Effects of modifications near the 2-, 3- and 4-fold symmetry axes on human ferritin renaturation

Paolo SANTAMBROGIO*||, Patrizia PINTO*, Sonia LEVI*, Anna COZZI*, Ermanna ROVIDA†, Alberto ALBERTINI‡, Peter ARTYMIUK§, Pauline M. HARRISON§ and Paolo AROSIO‡

*DIBIT, San Raffaele Scientific Institute, Via Olgettina 58, Milano, Italy, †ITBA-CNR, Via Ampere 46, Milano, Italy, ‡Cattedra di Chimica Applicata alle Scienze Biomediche, University of Brescia, Italy, and §Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, U.K.

Ferritin is a protein of 24 subunits which assemble into a shell with 432 point symmetry. It can be denatured reversibly in acidic guanidine hydrochloride, with the formation of poorly populated renaturation intermediates. In order to increase the accumulation of intermediates and to study the mechanism of ferritin renaturation, we analysed variants of the human ferritin H-chain altered at the N-terminus (Δ_{1-13}), near the 4-fold axis (Leu-169 → Arg), the 3-fold axis (Asp-131 → Ile + Glu-134 → Phe) or the 2-fold axis (Ile-85 → Cys). We also carried out specific chemical modifications of Cys-130 (near the 3-fold axis) and Cys-85 (near the 2-fold axis). Renaturation of the modified ferritins yielded assembly intermediates that differed in size and physical properties. Alterations of residues around the 2-, 4- and 3-fold

axes produced subunit monomers, dimers and higher oligomers respectively. All these intermediates could be induced to assemble into ferritin 24-mers by concentrating them or by co-renaturing them with wild-type H-ferritin. The results support the hypothesis that the symmetric subunit dimers are the building blocks of ferritin assembly, and are consistent with a reassembly pathway involving the coalescence of dimers, probably around the 4-fold axis, followed by stepwise addition of dimers until the 24-mer cage is completed. In addition they show that assembly interactions are responsible for the large hysteresis of folding and unfolding plots. The implications of the studies for *in vivo* heteropolymer formation in vertebrates, which have two types of ferritin chain (H and L), are discussed.

INTRODUCTION

The native structure of a monomeric globular protein is determined by its amino acid sequence [1] and, in the case of a multimeric protein, by the positioning of amino acid residues in both inter- and intra-subunit contacts [2]. Ferritin is a tetra-cosamer which assembles as an almost spherical protein shell, of molecular mass about 500 kDa, enclosing a large cavity where iron is deposited as a 'core' of ferrihydrite [3–5]. It can be reversibly denatured from acidic guanidine hydrochloride (GdnHCl) or strongly acidic solutions [6,7]. Its simple construction from 24 structurally equivalent subunits in 432 symmetry and its lack of disulphide bridges make this protein a suitable model for renaturation studies.

Vertebrate ferritins are composed of two subunit types, named H and L, with about 50% amino acid sequence identity, which co-assemble in different proportions *in vivo* [8] and *in vitro* [9,10]. Within the assembled molecule each subunit is folded as a long four-helix bundle with a long loop (BC) connecting helix pairs A and B and C and D. There is a short fifth helix (E) at the C-terminus and a short non-helical extension at the N-terminus. In the protein shell, subunits make extensive contacts with five neighbours [3,5]. The long intersubunit interfaces around the 2-fold axis involve numerous hydrogen bonds and apolar interactions [3,4]; for example, two Ile-85 side chains from the BC loop face each other across the axis (Figure 1). At the 3-fold axis there are small metal-binding channels (Figure 2, top). Around these axes there are hydrophobic interactions of D-helix residues and extensive hydrogen-bonding between N-terminal side- and main-chain atoms with those of residues in helices C and D (Figure 2, bottom). The N-terminal residues 1–13 are involved in both inter- and intra-chain interactions. There are many further

interactions involving helices E and D and the DE turn around the 4-fold axis (Figure 3). The extensive co-operative network of inter- and intra-subunit interactions explains the unusual stability of ferritin and the observation that dissociation is accompanied by subunit unfolding [12].

Recombinant human H ferritin chain (rHF) has been subjected to numerous mutational studies to investigate *in vivo* renaturation after overexpression in the bacterium (*Escherichia coli*) [13,14]. Although *in vitro* stability was markedly decreased, assembly was not prevented by the deletion of the first 13 N-terminal amino acids (Δ_{1-13}), of the last 22 C-terminal amino acids ($\Delta_{161-182}$) [15], or by various substitutions in the four-helix bundle, in the hydrophilic channels, or in the inner and outer surfaces of the shell [16]. The deletions of Leu-82 and Ile-85 on the BC loop near the 2-fold axis gave proteins that were degraded during expression in the bacterium [15], as did the deletion of the last 28 residues, which includes the DE turn and E-helix [13]. The introduction of charged residues in the hydrophobic 4-fold channels [13] of basic residues in certain positions of the short DE turn [14] or the fusion of a 10 kDa peptide at the C-terminus [13,17] favoured an unnatural 'flop' conformation of the E-helix pointing outside the ferritin shell. Some mutations near the C-terminus gave insoluble aggregates [14]. However, the finding of functional activity in subassembly intermediates with the substitution Leu-169 → Arg in the E-helix implied correct folding [18]. The homopolymers of L-chains (rLF) are significantly more stable than those of H-chains [16], but the variants with the substitution Lys-62 → Glu within the four-helix bundle do not fold and assemble, either *in vivo* in *E. coli* or *in vitro*, unless they are co-renatured with wild-type H- or L-chains [19].

Renaturation of ferritin generally yields poorly populated intermediates which have been analysed in detail by two groups

Abbreviations used: rHF, recombinant human ferritin H-chain; rLF, recombinant human ferritin L-chain; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; 5-IAF, 5-iodoacetamidofluorescein; IAA, iodoacetic acid; IAM, iodoacetamide.

|| To whom correspondence should be addressed.

Figure 1 Schematic diagram of the interface between two subunits around the 2-fold axis, viewed from the centre of the molecule

One subunit is at the top left and the other at the bottom right. Ile-85 residues from the two chains are shown in van der Waals contact with one another, forming important hydrophobic contacts within the intersubunit interface. Replacement of Ile-85 by Cys with a bulky or charged modifying group will be expected to interfere with interactions around the 2-fold axis and destabilize dimer formation. Drawings in Figures 1–3 were made using the program MOLSCRIPT [11].

[20–22]. Both groups agree that the starting building blocks are subunit dimers, but they differ in the order of the subsequent steps. Sedimentation velocity and near-UV CD data were interpreted as renaturation via tetramers and octamers, i.e. with dimer aggregation around the 4-fold axis [20]. In contrast, cross-linking experiments [7,22] indicated that aggregation occurs by a pathway involving dimers, trimers, transient hexamers and dodecamers:

where the subscript refers to the number of subunits in the intermediate.

In the present experiments we applied selected modifications in the neighbourhood of the 2, 3- and 4-fold symmetry axes of human H-chain ferritins to increase the populations of renaturation intermediates. The conformation of the sub-assembly species so isolated was studied by UV and CD spectroscopy and their oligomeric status and capacity to reassemble under different conditions have been analysed by gel electrophoresis.

MATERIALS AND METHODS

Ferritins and variants

Human ferritin H-chain variants were obtained by oligonucleotide-directed mutagenesis of the plasmid pEMBLEx2HFT, as described previously [15,23]. The variants used in this study are listed in Table 1. Variants Δ_{1-13} and L169R have been described previously [15]. In variant rHF-C130, Cys-90 and Cys-102 were substituted with residues found in human ferritin L-chain (Glu and Ala respectively) leaving a single cysteine in position 130, near the 3-fold axis. Variant rHF- Δ C is cysteine-free, as the result of the further substitution of Cys-130

Table 1 Human ferritin H-chain variants

The stability is expressed as the GdnHCl molarity of the 50% transition from native to denatured states at pH 7.4, detected by fluorescence spectra with excitation at 295 nm, as indicated in Figure 4.

Protein	Substitutions	Stability (M GdnHCl)
rLF	Wild-type	> 8
rHF	Wild-type	6.0
rHF- Δ_{1-13}	Deletion of the first 13 N-terminal residues	3.8
rHF-L169R	Leu-169 $\rightarrow$ Arg on the 4-fold axis	3.2
rHF-D131I + E134F	Asp131Ile + Glu134Phe on the 3-fold axis	3.8
rHF-C130*	C90E + C102A; a single Cys-130 near the 3-fold axis	6.8
rHF- Δ C*	C90E + C102A + C130A; cysteine-free	7.0
rHF-I85C*	rHF- Δ C + I85C; a single Cys-85 near the 2-fold axis	5.9

* These mutants also carry the substitution K86Q to enable crystallization.

with Ala, and in rHF-I85C a single cysteine was inserted into rHF- Δ C in place of Ile-85, near the 2-fold axis. In variant rHF-D131I + E134F the residues lining the 3-fold channel (Asp-131 and Glu-134) were substituted with the hydrophobic Ile and Phe respectively [24]. Homopolymers of human H-chain and its variants were expressed by transformed *E. coli* strains as described by Levi et al. [15] and purified as described by Santambrogio et al. [16]. Yields of purified proteins were in the range of 10–15 mg/l of culture. Briefly, expression was induced by heat shock at 42 °C, cells were disrupted by sonication and the soluble homogenates were heated at 75 °C for 10 min, precipitated with $(\text{NH}_4)_2\text{SO}_4$ (520 g/l) and treated with DNase and RNase. The final purification steps consisted of gel filtration on a Sepharose 6B column followed by ion-exchange chromatography on Hi-Trap Q (Pharmacia). Ferritins were judged to be pure by gel electrophoresis. Protein concentration was determined with BCA reagent (Pierce) using BSA as standard.

Electrophoresis

Non-denaturing electrophoresis was carried out on 7.5% polyacrylamide gels, and proteins were stained with Coomassie Blue or silver stain (Bio-Rad) [10,16]. Approximate molecular mass estimations were made for some oligomers by calibration with the assembled 24-mer ferritins and subunit dimers. For electrophoresis in 7.5% polyacrylamide gels containing 3.5 or 5 M urea, the samples were diluted with an equal volume of 0.25 M Tris/HCl buffer, pH 6.8, containing 7 or 10 M urea and 10% glycerol. The gels were stained with Coomassie Blue, and, after destaining, if needed, densitometry was performed on a Computing Densitometer (Molecular Dynamics).

Spectrophotometric analysis

Far-UV CD spectra were measured with a JASCO J500-A spectropolarimeter equipped with a JASCO DP-500/PC data processor. The samples (0.05–0.2 mg/ml) were placed in 0.1 cm path length cells. Fluorescence spectra were measured with a Kontron SFM25 spectrofluorimeter with an excitation window of 5 nm and emission window of 10 nm. Protein concentrations were in the range 0.02–0.07 mg/ml. In the CD and fluorescence experiments, backgrounds resulting from GdnHCl or urea were subtracted.

Figure 2 Schematic diagrams of intersubunit contacts in the neighbourhood of a 3-fold axis

Top, 3-fold channel viewed from outside the molecule. The six carboxy groups of Asp-131 and Glu-134 residues shown in the diagram can ligate metal ions (not shown); their substitution with Ile-131 and Phe-134 may be expected to affect assembly. Cys-130 residues (large spheres) are also near to the 3-fold symmetry axis. They lie within the subunits between helices C and D (front and back respectively) of their four-helix bundles. Modification of Cys-130 with bulky or charged groups will be expected to interfere with assembly around the 3-fold axis. Bottom, stereodiameters of the region adjacent to a 3-fold channel showing the N-terminal sequence and its neighbourhood. N-Terminal residues 5 to 13 (black subunit) make hydrogen bonds (dotted lines) to both side-chain and main-chain atoms of two neighbouring subunits and of the same subunit. There are numerous apolar interactions which are not shown. The view is contiguous with that of the top diagram but is from a slightly different direction. N-Terminal residues 1–4, which are not clearly visible in the electron-density map, are not represented.

Ferritin denaturation and renaturation

Denaturation of ferritin was examined in samples (50 $\mu\text{g/ml}$) that had been incubated for 18 h at 4 $^{\circ}\text{C}$ with various GdnHCl concentrations in 0.1 M phosphate buffer, pH 7.4, containing 3 mM dithiothreitol (DTT) [10]. Conformational status was estimated from the ellipticity values at 222 nm and from fluorescence spectra (excitation at 295 nm), using as standards ferritin in either 0.1 M phosphate, pH 7.4 (native status) or 6 M GdnHCl/0.1 M phosphate buffer, pH 3.5 (denatured status) [16]. Fluorescence spectra were collected in the interval 310–370 nm, and used to calculate the average emission wavelength [25]. To examine renaturation, denatured ferritins were diluted at least 10-fold in 0.1 M sodium phosphate buffer, pH 7.4/3 mM DTT, containing various concentrations of GdnHCl or urea, and after 2 h incubation the fluorescence spectra were recorded. When needed, the renatured samples were concentrated on Centricon 30 membrane (Amicon).

Co-assembly

Co-assembly experiments were performed as described [10]: the homopolymers were unfolded in 6 M GdnHCl, pH 3.5, for 2 h, mixed in different proportions, and diluted in the renaturing buffer. In other experiments, the denatured rHF in 6 M GdnHCl was diluted in the renaturation buffer containing equal amounts of preformed subassembly and derivatized species of ferritin.

Cysteine modifications

Iodoacetic acid (IAA), iodoacetamide (IAM; Sigma) and 5-iodoacetamidofluorescein (5-IAF, Molecular Probes) were used for specific cysteine modification. The reactions were performed on ferritin previously denatured by incubation in 6 M GdnHCl/0.1 M phosphate buffer, pH 3.5, and titrated to pH 8.0. Typically, the proteins were incubated with 3 mM DTT, thiol

Figure 3 Diagram of parts of four subunits adjacent to a 4-fold axis

Residues Leu-165, Leu-169 (dark atoms) and His-173 from each of four E-helices point towards a 4-fold axis and make predominantly apolar contacts. Leucines 169 lie in the central part of the interface. The view is from the outside of the molecule. Parts of the D-helices are seen in the corners of the diagrams. Replacement of Leu-169 by Arg would lead to a concentration of bulky positively charged groups in the region and destabilize 4-fold contacts.

reagents (10 mM) were added, and the samples incubated for 1 h at 25 °C. Excess reagent was then removed by ultrafiltration.

RESULTS

Ferritin denaturation

We analysed human rHF and rLF wild-type ferritins and the H-chain variants listed in Table 1; the structural localizations of the substitutions are shown in Figures 1–3. The relative stability of the proteins (Figure 4) was analysed by incubating the apo-ferritins with increasing concentrations of GdnHCl at pH 7.4 and by monitoring the shift of fluorescent emission (excitation at 295 nm) of the single and conserved Trp-93. Proteins remained fully soluble in all the denaturing conditions used. Fluorescence was expressed as average emission wavelengths [25] and spectra were collected in the range 310–370 nm. The emission of rLF was blue-shifted with respect to that of rHF (maxima at 320 and 327 nm respectively), and its unfolding transition started at GdnHCl concentrations above 6 M, whereas that of rHF occurred in the range 4–8 M GdnHCl, as previously reported [16]. The denaturation plots of rHF- Δ C (not shown) and rHF-C130 (Figure 4) were similar and right-shifted with respect to that of rHF. The substitution Ile-85-Cys introduced into rHF- Δ C to give a new variant (rHF-I85C) with a single cysteine appeared to affect the slope of the denaturation curve more than the midpoint of unfolding transition. The variants rHF-L169R, rHF- Δ _{1–13} and rHF-D131I + E134F were significantly less stable than rHF (Δ midpoint about 2 M GdnHCl; Table 1) and the slopes of the plots indicated decreasing co-operativity in the order: L169R > D131I + E134F > Δ _{1–13}.

Figure 4 Denaturation plot of ferritin variants

Denaturation plots obtained by diluting the assembled 24-mers into 0.1 M phosphate buffer (pH 7.4)/3 mM DTT, containing various concentrations of GdnHCl. The final protein concentration was 50 μ g/ml. After 2 h incubation the fluorescence emission spectra in the interval 310–370 nm were collected (excitation at 295 nm) and the average emission wavelength was calculated. Den indicates the fully denatured samples in 6 M GdnHCl at pH 3.5.

Reassembly

The proteins were denatured in acidic 6 M GdnHCl, renatured by 10-fold dilution in renaturation buffer and analysed by non-denaturing PAGE. Protein precipitation was undetectable in all samples, and the recovery of the renatured ferritins was above 90%, as judged by A_{280} . Figure 5 (top) shows that rHF, rLF, rHF-I85C and rHF-D131I + E134F quantitatively reassembled to the 24-mer, as did rHF-C130 (not shown) whereas rHF- Δ _{1–13} and rHF-L169R formed single subassembly species of fast electrophoretic mobility (Figure 5, top, lanes 5 and 6). Thus the genetic modifications on the 2- and 3-fold axes did not show detectable effects on protein renaturation, under the analytical conditions used. Variant rHF-I85C has cysteines in adjacent subunits symmetrically located near the 2-fold axis (Figure 1), and variant C130 has three cysteines close to the 3-fold axis (Figure 2, top). These variants provided an opportunity for specific chemical modification of the contacts around the two types of axis. Preliminary data showed that in the assembled protein Cys-85 and Cys-130 are not easily accessible to 5,5'-dithiobis-(2-nitrobenzoic acid) or other thiol-specific reagents (not shown). Therefore, the variant was denatured, allowed to react with IAM (uncharged), IAA (single negative charge) or 5-IAF (three negative charges) and treated with renaturation buffer as above. IAM modification showed little effect on the renaturation of either protein (not shown), IAA prevented the assembly of rHF-I85C (Figure 5, middle, lane 3), but not of rHF-C130 (not shown), and IAF reduced the assembly of both (Figure 5, middle, lanes 2 and 4). The non-assembled proteins formed single electrophoretic bands, the mobility of which was related to the variant and to the type of modifying group. The subassembled rHF-L169R-IAF or -IAA moved faster than the unmodified rHF-L169R but remained slower than rHF-I85C-IAF or -IAA (Figure 5, middle, compare lanes 2 with 6, and 3 with 7). This suggested that the rHF-L169R species is in an

Figure 5 Non-denaturing PAGE of the renatured samples

The renatured samples (0.2 mg/ml in 0.6 M GdnHCl) were subjected to PAGE (7.5% gels; 3 μ g of sample per well) and stained with Coomassie Blue. Top, analysis of non-derivatized ferritins and variants. Lane 1, rLF; lane 2, rHF; lane 3, rHF-I85C; lane 4, rHF-D131I + E134F; lane 5, rHF- Δ_{1-13} ; lane 6, rHF-L169R. Derivatized samples were analysed before (middle) and after (bottom) concentration to about 1 mg/ml on Amicon membrane. Lane 1, rHF; lane 2, rHF-I85C-IAF; lane 3, rHF-I85C-IAA; lane 4, rHF-C130-IAF; lane 5, rHF-L169R; lane 6, rHF-L169R-IAF; lane 7, rHF-L169R-IAA. The arrows indicate the position of the assembled ferritins.

oligomeric state larger than that of rHF-I85C, since the chemical modifications would be expected to increase the electrophoretic mobility of rHF-L169R (with three available cysteines) more than that of rHF-I85C (with a single cysteine). Possibly the rHF-L169R species is a dimer and that of rHF-I85C is a monomer, the modification preventing association around the 2-fold axis under these conditions. The slow moving subassembly species of rHF-C130-IAF appears to be a large oligomer (Figure 5, middle, lane 4).

Renaturation experiments were normally performed at protein concentrations below 0.2 mg/ml to prevent protein precipitation; attempts at higher concentrations (up to 1 mg/ml) produced aggregation of rHF and, with unmodified rHF-L169R, a ladder of bands ranging from the fast moving species to the fully assembled protein (not shown). In contrast, when the renatured rHF-L169R subassembly species were concentrated by mem-

Table 2 Properties of the isolated subassembly species

Subassembly oligomerization was estimated by PAGE. The capacity to autoassemble is the capacity to assemble into the 24-mer protein after concentration on an Amicon membrane or 1 month incubation at 4 °C. The capacity to co-assemble with rHF was deduced from the experiments in which the variants were co-refolded with equivalent amounts of renaturing wild-type rHF. In parentheses are the results obtained from similar experiments in which rHF was renatured with the subassembly species of the variants. Assembled shells were recognized by electrophoretic mobility on non-denaturing PAGE of the fluorescent derivatives or after Coomassie Blue staining. Yes, complete assembly of the variants; partial, equivalent proportion in the assembled and non-assembled species; limited, assembled species present in minor proportions. The ellipticity at 222 nm is the percentage of the ellipticity values at 222 nm of the subassembly species compared with those of the same protein in the assembled state. n.d., not determined.

Variant	Subassembly oligomerization	Capacity to autoassemble into 24-mer	Capacity to co-assemble with rHF	Ellipticity at 222 nm (%)
rHF-I85C-IAF	Monomer	Yes	Yes (yes)	n.d.
rHF-I85C-IAA	Monomer	No	Yes (yes)	60
rHF- Δ_{1-13}	Dimer	Yes	Yes (yes)	90
rHF-L169R	Dimer	Yes	Partial (partial)	90
rHF-L169R-IAF	Dimer	No	Limited (limited)	80
rHF-C130-IAF	8–10-mer	Partial	Yes (partial)	80

brane ultrafiltration, they readily reassembled (Figure 5, bottom, lane 5). The same occurred for unmodified rHF- Δ_{1-13} (not shown), rHF-I85C-IAF and rHF-C130-IAF (Figure 5, bottom, lanes 2 and 4), although the latter reassembled only partially (about 50%). rHF-L169R-IAF remained soluble after concentration, but did not assemble and rHF-I85C-IAA produced insoluble aggregates (Figure 5, bottom, lanes 6 and 7).

In other experiments the denatured rHF was renatured in solutions containing equimolecular amounts of the refolded subassembly species of the variants. In parallel, the same unfolded variants were co-renatured with rHF. The results (Table 2) show that for the variants modified around the 2-fold (i.e. rHF-I85C-IAA and -IAF) and the 4-fold (i.e. rHF-L169R and its -IAF derivative) axes the two renaturation conditions (i.e. starting from unfolded or from subassembly states) were equally effective for co-reassembly. In contrast, the variant modified around the 3-fold axis (i.e. rHF-C130-IAF) co-reassembled with rHF more efficiently when added to rHF in the unfolded than in the subassembly state, although a significant shift from the subassembly species to the fully assembled proteins was induced by rHF.

Renaturation plots

The denatured proteins were diluted in renaturing buffer with various urea concentrations, and the intrinsic fluorescence determined as above. The renaturation plots of the ferritins (Figure 6, top) showed similar transition midpoints around 3.5–4.0 M urea but had different shapes. rLF showed a sharp two-state transition, whereas variant rHF-D131I + E134F showed two transitions, with an equilibrium intermediate at urea concentrations around 4 M. The plots of rHF and the other H-variants demonstrated trailing at low urea concentrations, suggesting the presence of intermediates (most evident in rHF-I85C). Electrophoretic analysis on non-denaturing urea-free gels showed that for rHF (Figure 6, middle) and rLF the formation of the assembled 24-mer protein occurred in parallel with refolding (monitored by intrinsic fluorescence) and the disappearance of various fast and slow moving subassembly species (which differed

UREA (M)

UREA (M)

Figure 6 Renaturation plots of the variants in urea

Top, the denatured samples were diluted 10-fold in renaturing buffer; after 2 h incubation, the fluorescence emission spectra in the interval 310–370 nm were collected (excitation at 295 nm) and the average emission wavelength was calculated. The data are presented as fraction of unfolded protein using as references the native conditions and unfolded protein in 8 M urea. Nat indicates the native samples and Den the fully denatured ones. The results of urea-free gel electrophoresis (silver stained) of denatured samples diluted in various urea concentrations are also shown: middle, rHF; bottom, variant rHF-D131I + E134F. The arrows indicate the mobility of assembled ferritins.

Figure 7 Renaturation and denaturation plots in urea of variant rHF-L169R subassembly species

Denaturation (□) and renaturation (●) plots of the subassembled rHF-L169R are shown. Conditions were as in Figure 4.

between rHF and rLF; not shown). In rHF-D131I + E134F, a ladder of subassembly species was evident mainly in the interval between the two transitions, and ferritin assembly appeared to occur mainly in the second transition (Figure 6, bottom). More interestingly, rHF-L169R and rHF- Δ_{1-13} (not shown) showed only a single subassembly species at all urea concentrations, together with some non-specific aggregates which decreased at low urea concentration. Electrophoretic analysis of the samples in 3.6 M urea performed in 3.5 M urea-containing gels showed equivalent amounts of the assembled 24-mer and a fast subassembly species in rHF and rLF and all derivatives, except rHF-L169R and rHF- Δ_{1-13} , where only the fast species was present. Also in rHF-D131I + E134F the assembled 24-mer could not be detected, and it was replaced by a large unresolved band (not shown).

The data indicate that the unfolded proteins have undergone refolding and subunit aggregation during the electrophoresis in urea-free gels. To analyse this further, we examined equimolar mixtures of denatured rHF-L169R and rHF- Δ_{1-13} in 8 M urea in urea-free gels. In addition to the bands of subassembled rHF- Δ_{1-13} and rHF-L169R, a new intermediate band of similar intensity appeared, which was absent from 5 M urea/PAGE (not shown). The band must be a heterodimer of the two subunits, since it is single and has a mobility intermediate between those of rHF- Δ_{1-13} and rHF-L169R, and only appeared when the two variants were mixed. Consequently, the subassembled rHF- Δ_{1-13} and rHF-L169R species must be dimers. The fastest moving subassembly species of rHF in urea-free gels has the same mobility as refolded rHF-L169R (not shown) and must be dimers as well, whereas bands due to rHF-I85C derivatized with IAF or IAA, which have faster mobilities than the corresponding derivatized rHF-L169R (Figure 5, middle, lanes 2 and 3), are probably monomers. Analysis by far-UV CD showed the subassembled rHF-L169R and rHF- Δ_{1-13} to have more than 90% of the ellipticity at 222 nm of the native conformation, whereas the rHF-I85C-IAA had 60% (Table 2).

Finally, the subassembled rHF-L169R (dimer) was incubated in various urea concentrations, and the intrinsic fluorescence analysed as above. The unfolding plot so obtained overlapped the refolding plot in all points except for minor differences at low urea concentrations (Figure 7). Similar results were obtained with the subassembled rHF- Δ_{1-13} (not shown). Assembled rHF did not unfold even at the highest urea concentrations under these conditions.

DISCUSSION

Ferritin renaturation is a complex process involving subunit folding and various steps of subunit aggregation leading to the 24-mer protein [20,22]. Although general models have been proposed, the relationship between subunit folding and assembly, and the parts played by interchain interactions around the 2-, 3- and 4-fold axes have not been analysed in detail. Using rHF variants we have now found that changes in local charge and hydrophobicity at the 3-fold (D131I + E134F) or 4-fold (L169R) axes led to a reduction in shell stability, as did deletion of the 13 N-terminal residues that form numerous interactions at subunit interfaces near the 3-fold axis (Table 1 and Figure 4). In contrast, neither the more conservative substitution Ile85Cys near the 2-fold axis nor the changes Cys90Glu + Cys102Ala (in which the substituted residues correspond to those in rLF) have a significant effect on the stability of the assembled 24-mer. However, introduction of negative charges by chemical modification could have a dramatic effect on oligomerization (rHF-I85C-IAA, rHF-L169R-IAF and rHF-C130-IAF). The addition of wild-type rHF improved the capacity of the altered subunits to assemble into shells.

Contacts near the 2-fold axis and dimer formation

The data emphasize the importance of the extensive contacts around the 2-fold axis in several ways. Our results show that renaturation of rHF-L169R and rHF- Δ_{1-13} separately or together yields subunit dimers. Introduction of adjacent negative changes into this interface in the IAF and IAA derivatives of variant rHF-I85C not only destabilizes the dimer, but also lessens the ability of the subunit to regain its native conformation, as judged by the reduced ellipticity of the resultant monomer. Furthermore the failure of the IAA derivative to assemble into shells (except in the presence of wild-type rHF) suggests that structured dimers are key intermediates in the assembly process. This confirms the previous experimental data and the prediction of Ford et al. [3] that the antiparallel subunit pairs, formed through contacts around the 2-fold axis, are the building blocks of the ferritin shell [20,22]. The lack of hysteresis in the denaturation-renaturation plots of the dimeric rHF-L169R (Figure 7) supports the view that the hysteresis seen with wild-type rHF is a property of the shell structure.

Further steps in renaturation involving contacts around 3-fold and 4-fold axes

The renaturation experiments of Figures 5 and 6 indicate that the variants with changes in or near the 3-fold or 4-fold axes can acquire native secondary and quaternary structure (also shown by the isolation of variant 24-mer from *E. coli*). The assembly of rHF-L169R dimers is an exceedingly slow process (half-time of more than 2 days compared with a few minutes for wild-type rHF; not shown). Although the stable intermediates of rHF-L169R or rHF- Δ_{1-13} were only dimers, that of rHF-C130-IAF was larger, probably an octamer or decamer from its relative

mobility. This may have arisen by association of four dimers at a 4-fold axis, an unmodified region. These intermediates were competent to reassemble, at least partially, to the 24-mer when concentrated (Figure 5, bottom, lane 4) or when co-renatured with rHF (Table 2). The double 3-fold axis variant rHF-D131I + E134F, which reassembled at a rate comparable with that of wild-type rHF, appeared to give a new uncharacterized equilibrium intermediate on renaturation in decreasing urea concentrations (Figure 6, top). When examined by urea-free PAGE it showed a ladder of bands ranging from the dimer to the 24-mer (Figure 6, bottom). Short ladders of higher oligomers were observed also with rHF (Figure 6, middle), rLF and rHF-L169R (not shown) immediately after renaturation, indicating that oligomers of increasing size are formed by stepwise addition of a small species (presumably dimers) on to a larger intermediate. It has previously been pointed out [3] that, if dimers associate around the 4-fold axis to give octamers, then completion of the shell by confluence of three such octamers is impossible for topological reasons, and stepwise assembly must be envisaged in this case. It is plausible that such 4-fold association is occurring here, especially in the case of variant rHF-D131I + E134F, where there is interference with the normal 3-fold axis interactions. However, proof that the subassembly ladder species are productive intermediates is lacking, and they may be produced by aggregation of misfolded subunits. Of the oligomers isolated by Gerl and Jaenicke [22] by cross-linking of horse spleen ferritin, the dimer fraction gave 100% regain of tetracosamer, whereas the trimer yielded only 60% regain after 90 h incubation and the tetramer only 35%. We could not find evidence for trimer accumulations during dissociation or reassociation; they may have been destabilized by the modifications or may be an insignificant population. The formation of large oligomers (12–24-mers) contrasts with the mechanism proposed by Gerl and Jaenicke [22] where the last assembly step was the association of two dodecamers.

Subunit co-assembly

When mixed with H- or L-chains all the chemically or genetically modified rHF chains were capable of complete or partial copolymerization to shells, including those incapable of reassembling on their own. Presumably the introduction of charged or bulky groups near symmetry axes causes repulsion, but when 'diluted' by wild-type residues the groups can be tolerated. When located on the 2-fold axis (e.g. rHF-I85C-IAA) the groups do not prevent the formation of heterodimers with rHF, which are probably the prevalent first-assembly intermediates in this case. The folded rHF-L169R dimers readily co-assemble with rHF (Table 2), indicating that the ferritin shell can be built from different homodimers. In contrast, the large oligomeric species of rHF-C130-IAF co-assemble only partially with rHF, probably producing co-polymers in which the two subunit species form clustered patches. However, when this variant is co-refolded with rHF, it reassembles quantitatively, probably because the formation of heterodimers reduces the probability of the interfering groups coming together at the 3-fold axis. Thus we propose that the quaternary structure of the heteropolymers may be affected by intersubunit contacts and by the kinetics of co-assembly.

Implications for folding and assembly *in vivo*

Our data suggest that ferritin renaturation starts with the subunit folding into a partially structured conformation followed by the

formation of fully structured dimers. The subsequent steps in the reassembly pathway may be clarified by detailed kinetic studies and further characterization of intermediates. The suggested mechanism, in which the subunit dimers coalesce to give an oligomeric nucleus on to which further dimers are added stepwise, resembles that observed for virus procapsid shells [26]. The time course of the various steps is unclear except that we expect dimer formation to be the fastest. If so, then the H- and L-chains being synthesized on different polysomes may form homodimers *in vivo* and the naturally assembled heteropolymers would be assemblages of homodimers. This would reduce the heterogeneity of isoferritins and could also have consequences for their functionality. The putative centres for iron-core nucleation lie on inner-surface carboxy groups near the 2-fold axis [3,4]. L-chains have more extended clusters of such groups [27], and pairs of L-chains could co-operate in the nucleation and growth of the ferrihydrite mineral. The observations that L-chains exhibit more co-operative renaturation plots (Figure 6, top), populate different refolding intermediates and have a higher stability than H-chains (Figure 4) are consistent with the hypothesis that the two chains renature via different pathways and/or kinetics. If one chain type gives a relatively stable assembly nucleus, then the two chains may be partially segregated into patches within the 24-mer, as suggested previously [28].

The project was partially supported by CNR Target Project Biotechnology and Bioinstrumentation and by CEC-BRIDGE grant BIOT-CT91-0262.

REFERENCES

- Anfinsen, C. B. (1973) *Science* **181**, 223–230
- Ziegler, M. M., Goldberg, M. E., Chaffotte, A. F. and Baldwin, T. O. (1993) *J. Biol. Chem.* **268**, 10760–10765
- Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L. and Yariv, J. (1984) *Philos. Trans. R. Soc. London Ser. B* **304**, 551–565
- Lawson, D. M., Artymiuk, P. J., Yewdall, S. J., Livingstone, J. C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C. D., Shaw, W. and Harrison, P. M. (1991) *Nature (London)* **349**, 541–544
- Harrison, P. M. and Arosio, P. (1996) *Biochim. Biophys. Acta* **1275**, 161–203
- Stefanini, S., Finazzi-Agro', A., Chiancone, E. and Antonini, E. (1979) *FEBS Lett.* **100**, 296–300
- Gerl, M. and Jaenicke, R. (1987) *Biol. Chem. Hoppe-Seyler* **368**, 387–396
- Arosio, P., Adelman, T. G. and Drysdale, J. W. (1978) *J. Biol. Chem.* **253**, 4451–4458
- Otsuka, S., Maruyama, H. and Listowsky, I. (1981) *Biochemistry* **20**, 5226–5232
- Santambrogio, P., Levi, S., Cozzi, A., Rovida, E., Albertini, A. and Arosio, P. (1993) *J. Biol. Chem.* **268**, 12744–12748
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950
- Listowsky, I., Bethel, J. J. and Englard, S. (1967) *Biochemistry* **6**, 1341–1348
- Luzzago, A. and Cesareni, G. (1989) *EMBO J.* **8**, 569–576
- Jappelli, R., Luzzago, A., Tataseo, P., Pernice, I. and Cesareni, G. (1992) *J. Mol. Biol.* **227**, 532–543
- Levi, S., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. and Arosio, P. (1988) *J. Biol. Chem.* **263**, 18086–18092
- Santambrogio, P., Levi, S., Arosio, P., Palagi, L., Vecchio, G., Lawson, D. M., Yewdall, S. J., Artymiuk, P. J., Harrison, P. M., Jappelli, R. and Cesareni, G. (1992) *J. Biol. Chem.* **267**, 14077–14083
- Sidoli, A., Tamborini, E., Giuntini, I., Levi, S., Volonté, G., Paini, C., De Lalla, C., Siccardi, A. G., Baralle, F. E., Galliani, S. and Arosio, P. (1993) *J. Biol. Chem.* **268**, 21819–21825
- Levi, S., Santambrogio, P., Albertini, A. and Arosio, P. (1993) *FEBS Lett.* **336**, 309–312
- Levi, S., Corsi, B., Rovida, E., Cozzi, A., Santambrogio, P., Albertini, A. and Arosio, P. (1994) *J. Biol. Chem.* **269**, 30334–30339
- Stefanini, S., Vecchini, P. and Chiancone, E. (1987) *Biochemistry* **26**, 1831–1837
- Gerl, M. and Jaenicke, R. (1987) *Eur. J. Biophys.* **15**, 103–109
- Gerl, M. and Jaenicke, R. (1988) *Biochemistry* **27**, 4089–4096
- Levi, S., Cesareni, G., Arosio, P., Lorenzetti, R., Soria, M., Sollazzo, M., Albertini, A. and Cortese, R. (1987) *Gene* **51**, 267–272
- Levi, S., Santambrogio, P., Corsi, B., Cozzi, C. and Arosio, P. (1996) *Biochem. J.* **317**, 467–473
- Royer, C. A., Mann, C. J. and Matthews, C. R. (1993) *Protein Sci.* **2**, 1844–1852
- Prevelige, P. E., Thomas, D. and King, J. (1993) *Biophys. J.* **64**, 824–835
- Levi, S., Santambrogio, P., Cozzi, A., Rovida, E., Albertini, A., Yewdall, S. J., Harrison, P. M. and Arosio, P. (1992) *Biochem. J.* **288**, 591–596
- Luzzago, A., Arosio, P., Iacobello, C., Ruggeri, G., Capucci, L., Brocchi, E., De Simone, F., Gamba, D., Gabri, E., Levi, S. and Albertini, A. (1986) *Biochim. Biophys. Acta* **872**, 61–71