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Recombinant H-chain ferritins: effects of changes in the 3-fold channels

Amyra Treffry, Pauline M. Harrison, Alessandra Luzzago[+] and Gianni Cesareni[+]*

Biochemistry Section, Department of Molecular Biology and Biotechnology, The University, Sheffield S10 2TN, England and [+]*European Molecular Biology Laboratory, Postfach 10.2204, Meyerhofstrasse 1, 6900 Heidelberg, FRG*

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Human H-chain ferritins bearing sequence changes in the 3-fold channels have been expressed in *E. coli* to investigate the role of these channels in iron-storage processes. The proteins assemble into shells resembling those of native ferritins. Iron uptake measurements indicate that residues in the 3-fold channels are involved neither in initial Fe(II)-oxidation nor in iron-core nucleation.

Ferritin; Ferritin channel; Iron storage; H chain; Recombinant ferritin; Iron(III)-apoferritin

1. INTRODUCTION

The iron-storage protein, ferritin, is a 24-mer of two types of subunit, H and L, coassembled into a hollow shell that provides a cavity in which iron is deposited as the mineral ferrihydrite [1]. In vitro ferritin reconstitution can be achieved by the addition of Fe(II) to apoferritin in the presence of an oxidant [2,3]. Apoferritin facilitates ferrihydrite formation by providing sites at which Fe(II) oxidation is accelerated [2–5] and those at which the iron-core mineral is nucleated [3] which may or may not be the same. It has recently been suggested that there is a 'ferroxidase' site on the outside of the molecule, since Fe(III) can be transferred to added apotransferrin [5]. Fe(III) (or Fe(II)) must be able to traverse the protein shell to reach the storage cavity.

We have previously observed, by UV difference spectroscopy, the formation of an initial Fe(III)-apoferritin complex, when 4–8 Fe(II) atoms are added to apoferritin at pH 6.5 [6]. The spectrum changes with time due, we believe, to the migration of Fe(III) atoms from isolated sites to give nucleation clusters. Comparable changes are seen by EPR [7] and Mössbauer [8] spectroscopy. The three-dimensional structures of horse spleen apoferritin [1] and of recombinant L-chain and H-chain homopolymers as well as of native rat liver apoferritin (Lawson, D.M., Artymiuk, P.J. and Harrison, P.M., unpublished work) show the presence of eight channels at 3-fold symmetry axes. We investigate here the hypotheses that these channels provide the route by which iron traverses the shell and that they contain the initial Fe(II)-oxidation site [6,9,10]. Our approach is to make mutant proteins by over-expression in *Escherichia coli* of human H-chain genes containing appropriate codon changes. We then examine the formation of the initial Fe(III)-apoferritin complex at a pH (5.9) at which the ferrihydrite core formation is inhibited, and separately, the rate of core formation when 500 Fe(II) atoms/apoferritin molecule are added at pH 6.8. The 3-fold channels consist of an inner narrow region surrounded by three highly conserved aspartates (sequence no.131) and three glutamates (no.134) that are

Correspondence address: A. Treffry, Biochemistry Section, Department of Molecular Biology and Biotechnology, The University, Sheffield S10 2TN, England

* *Present address:* Dipartimento di Biologia, II Universita di Roma, Tor Vergata, Via O. Raimondo, 00173 Roma, Italy

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known to bind metal ions [11], and an outer, wider region in which several amino acid changes are found when H- and L-chain sequences are compared [12]. To investigate the two regions we have made the following changes: Asp131⟶His; Glu134⟶His; Asp131,Glu134⟶His; Asp 131⟶Ala; Glu134⟶Ala; Asp131,Glu134⟶ Ala; Ala-Thr-Asp-Lys-Asn(121–125)⟶Gly-Ser-Ala-Gln-Thr. All of these mutations have been superimposed on a separate single change, Lys-86⟶Gln, introduced to induce crystallization by formation of intermolecular metal bridges as in horse spleen apoferritin [11]. Apoferritin bearing the single Lys86⟶Gln change has been used as control in studies of iron uptake. For convenience, when describing the mutants, the control will be described as Lys86⟶Gln and the others simply as Asp131⟶His etc. and H(121–125)⟶L.

2. METHODS

2.1. *Expression and isolation of recombinant H-chain ferritins*

To express the H-chain of ferritin we made use of plasmid p2HFt-91 that directs the synthesis of large quantities of ferritin mRNA under the control of the λP_L promoter [13]. This plasmid is a derivative of p2HFt [14] in which the 3′-end of the ferritin gene has been fused, by site directed mutagenesis, to the gene encoding the α-peptide of β-galactosidase. The two genes, however, are separated by an amber codon and, in a su^- strain, translation stops at the carboxy-terminus of ferritin. The bacterial host used for ferritin production is GC382-Δ[*lac-pro*]/*F'* [*lacI*^{q} *lacZ*ΔM15*proA*B$^+$]*sup*^{o}/pCI857 where pCI857 is a plasmid conferring kanamycin resistance and carrying the temperature-sensitive allele of the λ repressor. Mutants have been constructed by standard site directed mutagenesis techniques.

Ferritin over-expression was achieved by thermo-inactivation of the λ repressor. A 60 l fermenter was inoculated with 3 l of an overnight saturated culture of GC382 containing p2HFt-91 or its derivatives. The temperature was maintained at 30°C till the culture reached an absorbance of 0.4 at 600 nm. At this point the temperature was raised to 42°C and, after 3 h of further incubation at this temperature, the cells were collected by centrifugation in a continuous flow rotor.

Cells were suspended in 0.02 M sodium borate buffered saline, pH 7.2, containing 0.02%, w/v, NaN_3 (BBS), 4 ml/g cell paste, and sonicated for 5 × 1 min. The homogenate was heated to 75°C for 5 min and centrifuged. The supernatant was applied to a Sepharose 6B column (5 × 70 cm) and eluted with BBS. The purest fractions (monitored by gel electrophoresis) were pooled and precipitated with ammonium sulphate (37%, w/v).

2.2. *Iron removal*

For iron removal, ammonium sulphate precipitates were dialysed against BBS and reduction was carried out in an 8MC ultrafiltration cell (Amicon, High Wycombe, Bucks, England) equipped with a YM100 Diaflo membrane. The reducing solution of 50 mM thioglycollic acid in 50 mM Mes buffer, pH 6.0, containing 0.15 M NaCl, was placed in the reservoir with the protein (usually 0.5–0.9 ml of a 10–20 mg/ml solution) in the sample compartment. Fe(II) in the filtrate was measured as its 2,2′-bipyridine complex.

2.3. *Monitoring of purity and assembly*

Protein was assayed with the Bio-Rad micro-assay kit with BSA as standard. Purity was checked and subunits analysed by electrophoresis in 12.5% polyacrylamide gels containing 1% SDS, the sample being first boiled for 10 min in 62.5 mM Tris containing 10% glycerol, 5% mercaptoethanol, 2.3% SDS. Subunit folding and assembly was monitored by electrophoresis in 6% polyacrylamide gels and by immunoassay. Microtest plates were coated overnight with the test ferritins (1 μg/ml) in 0.05 M sodium bicarbonate buffer, pH 9.5. Immunoassays were carried out with polyclonal antisera prepared in our laboratory [15] and with anti-human H-chain monoclonal antibody 2A4 (kindly provided by P. Arosio), serially diluted over a suitable range. Bound IgG was detected with either anti-rabbit or anti-mouse IgG coupled to peroxidase using 3,3′,5,5′-tetramethyl-benzidine as substrate [16].

2.4. *Iron uptake*

Formation of the initial Fe(III)-apoferritin complex was measured after addition of 4 Fe(II) atoms/molecule to apoferritin (1 mg/ml) in 0.05 M Mes buffer, pH 5.9, by UV difference spectroscopy in a Cary 219 double beam spectrophotometer [6]. Spectra in the wavelength range 250–400 nm due to the complex were measured repeatedly until they were fully developed. Relative rates were obtained by measuring the absorbance at

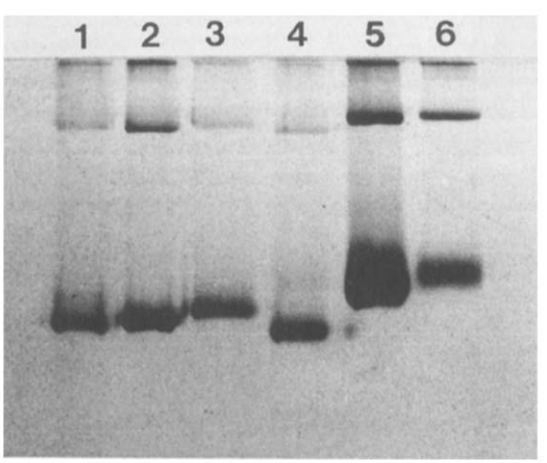

Fig.1. Electrophoresis in 6% polyacrylamide gel of human ferritins stained with Coomassie blue. Tracks 1–4 are mutant H-chain ferritins, 5 and 6 are native human brain and human liver ferritins, respectively. Mutants are: 1, H(121–125)⟶L; 2, Asp131,Glu134⟶Ala; 3, Asp131,Glu134⟶His; 4, Lys86⟶Gln.

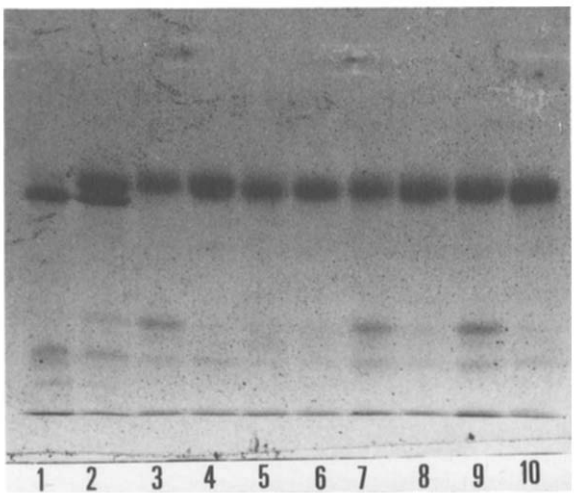

Fig.2. Electrophoresis in an SDS-12.5% polyacrylamide gel of human ferritin subunits stained with Coomassie blue. Tracks 1 and 2 are native human brain and liver ferritins, respectively. The lower bands in 1 and 2 are human L-chains. Mutant H-chains in tracks 3–10 are: 3, Lys86⟶Gln; 4, Asp131⟶His; 5, Glu134⟶His; 6, Asp131,Glu134⟶His; 7, H(121–125)⟶L; 8, Glu134⟶Ala; 9, Asp131⟶Ala; 10, Asp131, Glu134⟶Ala.

310 nm reached at 10 s and expressing this as a percent of the maximum absorbance. Formation of the iron core was followed by adding 500 Fe(II) atoms/molecule to apoferritin (50 μg/ml) in 0.05 M Mops buffer, pH 6.8, and monitoring the increase in absorbance at 310 nm. Iron was added as a $(NH_4)_2Fe(SO_4)_2$ solution in deoxygenated water for both types of experiment.

3. RESULTS AND DISCUSSION

On electrophoresis in 6% polyacrylamide gels (fig.1), all the recombinants showed bands at similar positions to those of native ferritins. The H-chain homopolymers migrate somewhat faster than ferritins from human brain (66% H-subunit) or liver (20% H-subunit). Although H-chains are slightly larger than L-chains [17], they are more acidic. The greater basicity of mutant Asp131,Glu134⟶His may explain why it is slightly retarded compared with the other recombinants (fig.1). Minor, more slowly migrating, bands like those attributed to molecular oligomers in native ferritins [18] are also visible on the gels. Fig.2 shows that each of the mutants gave a single

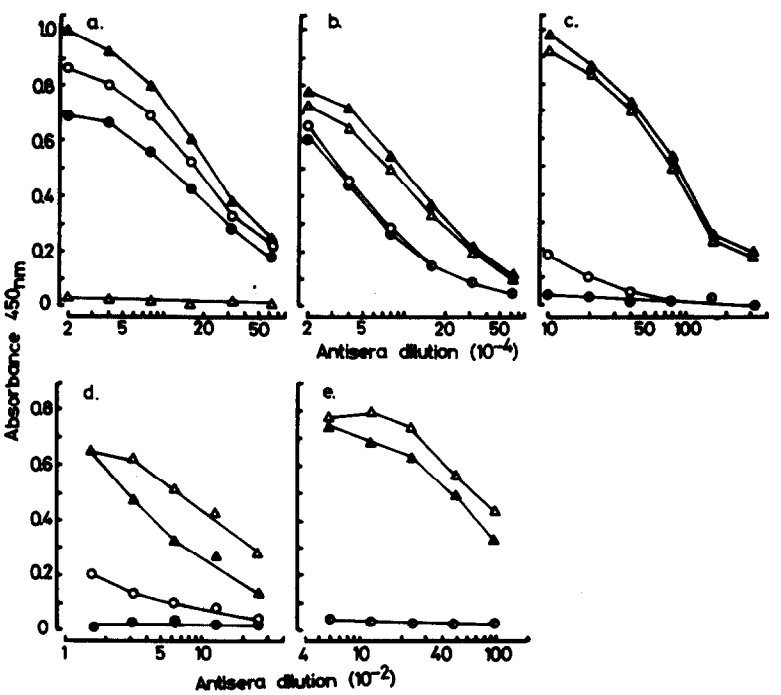

Fig.3. Immunoassay of human ferritins. Two human H-chain recombinant ferritins, Lys86⟶Gln (●) and H(121–125)⟶L (○) are compared with native human brain ferritin (▲) and liver ferritin (△). (a) Monoclonal 2A4 specific to the H-subunit; (b) antiserum to human brain ferritin; (c) antiserum to human spleen ferritin; (d) antiserum to the 3-fold channel residues 120–129; (e) antiserum to the 4-fold channel, residues 155–166.

H-subunit band, unlike the tissue ferritins which gave both H- and L-subunit bands. Minor low molecular mass bands, similar to those in native ferritins, probably result from proteolytic digestion of a small fraction of molecules within intact molecules.

Fig.3 shows the results of ELISA for two recombinants, Lys86⟶Gln and H(121–125)⟶L, and for native human brain and liver ferritins. The other mutants gave values close to those for Lys86⟶Gln. It can be seen that monoclonal 2A4 recognises brain ferritin and both recombinants (and this indicates that the epitopic centre it recognises is not at the outside exit of the 3-fold channels), but not liver ferritin (fig.3a). Anti-brain ferritin polyclonal (fig.3b) recognises the recombinants, whereas anti-spleen ferritin (fig.3c) does not, except for weak recognition of H(121–125)⟶L. The latter is also weakly reactive to polyclonal antisera directed to the 3-fold channel L-subunit peptides (fig.3d), although not to that directed to the 4-fold channel L-chain peptides (fig.3e). Neither of these antisera react with Lys86⟶Gln although they recognise both spleen and brain ferritins.

The above results indicate that the recombinants had assembled into molecules resembling native ferritins. This is borne out by the fact that several of the mutants have been crystallized and three of them, Lys86⟶Gln, Glu134⟶Ala and Asp-131⟶His have been found to give crystals that are isomorphous with those of rat liver ferritin and that diffract to high angles (Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Smith, J.M.A. and Harrison, P.M., unpublished results).

Initial Fe(III)-apoferritin UV difference spectra developed after adding Fe(II) to the various recombinants all show an absorbance shoulder with a maximum at 290–295 nm (fig.4), very similar to the 'initial spectrum' seen previously with horse spleen [6] and rat liver [19] apoferritin. Except for Asp131, Glu134⟶His which gave relatively low absorbance, the peak heights were approximately the same for all the mutants. From this we must conclude that the ligands of the Fe(III)-apoferritin complex are not Asp131, Glu134 or residues Ala-Thr-Asp-Lys-Asn(121–125) respectively in the narrow part or the wider mouth of the 3-fold channels. This conclusion is supported by work with recombinant rat L-chain ferritin [20], which has all

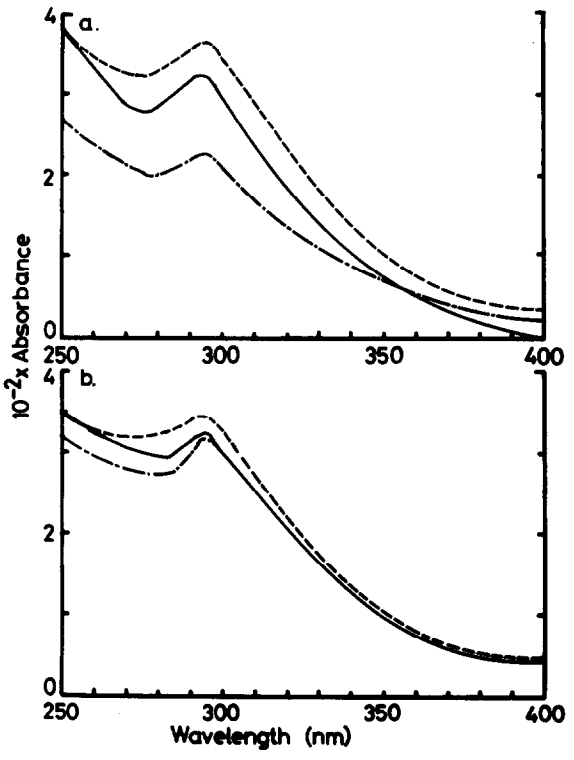

Fig.4. Initial UV difference spectra of 4 Fe(III)-apoferritin complexes of human H-chain ferritins in 0.05 M Mops buffer, pH 5.9. (a) Lys86⟶Gln (– – –), Asp131⟶His (——), Asp131,Glu134⟶His (·····); (b) Lys86⟶Gln (– – –), Glu134⟶Ala (——), Asp131,Glu134⟶Ala (·····).

of these residues, but which gives no initial Fe(III)-apoferritin complex.

The absence of the initial Fe(II)-oxidation site from the 3-fold channels does not rule out the use of these channels as the route or a route, by which iron traverses the shell. In principle it may do so either as Fe(II) or Fe(III), implying respectively oxidation on the inside or outside of the molecule. Table 1 gives the relative initial rates of formation of the iron core for the various mutants. Although all of them reached approximately the same final absorbance, some diminution of rate can be seen especially for Asp131,Glu134⟶His and Glu-134⟶His, but in no case was there complete inhibition. The results could mean that iron does indeed enter the molecule by the 3-fold channels, but if so, a specific mechanism involving ligand exchange by metal-binding carboxyls is evidently not required. Previous studies implicating the 3-fold

Table 1

Relative initial rates of formation of an initial 4 Fe(III)-apoferritin complex and a 500 Fe(III) atoms/molecule iron-core in recombinant human H-chain apoferritins

Recombinant	4 Fe(III)-apoferritin[a]	500 Fe(III)-apoferritin[b]
Lys86⟶Gln	100	100
Asp131⟶His	66	69
Glu134⟶His	46	42
Asp131,Glu134⟶His	48	30
Asp131⟶Ala	46	100
Glu134⟶Ala	72	69
Asp131,Glu134⟶Ala	76	76
H(121–125)⟶L	78	73

[a] 2 μM apoferritin, 8 μM Fe in 0.05 M Mes buffer, pH 5.9
[b] 0.1 μM apoferritin, 50 μM Fe in 0.05 M Mops buffer, pH 6.8

Iron was added as $(NH_4)_2Fe(SO_4)_2$ and the oxidant was dioxygen. Initial rates are expressed relative to those for mutant Lys86⟶Gln which were set at 100

channels have been based on the binding of several metal ions and on competition by iron for this binding [9,11] and also on the effects of chemical modification of residue Cys130 [10]. Table 1 shows very similar relative rates for the formation of the initial Fe(III)-apoferritin complex as for core. Assuming that iron is entering through the 3-fold channels, these results suggest that the initial Fe(III)-apoferritin complex is formed inside the molecule and hence that this iron enters as Fe(II) as had been proposed [3] and for which there is independent evidence from Mössbauer spectroscopy [21]. However, the different relative rates of table 1 could also reflect differences in fidelity of assembly of the various mutants. The apparently unimpaired ability of the mutants to form iron cores indicates that metal sites in the 3-fold channels are not directly involved in iron-core nucleation.

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