Loop mutations affect ferritin solubility causing non-native aggregation of subunits or precipitation of fully assembled polymers

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Received 23 July 1996

Abstract As a consequence of elevated expression rates, the intracellular aggregation of polypeptide chains is commonly observed in *E. coli*. Although wild-type human ferritin, a polymeric iron storage protein, accumulates in the soluble form at high level in the bacterial cytoplasmic fraction, some amino acid substitutions in an exposed loop direct the synthesis of a highly insoluble product. We found that two mechanisms can lead to the aggregation of ferritin. While some mutations prevent ferritin polymerisation, others cause the precipitation of molecules in the assembled state.

Key words: Ferritin; Inclusion body; Aggregation; Protein folding; Protein assembly; Loop

1. Introduction

Protein function depends on the ability of the polypeptide chains to fold correctly. In the case of polymeric proteins, further association steps add complexity to the process of folding-assembly in vivo and, in some cases, limit the yield of renaturation processes in vitro [1,2].

Human ferritin polymer is composed of 24 subunits (apoferritin) which assemble into a hollow shell and enclose a mineral iron nucleus [3]. Two chain types, H and L, are present in different proportions depending upon the tissue. After the human H gene cloning [4], the protein has been expressed at high levels in *E. coli* where it assembles correctly as a soluble homopolymer H_{24} [5]. The tridimensional structure of H_{24} has been determined at high resolution [6]. The subunits are arranged in 432 symmetry, and form hydrophilic and hydrophobic channels along the 3- and the 4-fold axes, respectively. The H subunit consists of 183 amino acid residues and folds as a 4-helix bundle. At the end of helix D the chain folds sharply back and continues into the helix E which forms an acute angle with the main axis of the bundle, with the carboxy-terminus pointing toward the cavity of the ferritin shell (Fig. 1). Ferritin can be unfolded and efficiently refolded in vitro and two detailed assembly maps have been reported [7,8]. The molecule has a very high stability to heat and other denaturing agents [5,9] and, because of its unique architecture, it has been used as a confined environment for the synthesis of nanomaterials [10].

In the past we have directed our attention to the loop connecting helices D and E of the H chain (G159–S164), using a random region mutagenesis approach combined with a sensitive screening test based on the α-complementation of β-galactosidase [11]. In this system the colour of bacterial colonies synthesising a hybrid ferritin-β-galactosidase depends upon the ability of the ferritin domain to fold and assemble properly [12] (Fig. 2). We have found that several mutations introducing positively charged residues in positions 159-160-161 result in the synthesis of an insoluble product that, after a brief centrifugation step, is largely recovered in the pellet fraction [11] (Fig. 3). Since these mutants form colonies of a very intense blue on indicator plates, we suggested that these insoluble aggregates are formed by molecules that are unable to fold or assemble. However, the mutants f38, f62 and f43, despite producing highly insoluble molecules, formed only very pale blue colonies [11]. A more accurate characterisation of f43 led us to the conclusion that this mutant directs the synthesis of a product that is essentially soluble. However, for the mutants f38 and f62, the inconsistency of the colony phenotype remains unexplained. Here we investigate the nature of the protein aggregates recovered from ferritin mutants and show that while some substitutions in the DE loop prevent ferritin assembly, others cause the precipitation of entirely assembled molecules.

2. Materials and methods

Bacterial strains, expression plasmids and production of recombinant ferritin have been described before [11]. GC382 bacterial cells expressing ferritin mutants were collected by centrifugation, resuspended in 1 ml of 20 mM Tris pH 7.4 and lysed by sonication. Cell debris were fractionated in two centrifugation steps of 5 min and 20 min, performed at 4°C in an Eppendorf centrifuge at 13 000 rpm. The corresponding pellets were termed P1 and P2. The supernatant resulting from the second centrifugation was kept in an Eppendorf tube for 10 min at 70°C. The precipitated heat-labile proteins (P3) were separated by centrifugation in an Eppendorf centrifuge for 15 min. Further purification was achieved by adding an equal volume of a solution containing 20% PEG (polyethylene glycol) 6000 and 2.5 M NaCl, leaving for 1 h in ice and precipitating the assembled apoferritin in an Eppendorf centrifuge (10 min). The pellet (P4) was washed with 20 mM Tris pH 7.4. Each of the four pellets was resuspended in 300 μl of the same buffer. The four fractions were analysed by fully denaturing polyacrylamide gel electrophoresis (15% acrylamide, 0.4% bisacrylamide, 0.1% SDS) [13].

Pellets P1 derived by the expression of mutants f15, f16, f38 and f62, after incubation for various times, at different temperatures and concentrations of SDS, were analysed by 0.1% SDS gel (stacking gel 7.5% acrylamide, running gel 15% acrylamide) electrophoresis, without preboiling the samples. A total soluble extract containing wild-type ferritin and corresponding to the supernatant fraction obtained from centrifugation for 5 min in the Eppendorf centrifuge was analysed as a control.

Pellet P1 buffer was replaced with solutions of urea (1–5–10 M). After 20 min incubation at room temperature, the samples were centrifuged for 5 min and equivalent aliquots of pellets and supernatants were analysed by SDS-polyacrylamide gel electrophoresis under denaturing conditions. The percentage of ferritin solubilized was scored by

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Abbreviations: BCIG, 5-bromo-4-chloro-3-indoyl-β-D-galactoside

PII S0014-5793(96)00979-9

visual inspection of the gel. The same fractions were also electrophoresed after incubation for a further 45 min at room temperature in 0.1% SDS (without boiling the sample).

Ferritin f62 pellet P1 buffer was replaced with 20 mM Tris pH 9.5 and/or in 2 M $NaClO_4$. After 10 min incubation at room temperature, the samples were centrifuged for 5 min and equivalent aliquots of pellets and supernatants were electrophoresed on SDS-polyacrylamide gel electrophoresis. Trypsin digestion of ferritins was performed as described before [11].

Recombinant DNA procedures were performed according to standard methods [14]. After site-directed mutagenesis [15], transformants in amber suppressor strain (71/72) were plated on BCIG indicator plates. Mutant f109 was obtained by oligonucleotide R73 (5'CAACTTGCGC(A/G)(A/C)GATGGGTAGG3'). Mutant f116 and f117 were obtained in a single mutagenesis experiment by oligonucleotide R105 (5'GTGACCAACTTGGAG(A/G)AGATGGGTAGGA3') which changes Arg-156 into Glu, and Lys-157 into either Lys or Glu.

3. Results

The differences between the two types of aggregates have been investigated by comparing two mutants displaying high β-galactosidase activity, f15 and f16, with f38 and f62 which only form faint blue colonies (Fig. 3).

Our previous experiments indicated that similar amounts of ferritin product could be recovered from all the DE loop substitutional mutants. Therefore, the phenotype of f38 and f62 cannot be ascribed to proteolytic activity affecting these particular ferritins. Instead, the two classes of mutants must accumulate aggregates of different nature inside the bacterial cell. Initially, we considered the hypothesis that both types of aggregates might be composed of misfolded ferritin chains. The pale blue colour of colonies formed by mutants f38 and f62 might be caused by the presence of more compact and less 'accessible' intracellular aggregates. Such inclusion bodies would hinder α-complementation and result in a lower β-galactosidase activity. However, observations by electron microscopy of the bacterial cells overproducing ferritins indicated that the mutants f15 and f16, despite the high enzymatic activity, produce large inclusion bodies. In contrast, f38 and f62 did not show the presence of a typical intracellular aggregate (P. Albertano, personal communication). These data suggested that the colony colour diversity exhibited by the two types of mutants is the consequence of a distinct difference in the folding-assembly state of the ferritin molecules.

Since the native ferritin polymer is highly thermostable and resists SDS at concentrations as high as 1%, it can be separated from the monomeric form by partially denaturing polyacrylamide gel electrophoresis. Aliquots of the soluble fraction of a bacterial extract containing wild-type ferritin H, were preincubated in 0.1% SDS at 37°C for times up to 160 min and analysed by SDS gel electrophoresis (7.5–15%). While most of the bacterial proteins appeared substantially denatured, H ferritin migrated in the 24-meric state (Fig. 4A). The sharpest electrophoretic band was observed when the extract was preincubated for 40 min.

We used this method to verify the assembly state of the ferritin chains present in the bacterial aggregates in comparison to the wild-type molecule. Aliquots of the insoluble fractions P1 (see Section 2) originated from mutants f15, f16, f38 and f62 were analysed. Ferritins f15 and f16 migrated in the monomeric form. Fig. 4B shows that, following 15 min incubation at temperatures as low as 30°C, ferritin f15 was completely denatured. Under similar conditions f38 and f62 appeared as high molecular mass aggregates which could barely penetrate the gel. Such aggregates could only be denatured by more drastic treatment. The mutant f62 was fully monomerised only if incubated at 75°C (Fig. 4B). No difference in the denaturation temperature was observed when the protein was preincubated in 2% SDS. Although we could not

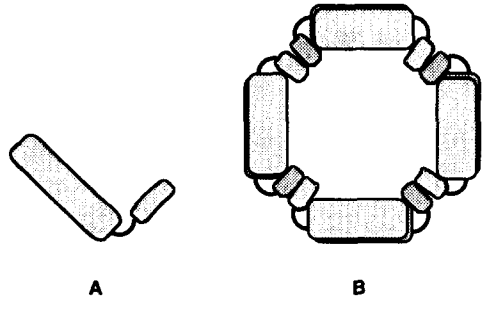

Fig. 1. (A) Schematic view of the ferritin subunit. The large box represents the 4-helix bundle while the small box represents the E helix. The narrow line corresponds to the D-E loop. (B) Model of the ferritin polymer drawn as an equatorial slice perpendicular to one of the six 4-fold axes.

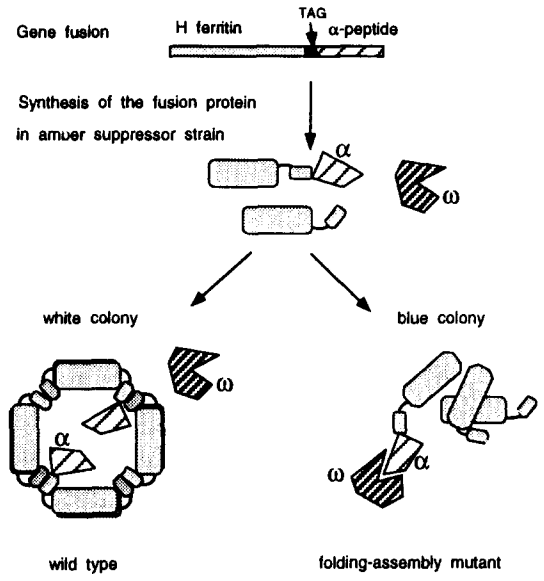

Fig. 2. Selection scheme for the isolation of ferritin folding-assembly mutants. α, α-peptide; ω, ω-peptide.

	D helix			DE loop				E helix			Colony colour
	156			159				165			
f14	R	K	M	R	C	E	M	L	T	L	A	E	intense
f15	R	K	M	R	K	K	A	T	G	L	A	E	intense
f16	R	K	M	L	R	K	A	I	T	L	A	E	intense
f67	R	K	M	R	E	Q	I	T	L	L	A	E	intense
f65	R	K	M	Q	S	R	I	I	G	L	A	E	intense
f38	R	K	M	G	I	R	A	S	G	L	A	E	faint
f62	R	K	M	G	R	K	P	S	G	L	A	E	faint
fwt	R	K	M	G	A	P	E	S	G	L	A	E	white

Fig. 3. DE loop sequence of mutants synthesising highly insoluble ferritin molecules. wt, wild-type ferritin.

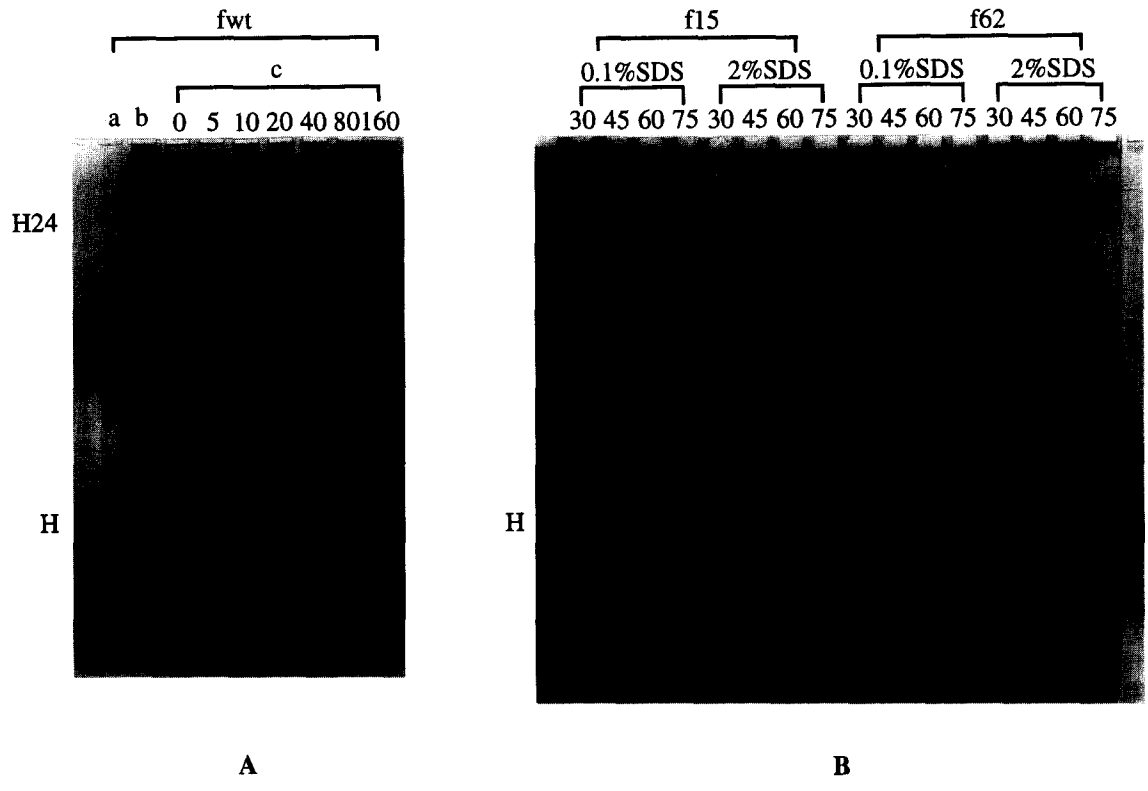

Fig. 4. Electrophoretic analysis of wild-type and mutant ferritins. (A) Purified wild-type ferritin fully denatured (a) and preincubated at room temperature in 0.1% SDS (b). Soluble fraction extracted from bacteria producing wild-type ferritin (c) preincubated in 0.1% SDS at 37°C for 0, 5, 10, 40, 80 and 160 min. (B) Soluble fractions extracted from bacteria producing f15 and f62 after 15 min of incubation in either 0.1 or 2% SDS at 30, 45, 60 and 75°C. H, ferritin monomer; H24, ferritin polymer.

resolve ferritins f38 and f62 in the form H_{24}, these experiments indicated that they have a very substantial resistance to denaturation. By contrast, the low resistance of f15 and f16 indicates that these ferritins do not assemble in the native state.

Urea at a concentration of 6–8 M has been used to release prochimosin, interferon and salmon growth hormone from inclusion bodies [16]. Equivalent amounts of the insoluble fractions extracted from bacteria expressing f15, f16, f38 and f62 were resuspended in solutions containing three different concentrations of urea. The percentage of ferritin solubilized was determined as described in Section 2. As in the case of other proteins, for the ferritin mutants forming typical inclusion bodies, the release of the protein required high urea concentration. Thus, the solubilization of ferritin f15 and f16 was incomplete at 5 M concentration (data not shown). Instead, most of the f38 and f62 chains were solubilized in 1 M urea (compare lanes c and e in Fig. 5A). To verify the assembly status of the protein solubilized following urea treatment, samples of the various supernatant fractions were electrophoresed under partially denaturing conditions. While f15 and f16 solubilized molecule migrated in the monomeric form (not shown) a sharp electrophoretic band corresponding to ferritin H_{24} was observed for f38 (Fig. 5A). Although urea treatment solubilized f62 as much as f38, the former was not clearly visualised in the 24-meric form. However, in both mutants the majority of the protein was not monomerised by urea concentrations as high as 10 M (Fig. 5A). In this respect, their resistance appears comparable to that of the wild-type molecule [1].

Inspection of the DE loop sequence of f38 and f62 (Fig. 3) shows that, in both cases, Glu-162 is replaced by an uncharged residue and a positively charged residue is present in position 161. In addition, in f62, a second positively charged residue is introduced at position 160. Wild-type recombinant H ferritin is a moderately acid protein with a p*I* value of 5 [5]. Because the residues in positions 160–162 are situated on the surface of the molecule, and there are 24 subunits in each ferritin polymer, the mutation f38 and especially the mutation f62 should lead to a substantial increase in p*I*. When the p*I* value becomes similar to that of the intracellular pH, proteins display the highest tendency to precipitate. Therefore, we attempted a better solubilization of the aggregate of f62 by increasing the pH and the ionic strength. Ferritin f62 precipitate was resuspended in 20 mM Tris-HCl pH 9.5 and/or in 2 M $NaClO_4$. Under the three conditions tested, f62 was almost entirely solubilized (Fig. 5B). However, we found that the effect of $NaClO_4$ was less specific toward the release of ferritin. In comparison, an attempt to solubilize f16 at pH 9.5 released only a small portion of ferritin (not shown). Wild-type ferritin polymer is highly resistant to trypsin [11]. To confirm the assembly state of the protein solubilized as a consequence of the alkaline treatment, samples of wild-type and mutant ferritins were incubated in the presence of the enzyme. As anticipated, f62 ferritin was resistant to proteolysis, indicating that it assembles in the polymeric form. Instead, f16 was completely degraded by the trypsin activity (Fig. 5C). The mutant f118 shown in Fig. 5C is an insertional mutant (LGIWGCSGKLIC in the DE loop of ferritin) constructed for other purposes (G.C., unpublished). It directs the synthesis of a highly insoluble product that as

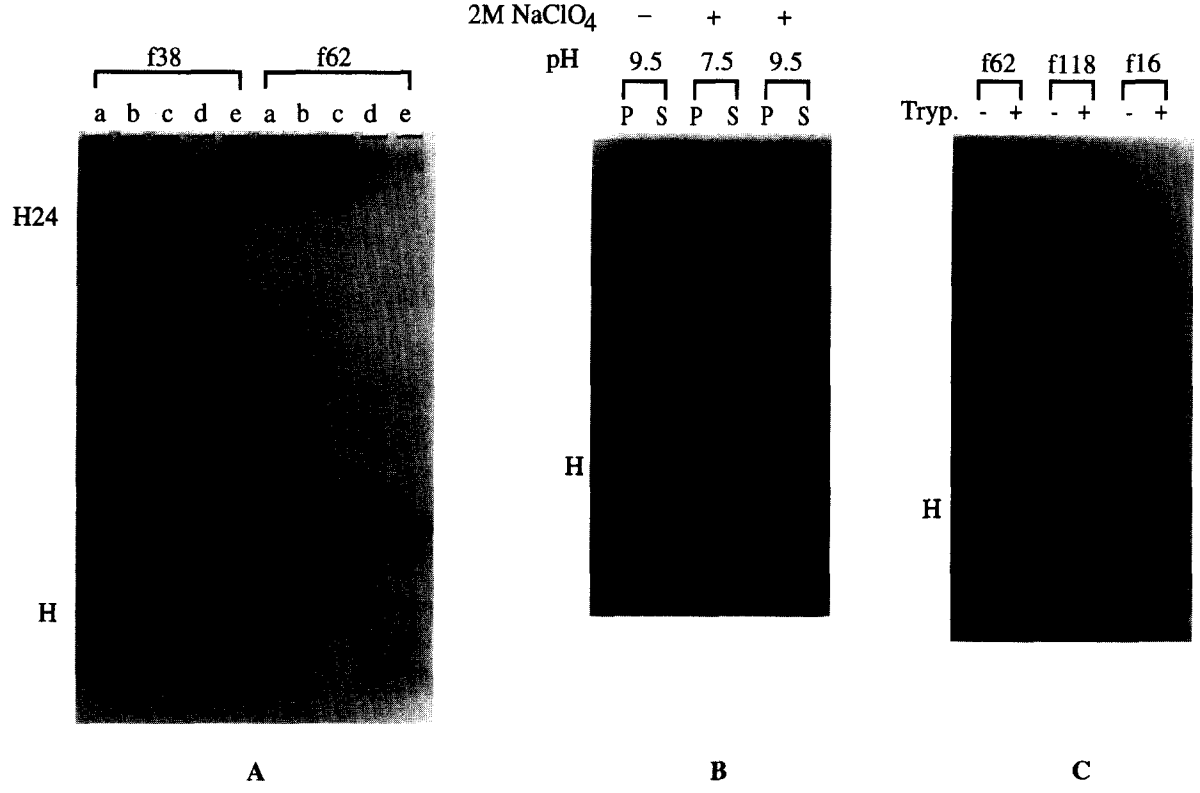

Fig. 5. (A) Electrophoretic analysis under partially denaturing conditions of samples derived from the P1 fraction of ferritins f38 and f62 after treatment with urea. Solubilization with 10 M (a), 5 M (b,d) and 1 M (c,e) urea and further separation into supernatant (a–c) and pellet (d,e) fractions by centrifugation. (B) Electrophoretic analysis under fully denaturing conditions after solubilization of samples of the P1 fraction of f62 in 20 mM Tris pH 9.5 and/or in 2 M $NaClO_4$ and further separation into pellet (P) and supernatant (S) fractions by centrifugation. (C) Electrophoretic analysis under fully denaturing conditions of supernatant of f62, f118 and f16 treated with (+) or without (−) trypsin. Supernatants were obtained by solubilization in 20 mM Tris pH 9.5 of P1 fractions and centrifugation. H, ferritin monomer; H24, ferritin polymer.

f16 was partially solubilized at pH 9.5 and sensitive to the action of trypsin.

In the next experiment we investigated whether the insolubility of ferritin f62 could be suppressed by mutations aiming at compensating the surface charge variation. This was carried out stepwise, by introducing Glu in place of Arg-156 and Lys-157 in f62. Characterisation of the new mutants, f117 and f109, indicated that the aggregation phenotype was largely suppressed (Fig. 6). However, a very substantial amount of ferritin was recovered in the fraction P3. This indicated either that the two mutants direct the synthesis of molecules more thermosensitive compared to the wild-type ferritin, or the presence of relatively low molecular mass aggregates requiring a prolonged centrifugation to precipitate. Finally, when in f62 both Arg-156 and Lys-157 were changed to Glu (f116), the thermostability of the protein was restored, becoming very similar to that of the wild-type molecule (Fig. 6).

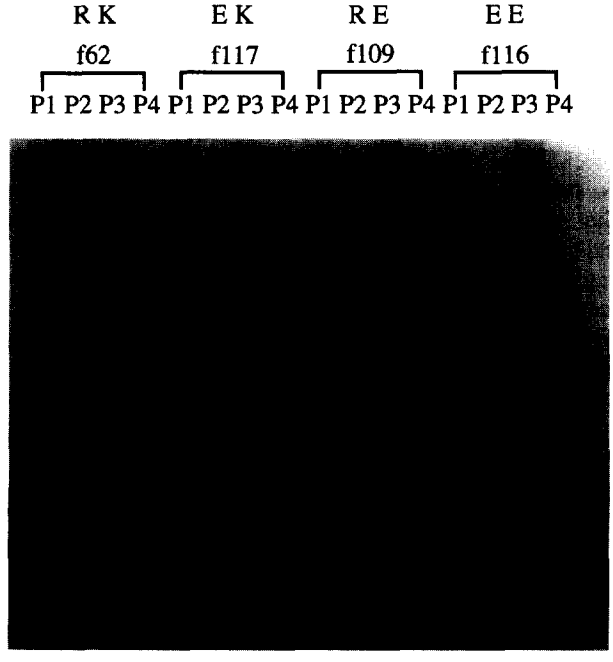

Fig. 6. Suppression of the insolubility of f62. Gel electrophoresis of the fractions P1–P4 of bacteria producing f62, f117, f109 and f116. The top line indicates the amino acid residues present in positions 156 and 157 in each mutant.

4. Discussion

The study of the mechanisms of aggregation of proteins in bacterial cells is important for biotechnological purposes and might also lead to the comprehension of primary pathological events. A number of human diseases are caused by the accumulation of proteins in an insoluble form. Overproduction of proteins in *E. coli* can lead to the accumulation of inclusion bodies [17]. These are large and dense aggregates consisting primarily of the recombinant protein that can be isolated by centrifugation and visualised by phase contrast or electron

microscopy [18]. Inclusion body formation is the consequence of a kinetic competition between the first-order process of folding and second-order process of association of unfolded or partially folded polypeptides. At high expression rate, aggregation dominates over folding [19].

Amino acid replacements can also influence the distribution of the product between bacterial cytoplasm and inclusion bodies [20]. Here we show that two mechanisms can determine precipitation of recombinant proteins in *E. coli*. First, some DE loop substitutions prevent the formation of the ferritin polymer. This is the case for many other proteins that form intracellular aggregates visualised as inclusion bodies. No information is available about the assembly level reached by this class of mutants. Second, we have found that two mutations cause the intracellular precipitation of fully folded and assembled ferritin molecules. This is indicated by their high grade of resistance to heat, urea and trypsin which approximates that of the wild-type molecule. The ferritin polymer, at least in the case of f38, was clearly visualised through partially denaturing gel electrophoresis. The observation that ferritin f38 and f62 extracts, but not f15 and f16, display a distinct red-brown colour indicates that only the former retain the ability to incorporate iron and is consistent with the different assembly state reached by the two classes of mutants.

By inspecting the sequences of DE loop random mutants we have previously identified a remarkable correlation between the production of insoluble ferritin and the presence of positively charged side chains at positions 159–161 [11]. The characterisation of mutants f38 and f62 indicates that the presence of positive charges at positions 160 and 161 is compatible with a wild-type-like ferritin assembly. Full solubility of ferritin f62 was restored by a second site mutation replacing two positively charged residues situated on the surface of the molecule with glutamic acid.

Beside the introduction of positively charged residues, further amino acid substitutions must lead to the impairment of the folding-assembly process of ferritins like f15 and f16. While these mutants have amino acid changes in at least five out of six positions of the DE loop, both f38 and f62 are mutated only in positions 160–162 (Fig. 3). In particular, f15, f16 and the other mutants forming intense blue colonies have bulky residues in position 159. In contrast, f38 and f62 maintain the Gly in this position (Fig. 3). To verify the possibility that this Gly could be responsible for the different behaviour of the two classes of mutants we have further characterised f45, a previously isolated insoluble mutant whose DE loop sequence (WRKPSL) differs from f62 with Trp in place of Gly-159 and Leu in place of Gly-164 [11]. Despite the absence of the two glycines, the nature of the aggregate formed by this mutant was quite similar to that formed by f62. The properties of f45 demonstrate that the presence of large residues in positions 159 and 164 is compatible with the assembly of the ferritin molecule and suggest that a combination of loop features that are not easily separable is responsible for the different behaviour of the two classes of mutants.

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