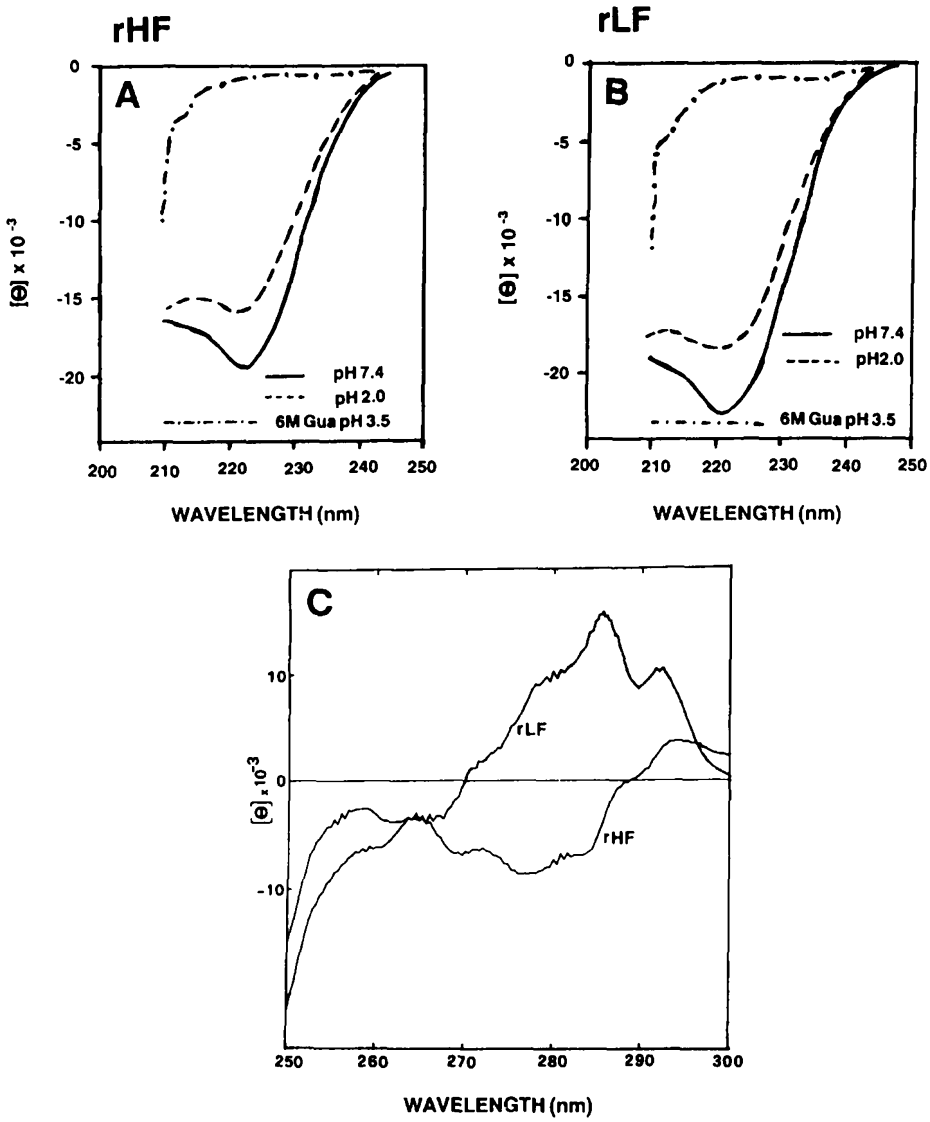

FIG. 1. **Circular dichroism spectra.** Far-UV CD spectra of rHF (*A*) and rLF apoprotein (*B*) at physiological pH (7.4) and under denaturing conditions (pH 2 and 6 M GdnHCl at pH 3.5). *C*, near-UV CD spectra of rHF and rLF apoproteins at pH 7.4.

analysis. Circular dichroism experiments were carried out in the UV region with a Jasco J500-A spectropolarimeter equipped with a Jasco model DP-500/PC data processor. The far-UV spectra were measured on apoferritin solutions, at room temperature, in 0.1-cm pathlength cells and the mean residue ellipticity values, $[\theta]$, expressed in degrees $\times$ cm^2 $\times$ dmol^{-1}, were calculated by the equation $[\theta] = (\theta \times M_o)/(10 \times l \times c)$ with θ = observed ellipticity, l = pathlength of the cuvette, c = protein concentration, and with a mean residue weight, M_o, of 114.4 for rLF and 115.9 for rHF and for all the H-variants. Near-UV spectra were determined on apoferritin samples, at room temperature, in 1.0-cm cells, and the molecular ellipticities were calculated on a 19,906 molecular weight for rLF and 21,097 for rHF (10).

ANS Fluorescence—Fluorescence measurements were performed with a Perkin-Elmer 650-40 fluorescence spectrophotometer furnished with an ordinate data processor accessory. To apoprotein samples equilibrated under the various denaturing conditions ANS was added to 0.1 mM final concentration (29, 30). The mixtures were excited at 380 nm and the emission spectra monitored.

RESULTS

H- and L-ferritins

Circular Dichroisms—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. 1*C*), 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^{93}, which is conserved in the two proteins (Fig. 1*C*). 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. 2*A*) show that rHF is less stable than rLF (50% unfolding transitions at 4.8 M 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-apofer-

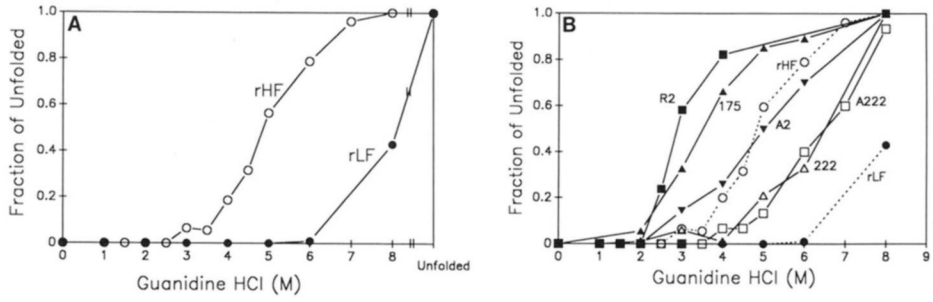

FIG. 2. **Guanidine HCl denaturation of ferritins and variants.** The ellipticity values at 222 nm of 50 μg/ml apoferritin at pH 7.4 and in various GdnHCl concentrations were measured. The data are plotted as fraction of unfolded (fU) = $(\theta N - \theta)/(\theta N - \theta D)$, using as references the ellipticity values of the proteins at pH 7.4 (native, θN) and at pH 3.5 in 6 M GdnHCl (denatured, θD). Unfolded, values obtained at 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.

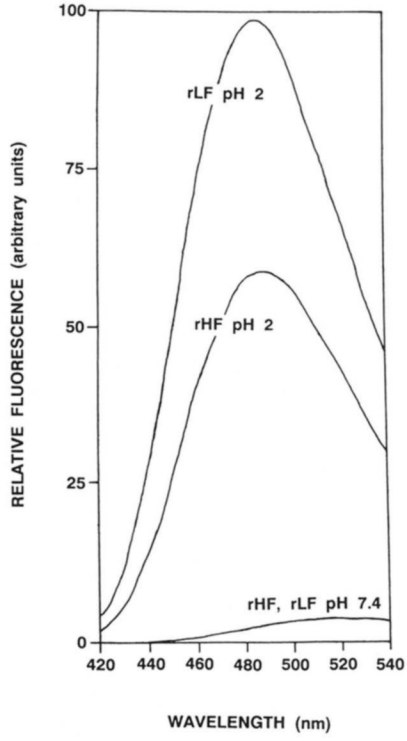

FIG. 3. **ANS binding.** Fluorescence emission spectra upon excitation at 380 nm of apoferritins (50 μg/ml) at pH 2 or at pH 7.4 in the presence of 0.1 mM ANS. The spectra of samples in 6 M GdnHCl, pH 3.5, are analogous to those of native apoferritins.

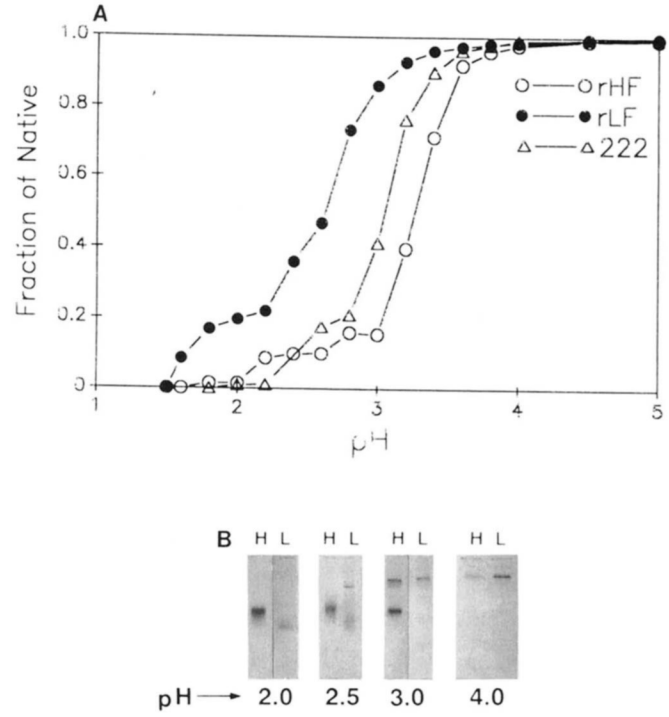

FIG. 4. **Acidic denaturation in absence of SDS.** *A*, monitored by ANS binding; rHF and rLF apoproteins and the H-variant $Glu^{62} \rightarrow Lys$, $His^{65} \rightarrow Gly$ (222) at 50 μg/ml were incubated at various pH, added of 0.1 mM ANS, excited at 380 nm, and the fluorescence emission at 480 nm was monitored. The plots are expressed as fractions of native ferritin (f_N) = $(F_D - F)/(F_D - F_N)$ using as references the fluorescence emissions of the samples at pH 7.4 (native, F_N) and at pH 1.5 (denatured, F_D). *B*, denaturation monitored by gel electrophoresis. rHF (H) and rLF (L) incubated at the pH indicated were run anodically on 6% polyacrylamide gels at the same pH values (5 μg/lane) and stained with Coomassie Blue.

ritin about twice that of H-apoferritin (Fig. 3). ANS did not bind to native or unfolded ferritins, and unfolding of the proteins at pH 2 by the addition of 3 M GdnHCl abolished ANS fluorescence (not shown). ANS was added to the apoferritins equilibrated at various pH values to study acidic denaturation. The 50% transition points were at pH 3.3 and 2.6 for rHF and rLF, respectively (Fig. 4*A*).

On gel electrophoresis at pH 2.0, rHF and rLF migrated anodically as single bands, the rLF having faster mobility; at pH 4.0 they had similar anodic migration patterns with the oligomers typical of the assembled ferritin (Fig. 4*B*). In the intermediate pH 2.5 and pH 3.0, rLF and rHF respectively showed the slow and fast bands assigned to assembled and denatured states (Fig. 4*B*). In order to distinguish with confidence the assembled from the denatured subunits, and to explore the pH range 4–6, we analyzed the samples at various pH values on SDS-electrophoresis. At neutral pH both ferritins were fully assembled, subunits began to appear at pH 5, and at pH 2.7 they were fully dissociated (Fig. 5*A*, *solid lines*).

Densitometry of the stained protein bands (Fig. 5*A*, *solid lines*), showed that massive dissociation occurs in the 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 pH 3.0 and 3.4 for rLF and rHF, respectively (Fig. 5*A*, *solid lines*). In similar electrophoreses performed on samples equilibrated in 8 M urea (Fig. 5*A*, *dashed lines*) the difference in stability between the two ferritins increased (50% transitions points at pH 3.8 and 5.2 for L and H, respectively) (Fig. 5*A*, *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

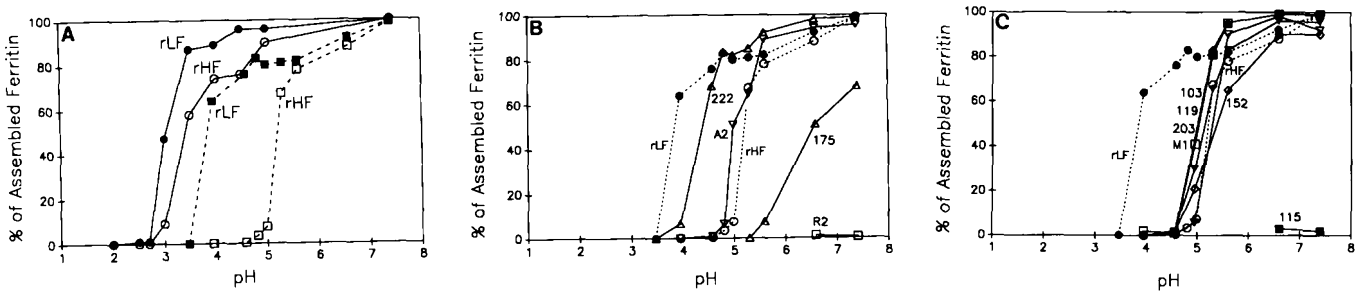

FIG. 5. **Acidic denaturation monitored by SDS-electrophoresis.** Panel ferritins were incubated at the pH values indicated, diluted 1:1 with 0.2% SDS, 0.25 M Tris-HCl, pH 6.8, run on SDS gels, stained with Coomassie Blue, and the proportion of assembled ferritin determined by densitometry. *A*, plots of rHF (*open symbols*) and rLF (*filled symbols*); the *solid lines* are for the experiments without urea and the *dashed lines* from experiments with 8 M urea. *B* and *C*, denaturation plots in 8 M urea of the H-chain variants, code names as in Table I.

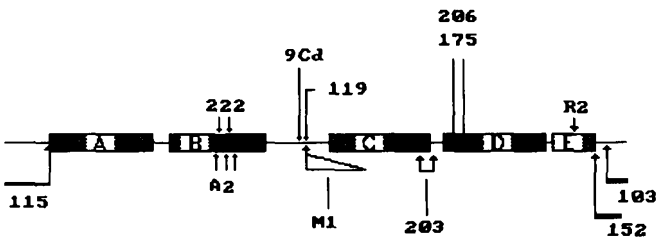

FIG. 6. **Scheme of the H-chain variants.** *Shaded boxes* indicate the α helices A-E; the *arrows* point to the site of alteration on the sequence and carry the code names and substitutions of the variants.

TABLE II

Stability of human H-ferritin variants

One-letter codes are used for amino acids.

	Stability (transition points (difference from rHF))		
	pH in 8 M urea[a]	Gua pH 7.4[b]	pH[c]
		M	
rLF	3.8 (+ 1.4)	8.3 (+ 3.5)	2.6 (+ 0.7)
rHF	5.2 (0)	4.8 (0)	3.3 (0)
N terminus			
115 (del. T1-H13)	Dis[d] (<<)	ND	3.2 (+ 0.1)
Loop			
9Cd K86Q	5.2 (0)	4.8 (0)	3.3 (0)
119 (del. P88)	5.3 (− 0.1)	ND	ND
M1 (dupl. P88-L106)	5.0 (+ 0.2)	ND	3.2 (+ 0.1)
Hydrophilic channel			
203 (subs. 121-125)	5.0 (+ 0.2)	ND	3.3 (0)
175 (D131H,E134H)	6.6 (− 1.4)	3.5 (− 1.3)	4.0 (− 0.7)
206 (D131A,E134A)	ND	2.6 (− 2.2)	4.2 (− 0.9)
C terminus			
R2 (L169R)	Dis[d] (<<)	2.9 (− 1.9)	3.3 (0)
152 (subs. C terminus)	5.4 (− 0.2)	ND	3.2 (+ 0.1)
103 (G176am)	5.1 (+ 0.1)	ND	ND
Four-helix bundle and cavity			
A2 (E61A,E64A,E67A)	5.0 (+ 0.2)	4.9 (+ 0.1)	3.2 (+ 0.1)
222 (E62K,H65G)	4.4 (+ 0.8)	6.5 (+ 1.7)	3.0 (+ 0.3)
A222 (A2 + 222)	ND	6.5 (+ 1.7)	3.0 (+ 0.3)

[a] pH of 50% transition from assembled into dissociated ferritin, in 8 M urea, detected by electrophoretic analysis as in Fig. 5.

[b] GdnHCl molarity of the 50% transition from folded into unfolded state in pH 7.4, detected by circular dichroism ellipticity values at 222 nm, as in Fig. 2.

[c] pH of 50% transition from native to denatured state detected by ANS fluorescence, as in Fig. 4.

[d] Dissociated by simple incubation in 0.1% SDS at room temperature.

recently overexpressed in *E. coli*,[3] and comparative studies showed that the substitutions had no detectable effects on 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-amino acid stretch with two mutations (variant M1), by the deletion of Pro^{88} near the 2-fold axis (variant 119), and by the substitution $Lys^{86} \rightarrow Gln$, 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^{131} and Glu^{134}) 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^{61}, Glu^{64}, and Glu^{67}) with Ala (variant A2), and the inner part of the four-helix bundle was modified by the substitutions of Glu^{62} and His^{65} with Lys and Gly, which are the residues found in the L-chain (variant 222). Alterations A2 and 222 were combined in the variant A222. In summary, variants 222, 203, and 152 carry the alteration H → L, while most of the others carry alterations of residues that are conserved in all mammalian ferritin sequences. All variants were shown to assemble into molecules that were electrophoretically similar to wild type. Three of them have been analyzed crystallographically and have been found to exhibit a high degree of structural isomorphism.[2] From the amino acid substitutions made and the positions of these substitutions, the other variants are assumed to have structures resembling wild type.

The CD spectra and the ANS fluorescence of the apo-

[3] S. Levi and P. Arosio, unpublished results.

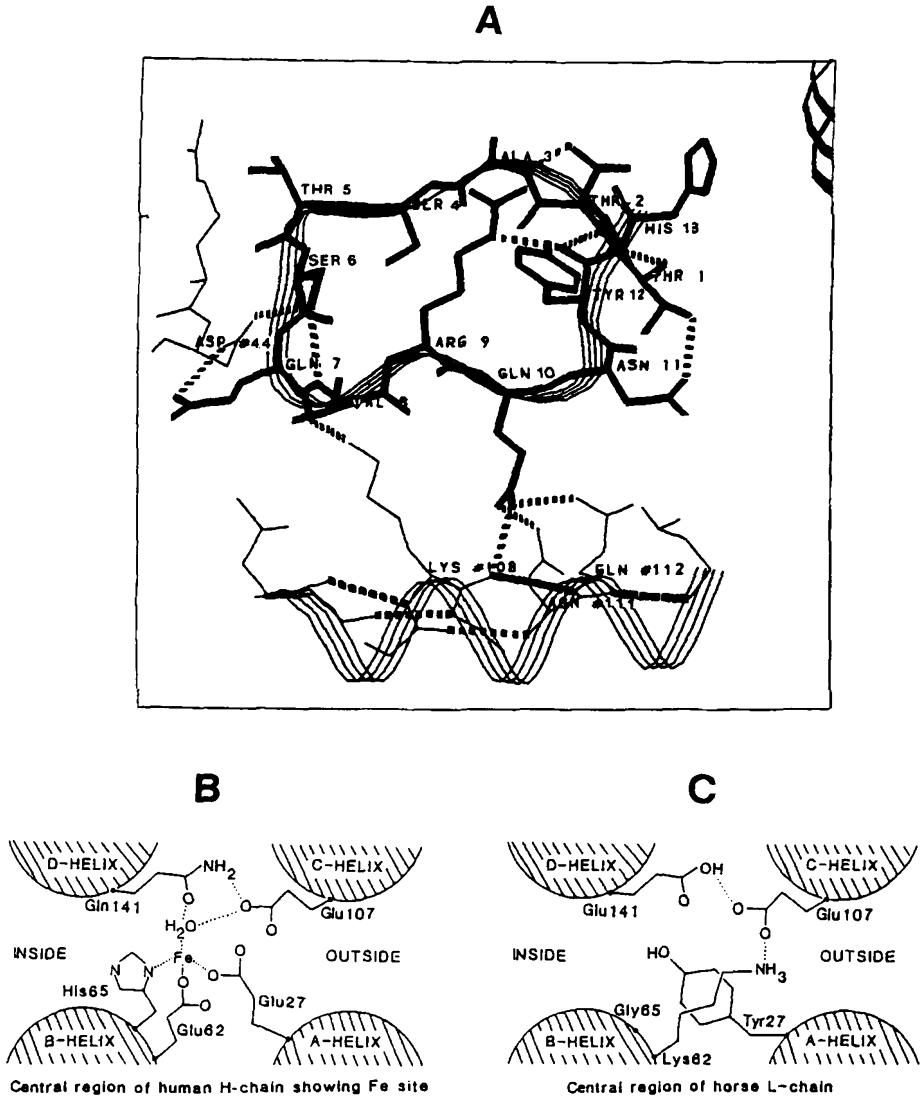

FIG. 7. **Computer graphics drawing of parts of the ferritin molecule.** *Panel A*, drawing of the N-terminal sequence deleted in variant Del T1-H13 (115) (*thick line*), the *dotted lines* indicate intra- and interchain interactions. *Panels B* and *C*, drawing of the central region of the ferritin subunits (from Ref. 41) showing the metal binding site of H-chain (*B*) and showing the salt bridge Lys^{62}-Glu^{107} in the L-chain (*C*), which has probably been reformed in the H-variant $Glu^{62} \rightarrow Lys$, $His^{65} \rightarrow Gly$ (222).

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. 2*B*) 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 intersubunit 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 $Glu^{62} \rightarrow Lys$, $His^{65} \rightarrow Gly$ (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. 4*A*, 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. 2*A*). 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. 5*A*). In constrast in the denaturation induced by pH alone (Figs. 4, *A* and *B*, and 5*A*) 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

ferritin at pH 2, they bind ANS, and they have native-like far-UV CD spectra and denatured-like aromatic-CD spectra (35). The finding that the H-chain alterations expected to stabilize the helix-bundle fold (see below) increased stability to acidic denaturation (Fig. 4*A*) 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. 4*B*) (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.[2] 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, while alterations of regions involved in inter- and intra-chain interactions (*e.g.* N terminus shown in Fig. 7*A* and hydrophobic and hydrophilic channels), strongly reduced ferritin stability (Figs. 2*B* 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 $Glu^{62} \rightarrow Lys$, $His^{65} \rightarrow Gly$ had a profound effect on H-ferritin stability and functionality; it abolished the ferroxidase activity typical of rHF 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 rLF in all denaturation experiments. The structure of H-ferritin has been recently obtained (crystal coordinates deposited in Brookhaven PDB, accession number 1FHA), and a metal binding site probably responsible for the rapid Fe(II) oxidation has been identified inside the four-helix bundle (13). The H-chain metal site ligands Glu^{27}, Glu^{62}, and His^{65} are Tyr^{27}, Lys^{62}, and Gly^{65} in L-chain, and the metal site is substituted with a Lys^{62}-Glu^{107} salt bridge (Fig. 7). The higher stability of the H-variants with $Glu^{62} \rightarrow Lys$ and $His^{65} \rightarrow Gly$ substitutions can be attributed to the reconstruction of this salt bridge Lys^{62}-Glu^{107}, which links helices B and C and stabilizes the subunit fold as in the L-ferritins (13). Crystallographic analyses now in progress confirm the presence of a salt bridge between Lys^{62} and Glu^{107} in variant A222.[2] The idea that the salt bridge contributes to the observed stability of the double variant is in agreement with recent studies on T4 lysozyme showing that a single salt bridge can give a strong contribution of 3–5 kcal/mol to the free energy of folding (36).

Present and previous (15, 18) studies on H-variant $Glu^{62} \rightarrow Lys$, $His^{65} \rightarrow Gly$ (named 222) show that a major component of the functional differences between the rLF and rHF lies inside the four-helix bundle: the H-chain uses this region for the formation of a catalytic site in a manner analogous to hemerythrin, which has a similar structural motif (37), while the L-chain uses it to increase stability. The greater stability of L-chains at acidic pH may have biological significance; L-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 unclear (25, 38–40); the roles of turnover, compartmentalization, and post-translational modifications have yet to be determined.

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