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Isolation of point mutations that affect the folding of the H chain of human ferritin in *E.coli*

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We have approached the problem of folding and assembly of the heavy (H) chain of human ferritin by isolating point mutations that affect this process. Apoferritin is an ideal model system to approach the problem of protein folding and assembly into multimeric structures. We have developed a recombinant hybrid molecule that allows us to select for ferritin mutants in which the folding-assembly process is altered or completely impaired. The selection procedure is based on a recombinant protein which consists of a fusion between the H chain of human ferritin and the α-peptide of β-galactosidase. In the wild type situation, the α-peptide domain is segregated inside the apoferritin shell upon assembly and is unable to interact with the substrate and perform its enzymic function. We show that by selecting for mutations that restore β-galactosidase activity we are able to identify ferritin mutations that affect the folding-assembly process. The selective procedure was applied to the analysis of the amino acid side chains that are important for the attainment of the correct conformation of the carboxy-terminal E helix in the 4-fold axis.

Key words: Escherichia coli/ferritin/heavy chain/tertiary structure

Introduction

The information required to determine the correct three dimensional structure of a protein is entirely contained in the linear sequence of amino acids of the peptide chain (Anfinsen, 1973). Most proteins can be unfolded *in vitro* in the presence of denaturing agents and then correctly refolded by diluting away the denaturant indicating that no complex machinery is essential (for a review see Jaenicke, 1987). Although a number of different experimental systems have been successfully approached by these *in vitro* techniques the rules that determine the folding pathway, the kinetics of the process, the configuration of the native structure and its stability are not understood. The possibility of deciphering a folding code that would allow the information encoded in the linear sequence of amino acids to be translated into three dimensions seems rather remote.

A further complication derives from the fact that not much information is available about the process of folding *in vivo*. It is conceivable that interaction with the complex cellular environment might interfere either negatively or positively with the kinetics of the process. At least two enzymes, proline isomerase and disulphide isomerase, have been shown to speed up the kinetics of folding of some proteins by accelerating the rate limiting process of proline isomerization and disulphide bond shuffling (Lang *et al.*, 1987; Freedman, 1984). Furthermore, the cell environment is rather different from an idealized buffered aqueous solution and it is unclear how the high protein concentration in the cytoplasm or the presence of small non-polar molecules might affect the folding process. A last controversial point concerns the issue of whether the directionality of the translation process, from the N- to the C-terminus, might confer some directionality on the folding process *in vivo* (Dunhill, 1967; Wetlaufer, 1984).

King and colleagues have pioneered the study of protein folding *in vivo* by developing, as a model system, the tail spike protein of phage P22 (King *et al.*, 1986). This system is amenable to genetic analysis and has allowed the identification of scattered amino acid sequence information, along the 666 amino acids of the polypeptide chain, that is required for correct folding and assembly of the trimeric protein. It seems unlikely however that the detailed study of a single system will lead to the complete understanding of protein folding *in vivo*.

Ferritin as a model system

During the past two years we have developed a system that should allow us to study the mechanism of folding and assembly of ferritin in *Escherichia coli*. Apoferritin is an attractive molecule to study protein folding and molecular assembly into complex structures because: (i) the monomer has a size (183 amino acids in the H chain of ferritin) which is comparable to the average size of known protein domains. (ii) More importantly the structure of horse spleen apoferritin is known to a high resolution, thus providing a structural basis for planning site-directed mutagenesis experiments and interpreting the results (Clegg *et al.*, 1980; Ford *et al.*, 1984). The monomer consists of a bundle of four antiparallel α-helices (A-D). Helices B and C are connected by a long loop traversing the whole bundle and making contacts with the A helix. The carboxy-terminal amino acids are arranged in a fifth shorter α-helix (helix E), that forms an acute angle with the main axis of the molecule (see Figure 1). Ferittin is an etheropolymer consisting of two homologous subunits, H and L, that assemble in different proportions in different tissues (Arosio *et al.*, 1978). An apoferritin molecule is composed of 24 subunits arranged in 432 symmetry forming a hollow shell pierced by six hydrophobic and eight hydrophilic channels along the 4-fold and 3-fold axes of symmetry respectively (Clegg *et al.*, 1980; Ford *et al.*, 1984). It is proposed that iron accumulates in the ferritin molecule by entry through these channels. (iii) The molecule can be unfolded and efficiently refolded *in vitro* and two detailed, but conflicting, assembly pathways have been proposed (Gerl

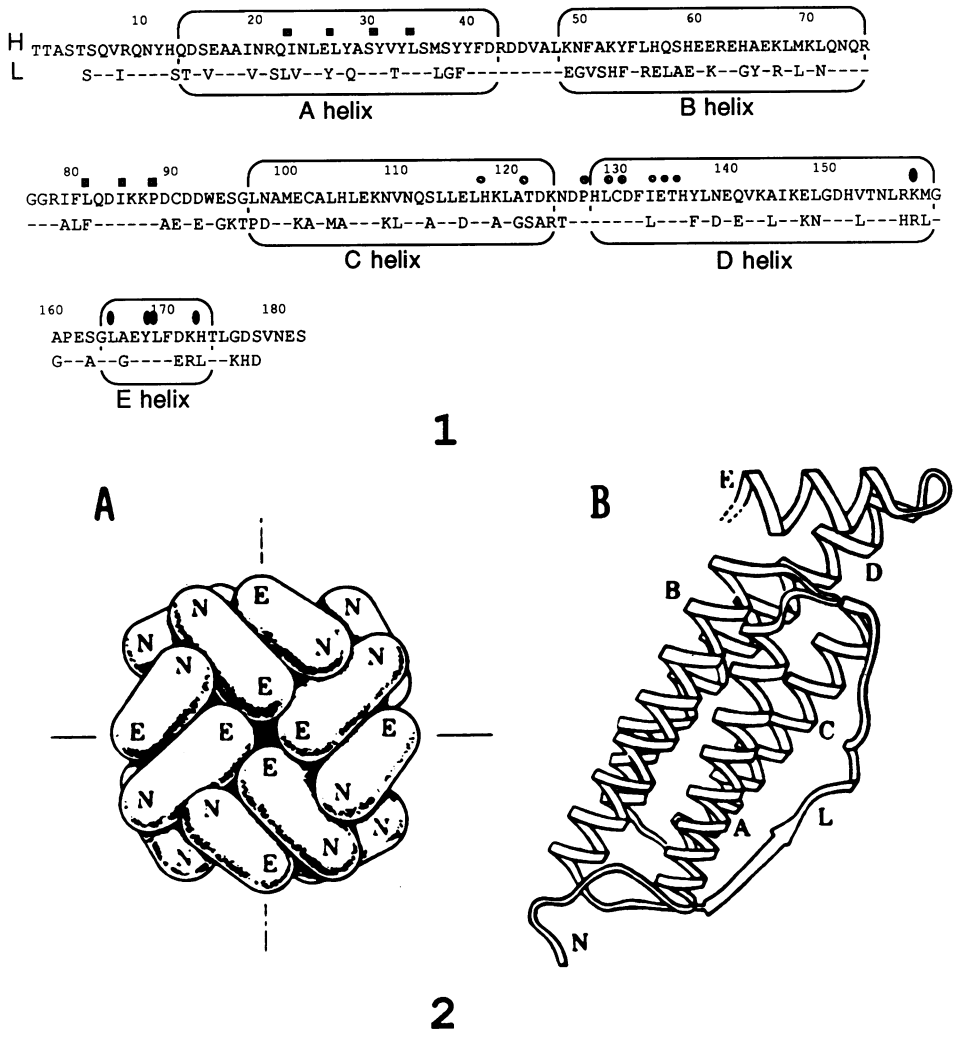

Fig. 1. Panel 1. Comparison between the amino acid sequence of the H chain (upper line) and L chain (lower line) of human ferritin. The residues that correspond to α-helices in horse spleen apoferritin are boxed. Small squares, circles and ovals indicate amino acid residues participating in subunit–subunit contacts in the 2, 3 and 4-fold axes respectively. **Panel 2A**, schematic representation of the horse spleen apoferritin viewed down a molecular 4-fold axis. **B**, ribbon type diagram of the α-carbon backbone of a horse spleen apoferritin subunit. **Panel 2** has been reproduced from Ford *et al.* (1984) with permission.

and Jaenicke, 1987; Stefanini *et al.*, 1987). Both models suggest that the starting building block is a ferritin dimer, but they differ in the proposed order of the subsequent assembly steps. The interpretation of sedimentation, velocity and circular dichroism studies lead Stefanini *et al.* (1987) to propose the octamer as the next intermediate structure, while Gerl and Jaenicke (1987) from crosslinking experiments, obtained evidence for the following pathway:

$$24M_1 > 8M_1 + 8M_2 > 8M_3 > 4M_6 > 2M_{12} > M_{24}$$

(Where M_1 to M_{24} represent the different aggregation states of apoferritin from monomer to 24mer.)

In our studies we use the H chain of human ferritin that was successfully expressed to high levels in *E.coli* by inserting its coding sequence in the vector pEMBLex2 (Levi *et al.*, 1987). The expression of apoferritin can be controlled by a temperature sensitive λ repressor encoded by a second plasmid present inside the cell. By inactivating the repressor at 42°C, ferritin accumulates up to levels corresponding to 15% of total *E.coli* proteins. In this manuscript we present a genetic screen that has been developed to identify and isolate ferritin folding–assembly mutants in *E.coli*, and its application to the analysis of the molecular interactions in the 4-fold axis of the ferritin cage.

Results and discussion

The three dimensional structure of horse spleen ferritin exhibits three regions of subunit contact along the symmetry axes that are proposed to be important in the assembly process. We are interested in determining which of these molecular contacts play an essential role and whether it is possible to re-design the assembly pathway by creating altered ferritins. Here we analyse the interactions along the channel formed by the four E helices along the 4-fold axis. Unlike the 3-fold channel this channel is essentially hydrophobic and formed by the close packing of the hydrophobic side of the E helices.

Contacts of E helices along the 4-fold axis are not essential for apoferritin assembly

To assess whether the hydrophobic interactions along the 4-fold channel are essential for subunit folding and/or protein

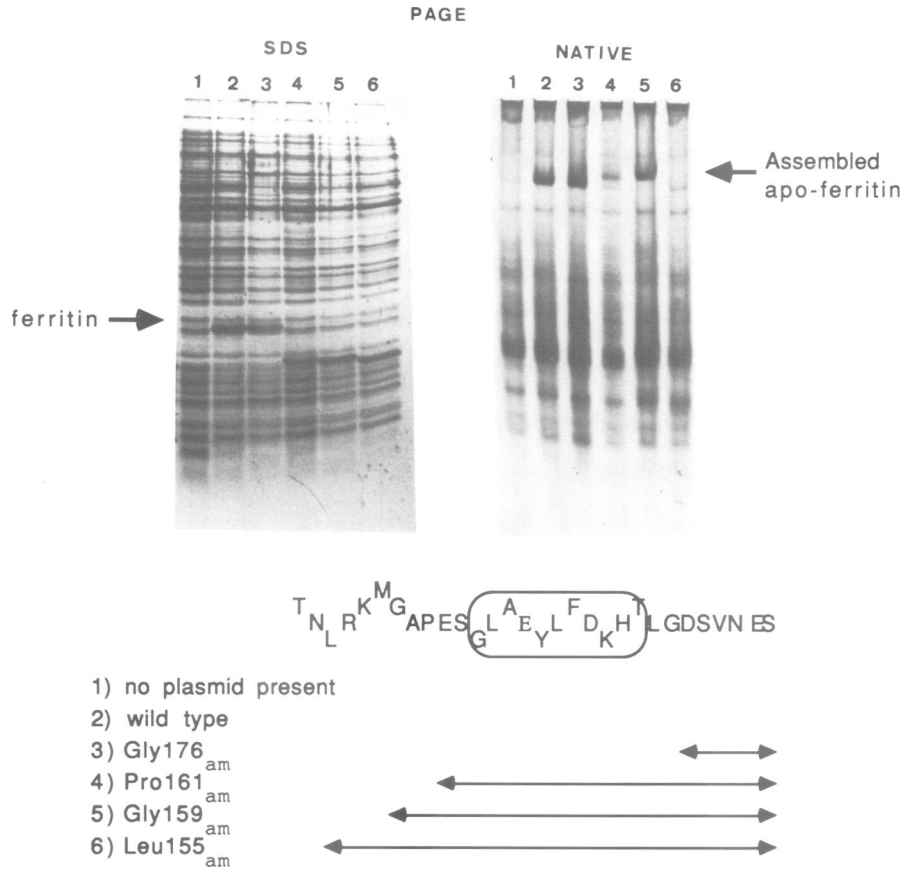

Fig. 2. Expression and assembly of ferritin amber mutants. 71/72 strains containing plasmid expressing various ferritin amber fragments were induced by raising the temperature to 42°C and further incubation at this temperature for 3 h. Total cell extracts or low speed supernatants of sonicated cells were analysed on denaturing (left) and native (right) polyacrylamide gels respectively. The extent of the peptides that are deleted in the amber fragments is indicated by arrows in the lower part of the figure below the amino acid sequence of the carboxy-terminal part of the protein.

assembly, we created a series of ferritin mutants carrying deletions extending from the carboxy-terminus for different lengths into the protein. This was achieved by inserting amber mutations at different positions in the expression plasmid. The proteins synthesized after induction of ferritin transcription were analysed by native and SDS−PAGE. As shown in Figure 2, deletions that extend up to Gly-159 still allow correct assembly, while removal of the last four amino acids in the D helix (up to Leu-155), prevented recovery of an assembled ferritin in native gels. These results prove that the carboxy-terminus, including the E helix, is neither essential for proper folding of the subunit nor for assembly of the 24mer. Our results do not determine whether the four amino acids that are missing in the Leu-155_{am} mutant are required for subunit folding or are responsible for some essential intersubunit contact. The crystal structure of horse spleen apoferritin indicates that the last Leu in the D helix forms van der Waals contact with the other subunits in the 4-fold axis. This Leu however is substituted by Met in the human H chain.

Despite the big deletions, Pro-161_{am} and Gly-159_{am} are still relatively resistant to heat denaturation and have a melting temperature of ~70°C, compared to 88°C for the wild type molecule. Although it is still possible that in the native molecule the interaction along the 4-fold axis plays an important kinetic role, our results prove that ferritin assembly can proceed to completion in the absence of most of these molecular contacts. Whether the folding−assembly

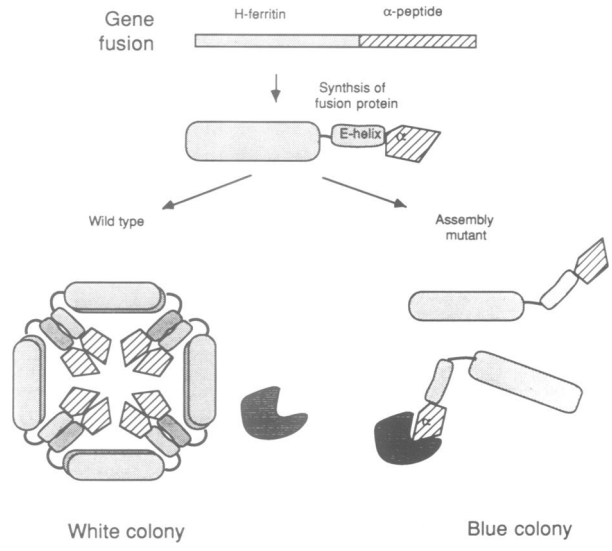

Fig. 3. Schematic illustration of the rationale behind the selection scheme that we have developed to be able to isolate ferritin assembly mutants. In the representation of the fusion protein, the big box represents the bundle of the four helices A, B, C, D while the small box represents the carboxy-terminal E helix. The α-peptide is illustrated as a more irregular polygon filled with slanted lines. The assembled apoferritin is illustrated schematically by slicing the shell along a plane identified by four 4-fold axes. The chromosomally encoded ω-peptide and its interaction with the α-peptide is also illustrated.

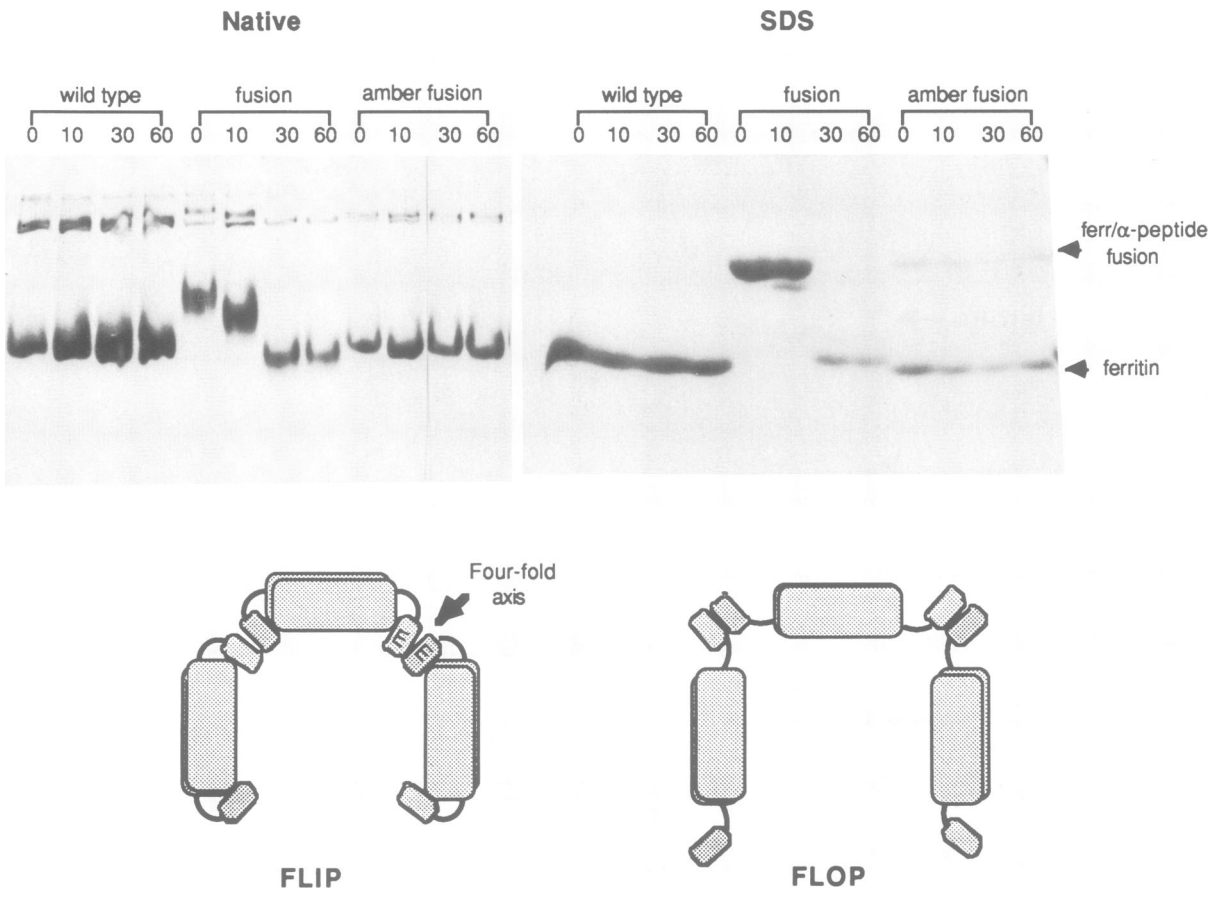

Fig. 4. Upper part. Purified ferritin molecules, overproduced in a *sup*E *E.coli* strain (71/72) containing the (i) wild type gene, (ii) the fusion between the H chain and the α-peptide and (iii) the fusion separated by an amber triplet (amber fusion) were incubated, as described in Materials and methods with trypsin for different time lengths (0, 10, 30 and 60 min). After stopping the reaction by addition of an excess of trypsin inhibitor the samples were analysed by electrophoresis on native and denaturing polyacrylamide gels. **Lower part**, schematic representation of our interpretation of the results. Assembled apoferritin is represented as in **Figure 3**. The position of the four helix bundle is the same in the two configurations, while the carboxy-terminal E helix *flips* outside in the *flop* model.

pathways of the wild type and deleted molecules differ in any respect has still to be determined.

To understand which sequences in the carboxy-terminal α-helix would be incompatible with the formation of the ferritin cage, we started to look for mutants that prevent this process. Since the high resolution structure of ferritin has been determined (Clegg *et al.*, 1980; Ford *et al.*, 1984), a site-directed mutagenesis approach is feasible in theory. We felt however, that given the present lack of understanding of the process of protein folding and the difficulty in planning intelligent modifications, a more classical (random) approach would be more productive. Thus we developed a scheme that would allow us to detect, by direct inspection of the colony phenotype, clones that express a ferritin peptide with folding−assembly problems.

Selection scheme

The rationale of the scheme is illustrated in Figure 3. We reasoned that, given the shell type structure of ferritin, an enzymic tag on the inside of the structure allowed its separation from the aqueous environment. A mutation that would prevent the formation of the ferritin cage however, would liberate the enzyme and allow it to interact with its substrate and perform its enzymic function. By choosing the enzyme and the substrate such that the enzymic reaction developed a colour product, we could distinguish colonies containing either assembled or disassembled ferritin by their colour.

This scheme could be realized by fusing the α-peptide of β-galactosidase to the carboxy-terminus of ferritin. Given the topology of ferritin (carboxy-terminus E helix pointing inside) we expected that the correct folding of ferritin would force the α-peptide into the inside of the cage. However, the empty volume inside the cage is not sufficient to contain 24 α-peptides (the radius of the cavity is ~37 Å corresponding to a volume of $\sim 2.1 \times 10^5$ Å^3. Assuming an average volume of 150 Å^3/amino acid the sum of the volumes of the 90 amino acids of the α-peptide would be $\sim 1.35 \times 10^4$ Å^3 and 24 of them, if very closely packed, would occupy a volume of 3.24×10^5 Å^3.) Indeed, we observed that the colonies formed by a clone containing this fused protein were blue on X-gal plates indicating that, in this hybrid protein, the α-peptide domain is exposed to the solvent and can complement the chromosomally encoded ω-peptide and react with the substrate. Since, as shown in Figure 3, this ferritin variant assembles into a molecule with the mobility of that of a 24mer, we predicted that, as the α-peptide is too large to be packed inside the shell, the E helix would be extruded outside. The topology of the protein was confirmed by trypsin digestion of the purified structure. In the range of protease concentration tested apoferritin was insensitive to proteolysis, but the ferritin−α-peptide fusion protein changes mobility in a native gel after mild trypsin

digestion (Figure 4). When denatured and electrophoresed on an SDS gel, the mobility of the treated protein was comparable with that of the H chain, indicating that the α-peptide subunit, being accessible to trypsin, had been digested by the protease (Figure 4). It is worth noting that, *a priori*, not all the ferritin−α-peptide protein population has to extrude outside and that few of the subunits of a given ferritin molecule could maintain the wild type conformation. Within the limits of sensitivity of our experiment however, it seems that ferritin−α-peptide fusion molecules are a homogeneous assembly of subunits in identical conformation with the carboxy-terminus pointing outside.

In order to tailor this hybrid protein for use in our screening procedure, we introduced an amber codon between the sequences coding for the H chain of ferritin and the α-peptide. By appropriately choosing the first nucleotide following the UAG termination codon it is possible to vary the efficiency of suppression of the amber triplet in various suppressor strains. In the combination that we are currently using, UAGG and a *sup*E strain, ~10% of the ferritin chains are fused to the α-peptide (Miller and Albertini, 1983). Thus, on average, each apoferritin molecule should contain approximately 2−3 α-peptide subunits which can be accommodated comfortably inside the ferritin cage. Confirming our prediction, the suppressor strain that contains this latter construction makes white colonies on X-gal plates.

The experiment in Figure 5 proves that the hybrid protein co-assembles, in the same molecule, with wild type H chain and that the fraction of fused subunits that are present in assembled apoferritin is comparable to the one that is synthesized. Furthermore, consistent with the model, the α-peptide is not sensitive to trypsin digestion suggesting that it is indeed inside the shell (Figure 4).

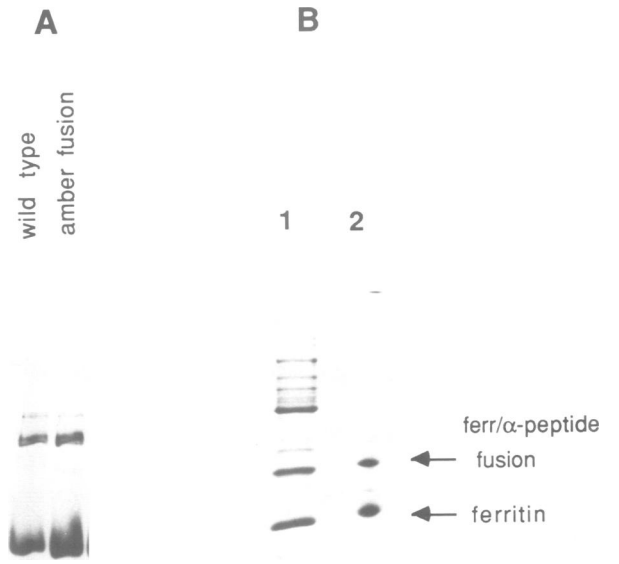

Fig. 5. Ferritin overproduced by a suppressor strain containing the amber fusion construction (H chain of ferritin and α-peptide of β-galactosidase separated by the amber codon) was electrophoresed on a native polyacrylamide gel in parallel with a wild type control and stained with Coomassie blue (**A**). The amber fusion protein was electro-eluted from an acrylamide slice and electrophoresed for a second time on a denaturing polyacrylamide gel (**B2**). In slot 1 of gel **B** we have loaded a sonicated extract of the amber fusion strain.

A folding decision

The flipping of the E helix fused to the α-peptide toward the outside of the shell indicates that both conformations, the native one (*flip*) with the carboxy-terminus pointing inside and the one with the E helix flipping outside (*flop*), are compatible with apoferritin assembly (see lower part of Figure 4). As a consequence, our screening procedure, especially when applied to the isolation of mutants in the carboxy-terminal portion of the molecule, is likely to yield mutants that assemble correctly but assume the *flop* configuration.

During the assembly process the ferritin monomer has to make the decision of whether to follow a path that leads to the native *flip* or to the less energetically favourable *flop* conformation. In the wild type molecule the *flip* conformation is highly preferred and the alternative conformation is not detectable. Characterizing the sequence perturbations that change the relative proportions of the two possible structures should help in the understanding of the rules that regulate a simple decision along a folding−assembly pathway.

As a first approach we tried to understand whether the interactions between the E helix and the main α-helical bundle are sufficient to cause a *flip* inside in the absence of helix−helix contacts along the 4-fold axis. In other words this is equivalent to asking whether the conformation of helix E that is observed in the monomer in the X-ray structure of the apoferritin crystal is present before the interaction in the 4-fold axis or whether this conformation is only taken during assembly. To this end we introduced an amber mutation at position Pro-161 in the gene that contains the fusion between the ferritin and the α-peptide. When this construction was introduced into an amber suppressor strain ~90% of the protein product terminated at Pro-161, while the remaining product is a fusion between the ferritin and the α-peptide (lane 3 of Figure 6). Thus the few ferritin molecules that maintain the E helix will fold and assemble in the absence of 4-fold interactions. Both the blue/white colony test and the trypsin digestion of assembled apoferritin (Figure 6) protein indicate that, in the Pro-161 amber fusion, the E helix conformation is the one indicated in the lower right part of Figure 6 suggesting that 4-fold contacts are essential for *flip* conformation and that the decision of whether to *flip* or to *flop* is taken upon assembly.

Isolation of point mutants, in the E helix, that affect the flip−flop folding decision

To understand which perturbations of the amino acid contacts along the 4-fold axis could alter the equilibrium between *flip* and *flop*, we have selected mutants that cause the E helix to *flop* outside. The blue/white screening procedure described above (see also Materials and methods) was used to identify putative mutants. For localized mutagenesis we used, as a primer for second strand synthesis, an oligonucleotide complementary to the sequence of the expression vector template encoding the E helix from Ser-163 to Thr-174. During the oligonucleotide synthesis however, we introduced at each position a fraction of contaminating non-complementary nucleotides, so that, on average, each oligonucleotide molecule contained a single mismatch with respect to

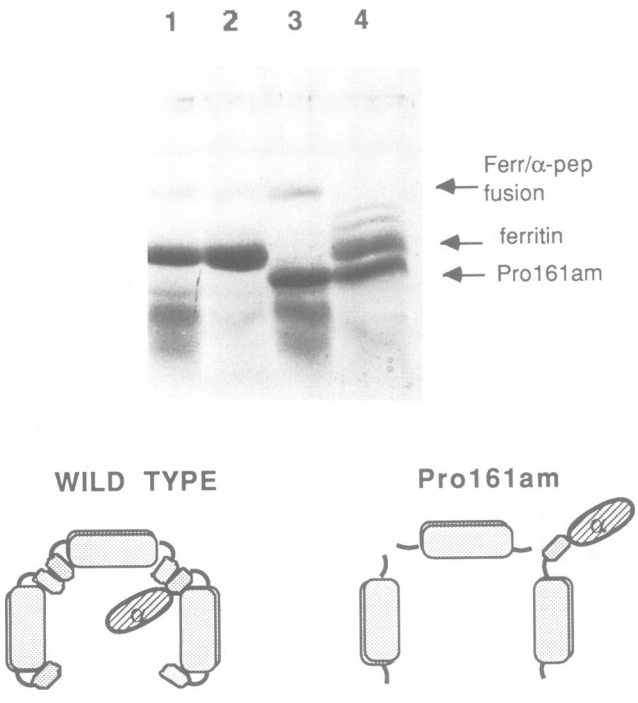

Fig. 6. Purified ferritin molecules, overproduced in suppressor strains containing the fusion construction with an amber codon either between the ferritin and the α-peptide coding sequences (**lanes 1** and **2**), or at position Pro-161 in the ferritin sequence (**lanes 3** and **4**), were analysed on a denaturing polyacrylamide gel before (**lanes 1** and **3**) and after (**lanes 2** and **4**) mild digestion with trypsin. The lower part of the figure is a schematic representation of our interpretation of the results.

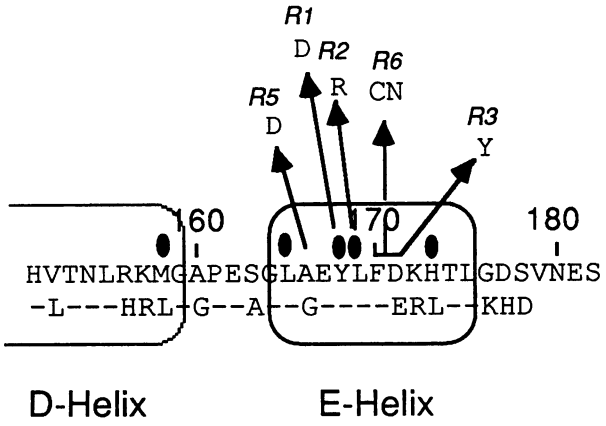

Fig. 7. The figure represents the carboxy-terminal 30 amino acids of the H chain of human ferritin. Residues that form α-helices are boxed. The small ovals indicate the residues that participate in 4-fold contacts in the three dimensional structure of horse spleen apoferritin (Ford *et al.*, 1984 and P.Harrison personal communication). Below the H chain sequence we have indicated the amino acid changes that occur in the L subunit. Figures above the sequence refer to residue numbers starting from the first amino acid in the H chain of human ferritin. The arrows point to amino acid changes that cause a blue phenotype in our selection scheme. The mutant name in our nomenclature is also indicated.

the wild type sequence. After the mutagenic procedure some blue colonies appeared on the selective plate containing X-gal. Five clones were analysed and they were all shown to contain a plasmid in which the sequence encoding the E helix was altered, four being single substitutions and one having both Phe-170 and Asp-171 changed to Cys and Asn

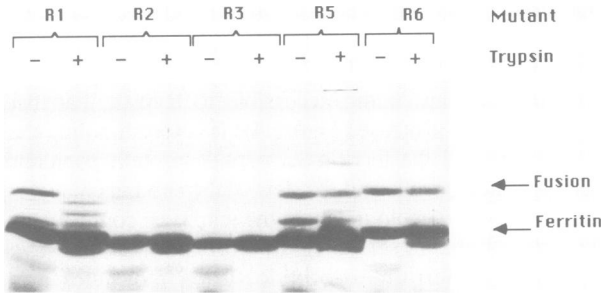

Fig. 8. Purified ferritin mutants (R1 to R6) were incubated in the presence of trypsin (see Materials and methods) to test whether the carboxy-terminal α-peptide extension could be digested by the protease. Undigested (−) and digested (+) samples were analysed on denaturing polyacrylamide (15%) gels. The extra bands in the lane of the R2 mutant correspond to partial digestions of the α-peptide subunit. They are a consequence of incomplete trypsin digestion and were not observed when more extensive digestions were performed.

(Figure 7). The most dramatic mutations seemed to be R1 and R2 which, having Tyr-168 and Leu-169 substituted by two charged amino acids like Asp and Arg, drastically altered the hydrophobic nature of the channel in the 4-fold axis.

The five mutant proteins were purified (both the products of su^+ and su^- strains) and all of them were found to assemble correctly, as judged from migration in non-denaturing polyacrylamide gels. The purified proteins (without the α-peptide tail) were also tested for resistance to heat denaturation and, with the possible exception of R2 (ΔT_m 4°C), they were found to have a stability comparable with the wild type protein.

The mutant ferritins cannot be folded in vitro

When the conformations of the mutant proteins (in the amber fusion version) were tested by mild trypsin digestion, the mutants could be grouped into two classes. Although all the mutant proteins could be recognized in the native assembled form by anti α-peptide antibody, only R1, R2 and R3 proteins had a carboxy-terminal α-peptide that could be completely digested by trypsin. In the remaining two mutants, however, a considerable fraction of α-peptides were protected from digestion indicating that their localization was probably inside the apoferritin shell. After trypsin digestion, none of the mutant proteins could be recognized by anti-α-peptide antibody, consistent with the hypothesis that the residual undigested α-peptide is hidden inside the apoferritin shell.

The wild type ferritin H chain, overproduced in *E.coli*, can be refolded efficiently after denaturation in 7.5 M GuHCl at pH 3.5 (not shown). The mutants obtained by our selection scheme however failed to do so in a variety of denaturation and renaturation conditions (see Materials and methods for details).

To summarize, the mutants that we have isolated show two phenotypes: (i) *in vivo*, they cause the extrusion of the E helix toward the oustide of the shell. The yield and stability of assembled protein, however, is not different from the wild type. (ii) *In vitro*, the mutations prevent the refolding and/or assembly of apoferritin in all the conditions tested till now. Whether this second phenotype is reflected by a slower rate of folding *in vivo* remains to be established.

Since the mutant ferritin are perfectly folded and assembled *in vivo*, this latter result indicates that the conditions that have been successfully used to reconstitute

unfolded wild type ferritin *in vitro* do not exactly reproduce the conditions *in vivo*.

Conclusions

Protein folding and assembly can be looked on as a successful walk through the multidimensional space of protein conformations. While folding, several decisions are taken by the polypeptide chain to avoid side paths that are incompatible with the native conformation. To understand which molecular interactions underlie this continuous decision cascade and how it is possible to influence them is fundamental to our understanding of the folding process.

Normally these side paths do not lead to alternative stable conformations but result in exposure to the solvent of hydrophobic surfaces and in the consequent formation of aggregates. We have shown however that the H chain of human ferritin can exist in two alternative conformations that differ in the position of the E helix in the 4-fold axis. Normally the carboxy-terminal E helix points inside the apoferritin shell and the alternative (E outside) conformation is not observed. By isolating mutations that favour the 'E outside' conformation we hope to identify the factors that influence this late folding decision. In this work we have shown that the hydrophobic interaction along the 4-fold channel is important in the attainment of the native conformation. By isolating similar mutants in the loop sequences that connect helix D with helix E we have recently identified the amino acid sequences that are incompatible with the sharp bending of the polypeptide chain (A.Luzzago and G.Cesareni in preparation).

Materials and methods

Bacterial strains and recombinant DNA methods

Two strains were commonly used in this work. They both contain pcI857, a plasmid compatible with p2HFT containing the temperature sensitive allele of the λ repressor and conferring resistance to the antibiotic kanamycin (Remaut *et al.*, 1983). This plasmid is used to control the λ_{pL} promoter in the expression vector p2HFT. 71/72 was used as a suppressor plus strain and derives from 71/18 (Δ*lac-pro*/F'[*lac*I^q*lac*ZΔM15 *pro*AB$^+$] *sup*E) (Messing *et al.*, 1977) by transformation with pcI857. When necessary plasmids carrying amber mutations were transformed into GC382, a derivative of GC76 (Δ*lac-pro*/F'[*lac*I^q*lac*ZΔM15 *pro*AB$^+$]*sup*^{o}) containing the plasmid pcI857.

Microbiological techniques, recombinant DNA, site directed mutagensis and DNA sequencing were according to standard protocols with minor variations.

Ferritin expression and purification

0.15 ml of an overnight culture of 71/72 containing plasmid p2HFT (or any of its mutant derivatives) were inoculated into a 100 ml flask containing 15 ml of L broth (50 μg/ml ampicillin and 25 μg/ml kanamycin) and incubated with shaking at 30°C till the culture reached an OD_{600} of ~0.7. The flask was then rapidly shifted to 42°C by adding 30 ml of pre-warmed L broth and incubated with shaking at that temperature for 3 h. The bacteria were collected by centrifugation, resuspended into 1 ml of 20 mM Tris pH 7.4 and lysed by sonication. Cell debris was eliminated by centrifuging for 10 min in an Eppendorf centrifuge. Heat labile proteins were denatured by heating in Eppendorf tubes for 10 min at 70°C (less stable mutants like those lacking the carboxy-terminal E helix and the fusion to the α-peptide were only heated up to 60–65°C). The precipitated heat labile proteins were eliminated by centrifugation in an Eppendorf centrifuge for 15 min. At this stage ~50% of the total protein in the supernatant is ferritin. A further purification (up to 95%) is achieved by adding an equal volume of a solution containing 20% PEG 8000 and 2.5 M NaCl, leaving 1 h in ice and pelleting the assembled apoferritin in an Eppendorf centrifuge for 10 min. The pellet is washed rapidly with 20 mM Tris pH 7.4 and resuspended in the same buffer. This washing step can be omitted if contaminations by PEG can be tolerated. This protocol yields reproducibly 0.5 mg of purified protein. The purified protein can be kept indefinitely at −70°C.

Mutant selection

Random 'region directed' mutagenesis was performed utilizing an oligonucleotide of sequence 5'-TGGCTTGGCGGAATATCTCTTTGA-CAAGCCA-3' complementary to the region encoding the E helix from Ser-163 to Thr-174. During the synthesis, however, each position of the oligonucleotide was contaminated (3%) with a mixture of the remaining three nucleotides. After site directed mutagenesis 71/72 transformants were selected by plating on ampicillin plates (100 μg/ml of ampicillin) containing X-gal (24 μg/ml) and IPTG (12 μg/ml). Blue colonies could be identified, at a frequency of ~1%, after overnight incubation at 30°C followed by 4 h at 37°C and ~20 h at 4°C.

Trypsin digestion

5 μg of purified ferritin were incubated with 0.5 μg of trypsin at 37°C in 20 μl of 20 mM Tris pH 7.5 and 100 mM NaCl. After one hour the reaction was stopped by adding 0.7 μg of trypsin inhibitor from soybean (Sigma) and the digestion products were analysed by polyacrylamide gel electrophoresis either in native (6% acrylamide, 0.16% bisacrylamide) or denaturing (15% acrylamide, 0.4% bisacrylamide, 1% SDS) gels. The protein bands were stained with Coomassie blue.

Unfolding and refolding ferritin

The standard conditions used for unfolding and refolding ferritin were essentially as described by Gerl and Jaenicke (1987). Ferritin was purified and diluted to a final concentration of 6 mg/ml in 20 mM Tris HCl pH 7.4. Complete denaturation was obtained by incubating for 10 min at 20°C with 7.2 M guanidinium−HCl at pH 3.5 in 0.1 M phosphate buffer, 1 mM EDTA, 1 mM DTT. Reassociation of wild type H chain could be obtained by diluting the denatured protein into 0.1 M triethanolamine pH 7.5, 1 mM EDTA, 3 mM DTT. The final protein concentration was 6 μg/ml and guanidinium−HCl was 0.15 M. The assembly process was stopped, at different times, by adding an equal volume of sample buffer (0.25 M Tris−HCl pH 6.8, 2% SDS, 10% β-mercaptoethanol). The assembled ferritins (wild type and mutants) are stable in these conditions. Aliquots were analysed (without pre-boiling the sample) by SDS gel electrophoresis in 15% polyacrylamide with a stacking gel (7.5% acrylamide) that represents half of the total gel. To attempt to refold the mutant proteins we have tried to carry out the denaturation step in milder conditions either by increasing the pH or by decreasing the guanidinium−HCl concentration. However we have not been able to find concentrations in which the protein can be reassembled after complete denaturation.

Preparation of anti-α-peptide antibody

Anti α-peptide antibodies were prepared according to the following protocol. A rabbit was immunized by serial injections of purified ferritin α-peptide fusion as described by Louvard (1982). Antibodies specific for the α-peptide were separated by anti-ferritin antibodies by immunoadsorption to wild type ferritin immobilized on a solid phase. 100 μg of purified ferritin were spotted on a nitrocellulose filter (Schleicher and Schüll) and incubated in 2 ml of antiserum (diluted 1/2000) in TBS (20 mM Tris pH 7.4, 0.9% NaCl) for 4 h at room temperature. The non-adsorbed antibodies were incubated a second time for 4 h with the same quantity of ferritin and tested for specificity by immunodotting against ferritin and ferritin−α-peptide fusion. The rabbit antibody was revealed by reacting with anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) and staining with nitro-blue-tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The reactivity of the immunoadsorbed antiserum with ferritin was indistinguishable from the background.

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