Ferritins are 24-mer proteins which store and detoxify intracellular iron. Mammalian ferritins are made of two subunit types, the H- and L-chains, with different functional specificity. The H-chain has a metal-binding site (the ferroxidase center) which confers ferroxidase activity to the protein and accelerates iron incorporation. In the L-chain the center is substituted by a salt bridge. We performed several site-directed mutageneses in the L-chain with the aim to construct the center and confer ferroxidase activity to the protein. Most variants were insoluble and did not refold into homopolymers, probably due to electrostatic repulsion introduced by the substitutions. However, they formed hybrids when they were renatured together with the L- or H-chains. The heteropolymers made of 90% L-chain and 10% of an L-variant with all the ligand residues of the H-chain center had 25–30% of the ferroxidase activity of the H-chain homopolymer. This corresponds to the activity of an H/L heteropolymer with 7% H-chain. It is concluded that: (i) it is possible to construct a ferroxidase center in the L-chain with an activity equivalent to that of the H-chain; (ii) the residues of the center interfere with the folding/assembly of the L- or variants of it; and form heteropolymers can be made even between ferritin subunits with large differences of refolding rates.

Ferritin is an iron storage protein made of 24 subunits which assemble with 4, 3, and 2 point symmetry (Ford et al., 1984). The protein is an almost spherical shell which delimits a large cavity about 8 nm across pierced by eight hydrophilic small channels along the 3-fold symmetry axes and by six hydrophobic small channels along the 4-fold axes (Ford et al., 1984). Iron enters the cavity via the hydrophilic channels (Levi et al., 1989b; Treffry et al., 1989, 1993; Desideri et al., 1991), and there it forms cores structurally similar to the mineral ferritrides (Wade et al., 1991). The ferritins found in bacteria and in plants are homopolymers of a single subunit species, while in mammals they are mostly heteropolymers made of variable proportions of at least two subunit types, the H- and L-chains (Harrison et al., 1991; Andrews et al., 1992). The two chains have similar sizes and about 50% amino acid sequence identity (Fig. 1A) (Lawson et al., 1991).

Crystallographic structures of horse, recombinant rat L-, and human H-chains are very similar and their backbones essentially overlap (Lawson et al., 1991). They fold into a bundle of four long α-helices with a long loop connecting helices B and C, a short E-helix near the C terminus, and non-helical extensions at the C and N termini (Ford et al., 1984). The major structural difference between the two chains is a metal-binding site inside the H-chain bundle, absent in the L-chain (Lawson et al., 1991; Harrison et al., 1991). The site has been named a ferroxidase center since it catalyzes the aerobic oxidation of Fe(II) (Lawson et al., 1988; Levi et al., 1988; Sun et al., 1993).

The three-dimensional structure of the human H-chain ferroxidase center is shown in Fig. 1B. It has Glu27, Glu65, His65, Glu141, and Gln141 as metal ligands. In addition, Glu65 and Tyr64 seem to participate in iron oxidation (Lawson et al., 1991; Treffry et al., 1992). These residues are highly conserved in mammalian H-chains and in bacterial and plant ferritins (Andrews et al., 1992) (except pea ferritin where Glu65 is substituted by His) (Lobreaux et al., 1992; Wade et al., 1993), but in mammalian L-chains Glu27, Glu65, His65, and Gln141 are substituted by Tyr or His37, Lys46, Gly69, and Gln141 (Fig. 1C). Lys46 forms a salt bridge with Glu65 which stabilizes ferritin (Santambrogio et al., 1992).

Mutational studies have been done mainly on the H-chain of human ferritin, showing that the substitution of the metal ligands of the center inactivated (E62K+H65G) (Lawson et al., 1989) or reduced (E27A, E107A) ferroxidase activity (Treffry et al., 1993). The ferroxidase activity accelerates ferritin iron incorporation with a mechanism that involves the production of hydrogen peroxide (Sun et al., 1993) and the transient formation of a dimeric Fe(III) species, likely in the ferroxidase center (Bauminger et al., 1991a, 1991b, 1993; Treffry et al., 1992). The functional specificity of the L-chain is less defined, and mutational studies have not been reported so far. L-homopolymers are more stable to denaturation, partially due to the contribution of the salt bridge between Lys46 with Glu107 (Santambrogio et al., 1992), and they incorporate iron at a slower rate than the H-type, due to the absence of a ferroxidase center (Levi et al., 1989a). The L-ferritins (and not the H variants with inactive ferroxidase centers) incorporate some of the iron oxidized by the H-homopolymer (Levi et al., 1992), and they reduce nonspecific iron oxidation outside the protein cavity (Levi et al., 1994), findings that suggested that L-chains are more efficient in promoting iron mineralization probably for some properties of the cavity (Levi et al., 1992). L- and H-chains or variants co-renature together in vitro and form heteropolymers with a restricted heterogeneity and the expected subunit composition (Santambrogio et al., 1993). Heteropolymers with a high L- and a low H-chain content incorporate iron more efficiently than the homopolymers, confirming that H- and L-chains have complementary roles in ferritin iron uptake (Levi et al., 1994).

The present report describes a mutational work for the construction of a ferroxidase activity in the L-chain. The results show that the introduction of Glu65 and Glu141, necessary for the activity, interfere with protein folding and assembly probably...
due to electrostatic repulsion. The L variants with most or all the residues of the H-chain ferroxidase center did not renature when alone, but they formed correctly assembled heteropolymers with H- or L-chains. A comparison with the H/L chain heteropolymers showed a comparable ferroxidase activity in an L variant and H-chain.

MATERIALS AND METHODS

Ferritins and Variants—Human ferritin L-chain variants were obtained by oligonucleotide-directed mutagenesis of the plasmid pEMBL12 FpRT described by Levi et al. (1992). Human H- and L-chain variants were expressed in Escherichia coli and, when soluble, were purified as described by Levi et al. (1987, 1992). Ferritins were electrophoretically pure. When needed they were treated to remove iron as described by Levi et al. (1988). Protein concentration was determined with BCA reagent (Pierce) using bovine serum albumin as a standard.

Purification and Renaturation of the Insoluble L Variants—Some of the L variants accumulated only in the insoluble fraction of E. coli extracts even using different strains and different induction procedures. Renaturation studies of the insoluble ferritins were performed on precipitates from 20 g of cell paste. They involved solubilization with GdnHCl or urea, and dilution followed by dialysis in renaturing buffers in the presence of 3 mM DTT, 1 mM EDTA. In method A, the precipitates were incubated in 60 ml of 6 x GdnHCl, pH 3.5, for 18 h, and after centrifugation the samples were diluted 10-fold into 0.1 x sodium phosphate, pH 7.4, at 4 C and then dialyzed against the same buffer. In method B the precipitates were resuspended in 20 ml of 2 x urea, pH 8.0, 1000 units of Benzonase (Merck) were added, and the samples were incubated at 37 C for 18 h to digest DNA and RNA. The variants were recovered by centrifugation, solubilized in 20 ml of 8 x urea, pH 3.0, at 4 C, diluted 10-fold into 0.1 x sodium phosphate, pH 7.4, and dialyzed at 4 C.

Heteropolymers—The H/L heteropolymers were constructed as described in Santambrogio et al. (1993). Denatured H- and L-chains (in 6 x GdnHCl, pH 3.5) were diluted in renaturing buffer (0.1 x sodium phosphate, pH 7.4, 3 mM DTT, 1 mM EDTA) and concentrated by ultrafiltration. A modified procedure was used for rLCr2 and rLCr4: they were incubated in 60 ml of 6 x GdnHCl, pH 3.5, for 18 h, and after centrifugation the samples were diluted 10-fold into 0.1 x sodium phosphate, pH 7.4, and dialyzed at 4 C.

RESULTS

Construction and Expression of L-chain Variants—Table I lists the L-chain variants constructed and analyzed. They derive from the variant rLCr (K86Q, N-terminal-Ser-Thr) which differs from rLF in electrophoretic mobility (see below) and not in functional properties. The metal ligand residues of the H-chain ferroxidase center (Fig. 1B) were progressively introduced with the substitutions of Lys62-Glu, Gly65-His, Tyr67-Glu, and Glu101-Gln. The additional mutations Glu77-His, Glu92-His were made to reduce the local number of negative charges, Phe177-Tyr to allow a hydrophobic bond with His82 and Leu144-Ala to remove a bulky residue. The variants were expressed by E. coli in similar yields (Fig. 2A), but the ones with the substitution Lys62-Glu were insoluble (Fig. 2B).

The use of different host strains and different induction procedures did not improve the solubility of the variants. A His was introduced in position 62 in the variants K62H+G65H and rLCr8, following the report that pea seed ferritin has His62 and ferroxidase activity (Llobreaux et al. 1992). and the two variants were soluble and without detectable ferroxidase activity (not shown). Similarly, the H-chain variant Glu82-His was soluble but without ferroxidase activity (not shown).

In Vitro Renaturation—We studied methods to renature in vitro the insoluble ferritins. The variants Lys62-Glu were solubilized by incubating the insoluble bacterial extracts with 8 M urea, pH 7.4, obtaining non-assembled species that reacted with antibodies to denatured H-ferritin (not shown). Since assembled ferritins do not denature under these conditions (Santambrogio et al., 1992), the finding suggests that the insoluble aggregates are made of misfolded subunits rather than aggregated ferritin shells. The variants Lys62-Glu were purified and analyzed as in Santambrogio et al. (1993). Denaturation plots were obtained by incubating ferritin for 18 h at 4 C with various GdnHCl concentrations, and protein conformational status was derived from fluorescence emission spectra with excitation at 296 nm, as in Santambrogio et al. (1993). Ferritin iron oxidation was monitored by the disappearance of Fe(II) with a discontinuous assays as described in Treffry et al. (1993). At intervals, 0.1 ml of the reaction solution was added to 0.1 ml of 0.24 mM ferroin in water and the Fe(II)-ferroin complex measured by reading at 570 nm. Iron uptake kinetics were performed as in Levi et al. (1988), monitoring the development of Fe(III) color at 310 nm on a Beckman DU 70 spectrophotometer. When iron uptake was to be monitored by gel electrophoresis, apoferritins (0.5 mg/ml) in 0.1 x HEPES buffer, pH 7.0, or 0.1 x MES, pH 5.5, were incubated for 2 h at room temperature with 1 mM ferrous ammonium sulfate (Levi et al., 1992, 1994). The reaction was stopped by addition of 10 ml 4,7-diphenyl-1,10 phenanthroline sulfonate and 1 mM desferoxamine. In the experiments at pH 5.5, the stacking gels were made at the same pH. The human L-chain structure was obtained by homology modeling from human H-chain crystallographic structure (Brookhaven PDB accession code 1FHA) based on the 55% sequence identity between the two chains. The residues Lys62 and Glu101 have been adjusted to fit the formation of a salt bridge as found in rat L-chain (Lawson et al., 1991). The software is Quanta/Charmm (Molecular Simulations Inc.) running on an IBM Risc6000 workstation.

Table I

<table>
<thead>
<tr>
<th>Code names</th>
<th>Substitutions</th>
<th>Helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLCr</td>
<td>S6T+K86Q</td>
<td>A</td>
</tr>
<tr>
<td>G65H</td>
<td>G65H</td>
<td>B</td>
</tr>
<tr>
<td>K62E</td>
<td>K62E</td>
<td>B</td>
</tr>
<tr>
<td>K62E+G65H</td>
<td>K62E+G65H</td>
<td>B</td>
</tr>
<tr>
<td>rLCr2</td>
<td>K62E+G65H+Y72E</td>
<td>A</td>
</tr>
<tr>
<td>rLCr3</td>
<td>K62E+G65H+Y27E</td>
<td>C</td>
</tr>
<tr>
<td>rLCr4</td>
<td>K62E+G65H+Y72E</td>
<td>C</td>
</tr>
<tr>
<td>rLCr8</td>
<td>K62E+G65H+Y27E</td>
<td>A</td>
</tr>
</tbody>
</table>

* The residues acting as metal ligands in H-chain ferroxidase centre are underlined (numbering on the human H-chain sequence).
* The substitutions K86Q (to enable crystallization) and N-terminal-S-T (for easier construction) are present in all the listed variants.

The abbreviations used are: MES, 4-morpholineethanesulfonic acid; GdnHCl, guanidine hydrochloride; rLF, recombinant human ferritin H-chain; rL, recombinant human ferritin L-chain; DTT, dithiothreitol.
Construction of a Ferroxidase Center in L-ferritin

A

H-c TIASTQYQ NIVKDEAAI NQGDLTEA SYLWGSTY FNEDEVALGN
L-c ----SQCQ NYDDEAAX NSWNLTEA SYLWGSTY FNEDEVALGN
51 61 71 81 91
H-c FAKYTVFQH EFMKAEL KMQLQGQGI FPLQKYPFCDMCGGLNM
L-c VENIFRELAE DQFRFQALL KMQNGQGTL FLPQKYPFCDMCGGLNM
101 111 121 131 141
H-c AMLALD RYYLNLNLKIL ATYEOFYLC FRKAIKLMGD
L-c KAMALDK NLQDLNLHAL GARDPKPHLC FRKAIKLMGD
151 161 171 181
H-c KYNLHDAG PESGLAEH HNLDDHHS EKLMKLONQRGGRI FLQDIKKNPDC DDWESGLNAM
EERLL KMQNQRGGRA LFQDIKKNPAE DEWGKTPDAM

B

A

B

C

D

FIG. 1. Sequences and three-dimensional structures of human ferritins H- and L-chains. A, comparison of the amino acid sequences of human H- (H-c) and L-chains (L-c). The α-helices (A–E) are marked by continuous lines, the residues which contribute to the ferroxidase center are boxed, and the amino acids substituted in the L-variants are labeled with stars (*). B, diagram of the ferroxidase center of the human H-chain showing the α-helices involved in metal coordination and some neighboring residues. C, the corresponding region in the human L-chain model. The amino acids are indicated by one-letter code and sequence number, and the ones substituted in the L-variants are in bold. The shaded circle represents the metal atom and the stars water molecules. Interhelical hydrogen bonds are indicated by dashed lines. The α-helices are shown by the letters A–D.

fied by a first incubation with 2 M urea, pH 8, to extract contaminants, followed by Benzonase digestion, and a second incubation with 8 M urea, pH 3.0, to solubilize ferritin. A final gel filtration on a Superose 12 column equilibrated in 5 M urea yielded electrophoretically pure proteins (not shown). Variants K62E+G65H and K62E could be refolded by a 10-fold dilution

in sodium phosphate buffer, pH 7.4, with 1 mM NaCl, 3 mM DTT, 1 mM EDTA, to a final protein concentration of 0.7–1 mg/ml, followed by 1–3 days dialysis against the same buffer. Transient renaturation intermediates accumulated after dilution and disappeared more rapidly in K62E+G65H than K62E, with the formation of the 24-mer ferritin (Fig. 3). The different rate of renaturation may account for the different recoveries of assembled protein shown in Table II. Preliminary analyses indicated that the non-assembled species were monomers with a degree of secondary structure intermediate between the native, assembled state, and the fully unfolded state (Table III). Under these conditions, and all the other tested, rLCr2, rLCr3, and rLCr4 produced only insoluble aggregates (Table II). It should be noticed that the procedure developed for renaturation of

FIG. 2. Expression gels of the L-ferritin variants. E. coli cells transformed with the plasmids were grown at 30 °C for 2 h, after raising the temperature to 42 °C for 10 min, the cells were grown at 37 °C for another 2 h, and collected. A, SDS gel electrophoresis (15% polyacrylamide) of cells from 50 ml of culture lysed by boiling in 1% SDS, 5% mercaptoethanol. B, non-denaturing gel electrophoresis (7.5% polyacrylamide) of the soluble fraction of cellular homogenates, heated at 75 °C for 5 min and corresponding to 100 μl of cell culture. The names of the variants are indicated above the gel slots; lane C is the control of purified rLCr (2 μg). The arrows indicate the position of the L-ferritin subunit (A) and of assembled L-ferritin (B).

FIG. 3. Renaturation of insoluble L-variants. The variants K62E+G65H and K62E were renatured following method B, described under “Materials and Methods,” and samples were collected immediately after dilution in renaturing buffer (lanes 1), after 18 h of dialysis in the same buffer (lanes 2). After an additional 18 h rest at 4 °C (lane 3), they were loaded (20 μg each) on non-denaturing 7.5% polyacrylamide gel electrophoresis. The upper arrows indicate the position of the assembled 24-mer ferritin and the lower arrows the position of the transient renaturation intermediate. The gels were stained with Coomassie Blue.
Construction of a Ferroxidase Center in L-ferritin

TABLE II

<table>
<thead>
<tr>
<th>From E coli</th>
<th>From in vitro refolding</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>rLF</td>
<td>+++</td>
<td>50%</td>
<td>2%</td>
</tr>
<tr>
<td>rLCr</td>
<td>+++</td>
<td>30%</td>
<td>2%</td>
</tr>
<tr>
<td>G65H</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K62E</td>
<td>No</td>
<td>No</td>
<td>2%</td>
</tr>
<tr>
<td>K62E+G65H</td>
<td>No</td>
<td>No</td>
<td>30%</td>
</tr>
<tr>
<td>rLCr2</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>rLCr3</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>rLCr4</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>K62H+G65H</td>
<td>+++</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>rLCr8</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Recovery of soluble and assembled ferritins detected by gel electrophoresis of the soluble fractions of bacterial homogenates. No, no detectable recovery.

b Percentage of soluble and assembled ferritins obtained after renaturation of the insoluble fractions of the cellular homogenates. Renaturation methods A and B are described under "Materials and Methods." No, no detectable recovery; ND, not determined.

table 2.

Properties of the renaturation intermediates of K62E and K62E+G65H

The intermediates were obtained by diluting 1:10 the variants denatured in 8 M urea, 0.1 M sodium phosphate, pH 3.0, 3 mM DTT, 1 mM EDTA, and were purified by gel filtration on a Superose 12 column in 0.8 M urea.

<table>
<thead>
<tr>
<th>Variant</th>
<th>M* (kDa)</th>
<th>Fluorescence (nm)</th>
<th>CD (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K62E</td>
<td>27.5</td>
<td>355/325 nm</td>
<td>0.75</td>
</tr>
<tr>
<td>K62E+G65H</td>
<td>27.1</td>
<td>1.30 (53%)</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a Apparent molecular mass determined by gel filtration on a Superose 12 column in 0.8 M urea, 0.1 M sodium phosphate, pH 7.4, 3 mM DTT, 1 mM EDTA.

b Ratio between the emissions at 355 and 325 nm (excitation at 295 nm), as described in Santambrogio et al. (1993). Co-renaturation of rLCr2 or rLCr4 with rLF gave the highest recovery of renatured heteropolymers. The proportion of rLCr2 or rLCr4 in the assembled state was estimated to be about 10%, by assuming a linear relationship between the electrophoretic mobility of ferritin and its subunit composition as with H/L heteropolymers (Santambrogio et al., 1993). The mobility of renatured ferritins did not change when rLF and rLCr4 (or rLCr2) chains exceeded 70%, and only L-homopolymers were obtained when rLF was denatured. Equimolecular amounts of L-chain and rLCr2 or rLCr4 gave the highest recovery of renatured heteropolymers.

FIG. 4.

Denaturation plots of renatured ferritins. The variants rLCr and K62E+G65H were denatured, renatured, and purified. Samples of the two proteins (50 μg/ml) were incubated in various GdnHCl concentrations in 0.1 M sodium phosphate, pH 7.4, 1 mM DTT, and the protein denaturation monitored by the ratio of fluorescence emission at 355 and 325 nm (excitation at 295 nm), as described in Santambrogio et al. (1993). The mobility of renatured ferritins did not change when rLF and rLCr4 (or rLCr2) chains exceeded 70%, and only L-homopolymers were obtained when rLCr2 or rLCr4 gave the highest recovery of renatured heteropolymers.

The proportion of rLCr2 and rLCr4 in the assembled protein was estimated to be about 10%, by assuming a linear relationship between the electrophoretic mobility of ferritin and its subunit composition as with H/L heteropolymers (Santambrogio et al., 1993). Co-renaturation of rLCr2 or rLCr4 with H-chains produced heteropolymers with 8−12% of L variants, as detected by densitometry of SDS-polyacrylamide gel electrophoresis. The recoveries of heteropolymers with L-chain were of about 25 and 50% for the rLCr2 and rLCr4, respectively, and the remaining protein was precipitated.

Heteropolymer Formation—The purified rLCr4 and rLCr2 variants in 8 M urea, pH 3.0, were mixed with equimolecular amounts of denatured rLF in the same buffer, dialyzed, and dialyzed as above obtaining renatured ferritins with electrophoretic mobility intermediate between rLF and that expected for the mutant homopolymers (Fig. 5). This indicated that heteropolymers had formed (Santambrogio et al., 1993). The mobility of renatured ferritins did not change when rLF and rLCr4 (or rLCr2) were mixed in different proportions (30−90% of rLCr4), but recovery decreased when rLCr4 (or rLCr2) chains exceeded 70%, and only L-homopolymers were obtained when 8−12% of L variants, as detected by densitometry of SDS-polyacrylamide gel electrophoresis. The recoveries of heteropolymers with L-chain were of about 25 and 50% for the rLCr2 and rLCr4, respectively, and the remaining protein was precipitated.

Characteristics of the rLCr4/rLF and rLCr2/rLF Heteropolymers—The renatured heteropolymers were further purified by heating at 75 °C. Addition of 1000 Fe(II) atoms/molecule at pH 5.5 formed iron cores in rLF and rLCr4/rLF but not in rLCr2/rLF and rLF (Fig. 6). The rate of iron oxidation was similar stability to GdnHCl denaturation (Fig. 4).
Fig. 7. Ferritins ferroxidase activity. Apoferritins of the homo- and heteropolymers (50 µg/ml) in 0.1 M HEPES, pH 7.0, were mixed with 0.1 mM ferrous ammonium sulfate, and iron oxidation monitored by Fe(II) disappearance with ferrozine (panel A) or by the increase of absorbance at 310 nm (panel B). The control in the absence of proteins is the dashed line in panel B. In panel A rHF(5), a sample of rHF at a concentration of 5 µg/ml, i.e. equivalent to the concentration of rLCr4 in the heteropolymer sample, is shown.

monitored as disappearance of Fe(II) by a discontinuous chelation assay with ferrozine. The activity of rLCr2/rLF was low, like rLCr, while that of rLCr4/rLF was about 25% of that of the H-homopolymer at the same protein concentration (Fig. 7A). This activity was higher than that of the H-chain homopolymer at 10-fold dilution, in which the H-chain concentration is the same as that of the rLCr4 in the heteropolymers. In other experiments we monitored at 310 nm the formation of the yellow ferric oxide (Fig. 7B); rLCr2/rLF and rLF were essentially inactive while rLCr4/rLF had an activity of about 25–30% of that of rHF. To compare the specific activity of rLCr4 with that of H-chain, we constructed and analyzed a range of H/L heteropolymers with different proportions of H- and L-chains were constructed as described in the text. The apo forms of the proteins (50 µg/ml) were mixed with 0.1 mM ferrous ammonium sulfate and the rate of iron oxidation monitored by the increase of absorbance at 310 nm. The initial rate of iron oxidation is plotted against the proportion of H-chain in the samples determined by SDS electrophoresis. The arrow indicates the activity of the rLCr4/rLF heteropolymer under the same conditions: it corresponds to that of a H/L heteropolymer made by 7% H-chain and 93% L-chains.

Fig. 8. Ferroxidase activity of H/L heteropolymers. Heteropolymers with different proportions of H- and L-chains were constructed as described in the text. The apo forms of the proteins (50 µg/ml) were mixed with 0.1 mM ferrous ammonium sulfate and the rate of iron oxidation monitored by the increase of absorbance at 310 nm. The initial rate of iron oxidation is plotted against the proportion of H-chain in the samples determined by SDS electrophoresis. The arrow indicates the activity of the rLCr4/rLF heteropolymer under the same conditions: it corresponds to that of a H/L heteropolymer made by 7% H-chain and 93% L-chains.

DISCUSSION

The mutational inactivation of the ferroxidase activity of human H-chain provided important indications of the functionality of H- and L-chains. The construction of an L-chain with ferroxidase activity should be similarly instructive. To this aim, up to 8 residues of the L-chain were substituted with the homologous of the H-chain, but they had a strong negative effect on protein folding and assembly. We found that the simple insertion of Glu, which is essential for H-chain ferroxidase activity (Lawson et al., 1991), produced insoluble, misfolded, proteins which could be renatured in vitro only at slow rate and with low recoveries (Fig. 3 and Table II). Other substitutions made to reduce the local differences between H- and L-chains reduced, instead of improving, the capacity of L-chain to fold and assemble, to the extent that rLCr2, rLCr3, and rLCr4 never assembled in homopolymers. The substitutions involving large modifications of the charges rather than of the hydrophobicity of the amino acids. In particular Lys302–Glu and Tyr127–Glu replaced positive or neutral residues with acidic ones and had the strongest negative effects on L-chain folding and assembly. The findings that renaturation of the variants was facilitated by high salt or by adjacent positive charged histidines (i.e. His62 or His65, in the variants K62E+G65H and rLCr8, Table II) support the hypothesis that renaturation was obstructed by electrostatic repulsion. These data may partially explain the failure of the rLCr2 and rLCr4 to renature, but they do not clarify how H-chain accommodates the same residues with no evident effects on folding. Probably, unidentified, long range interactions in H- and not in L-chains stabilize the ferroxidase center.

We have previously shown that H-, L-chains and variants readily co-assemble in vitro to form heteropolymers
(Santambrogio et al., 1993). Now we show that even rLCr2 and rLCr4 that do not renature when alone easily renature with L- and H-chains to form hybrids with two to three variant chains/molecule. Possibly, some productive contacts with the H- and L-chains accelerate the rate of refolding of the variants or modify the folding pathways. This finding indicates that the mechanism of ferritin folding somehow favors heteropolymer formation.

Heteropolymers of the inactive L-chain with rLCr4 showed ferroxidase activity, while those with rLCr2 did not. The two variants differ for 4 residues (Table I) and the substitutions His37 → Glu and His39 → Glu were found to have no effect on H-ferritin ferroxidase activity. Tyr112 and Ala144 do not appear to be involved in metal binding (Lawson et al., 1991), while Gln413 is a ligand of the ferroxidase center (Fig. 1B). Treffry et al. (1993) showed that the substitution Gln413 → Glu has minor effects on H-chain ferroxidase activity; we propose that the constructed ferroxidase center and L-chains as it occurs in H/L heteropolymer formation even of subunits with very large differences in rates of refolding.

In conclusion the results show that: (i) the construction of a ferroxidase center in ferritin L-chain is feasible although it interferes with protein folding, (ii) the constructed centers are almost as active as the ones of the H-chains, and (iii) the peculiar mechanism of ferritin renaturation permits co-reassembly even of subunits with very large differences in rates of refolding.


REFERENCES