Mutational analysis of the channel and loop sequences of human ferritin H-chain

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Human ferritin H-chain mutants were obtained by engineering the recombinant protein expressed by *Escherichia coli*. The mutageneses were directed to the C-terminal sequence forming the hydrophobic channel, to the hydrophilic channel and to the loop sequence. The mutants were analysed for extent of expression, for stability, for capacity to incorporate iron and for kinetics of iron uptake and iron oxidation. Of the 22 mutants analysed only two with deletions of single residues in the loop sequence and one with deletion of the last 28 amino acid residues did not assemble into ferritin-like proteins. The other mutants assembled correctly and showed similar chemical/physical properties to the wild-type; they included duplication of an 18-amino acid-residue stretch, deletion of the last 22 and the last seven residues and various mutations of single amino acid residues. Two mutants with extensive alteration in the C-terminal sequence had a diminished thermostability associated with incapability to incorporate iron though they still catalysed iron oxidation. The mutants with alterations of the sequence around the hydrophilic channel showed diminished iron uptake and oxidation kinetics, together with a slightly larger apparent molecular size. The results indicate (i) that two of the sequences are important for ferritin assembly/stability, (ii) that the presence of the hydrophobic channel is essential for formation of the iron core and (iii) that the sites of iron interaction and the path of iron penetration into ferritin remain unidentified.

INTRODUCTION

Ferritin is an exceptionally stable and ubiquitous iron-protein with the major functions of storing and detoxifying intracellular iron (Munro & Linder, 1978; Theil, 1987; Harrison *et al.*, 1987a). It is a 24-mer protein composed of various proportions of two subunit types named H and L (Arosio *et al.*, 1978), which are structurally and genetically distinct (Costanzo *et al.*, 1986; Santoro *et al.*, 1986). These two chains fold into a similar structure of four tightly packed α -helices (A–D) plus a loop sequence connecting the B–C helices and a short fifth E α -helix at an acute angle to the bundle, near the C-terminus (Ford *et al.*, 1984; Harrison *et al.*, 1987b). Crystallographic data have indicated that ferritin assembly and stability are ensured by many hydrogen bonds and salt bridges as well as by extensive hydrophobic interactions along the twofold, threefold and fourfold symmetry axes, where two, three and four subunits come into contact (Ford *et al.*, 1984; Harrison *et al.*, 1987b). The relative importance of these interactions remains to be experimentally defined (Luzzago & Cesareni, 1989).

The protein assembles to form a cavity, about 7 nm across, where the ferrihydrite-like iron cores of various sizes are accommodated. The cavity communicates with the solvent through six hydrophobic channels along the fourfold symmetry axis, each lined by eight to 12 leucine residues, and eight hydrophilic channels along the three-

fold axis, each lined by six carboxy groups. The strikingly different physical properties of the two channels are expected to play an important role in ferritin activity (Ford *et al.*, 1984).

The cDNAs and genes of both human ferritin subunit types have been sequenced: they are encoded on different chromosomes, and share 55% amino acid sequence homology (Boyd *et al.*, 1985; Costanzo *et al.*, 1986; Santoro *et al.*, 1986). In addition, the amino acid sequences of rat (Leibold *et al.*, 1984; Murray *et al.*, 1987), horse (Heustersprue & Crichton, 1981), mouse (Torti *et al.*, 1988) and hen (Stevens *et al.*, 1987) ferritin subunits have been determined, and they show above 85% homologies with the corresponding human H- and L-chains. The conserved H-chain $\rightarrow$ L-chain sequence substitutions are mainly localized on outer and inner protein surfaces and in the inner part of the hydrophobic channel. The sequence lining the hydrophilic channel is highly conserved in all ferritin sequences. Detailed analyses of the ferritin structure have been reviewed by Harrison *et al.* (1987a).

We have previously overexpressed human H- and L-chains in *Escherichia coli* and shown that they assemble in ferritins with biochemical properties analogous to those of natural ferritins, including the capacity to incorporate iron (Levi *et al.*, 1987, 1988, 1989). In addition, we found that the ferritin H-chain is able to promote iron oxidation even in conditions when it does not occur spontaneously, and that it can transfer oxidized

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iron to a macromolecule such as transferrin (Levi *et al.*, 1988). Since neither of these activities is present with ferritin L-chain (Levi *et al.*, 1989), it was deduced that the H-chain carries a specific ferro-oxidase activity, which may be important for the physiological regulation of cellular iron metabolism. The analysis of some H-chain mutant proteins showed that the last 22 amino acid residues are essential for formation of the iron core (Levi *et al.*, 1988). Thus it was concluded that the C-terminal sequence, the one forming the hydrophobic channel, is somehow involved in formation of the ferritin iron core. However, various studies on horse spleen ferritin indicated that iron binds and transits through the hydrophilic channel (Chasteen & Theil, 1982; Treffry & Harrison, 1984; Stefanini *et al.*, 1989). Mutational studies of the ferritin H-chain hydrophilic channel did not confirm its active involvement in iron uptake (Treffry *et al.*, 1989).

In the present study we extended the mutagenesis approach (1) to try to localize on the C-terminal sequence the iron-incorporation activity and to try to understand the functional roles of the H-chain → L-chain sequence substitutions in this region, (2) to try to localize the ferro-oxidase site, and (3) to try to identify some interactions important for ferritin assembly. We analysed some previously described mutants (Levi *et al.*, 1988; Luzzago & Cesareni, 1989; Treffry *et al.*, 1989) and some new ones in which accessible and interesting regions such as the loop sequence, along the twofold axis, the hydrophilic channel, along the threefold axis, and the C-terminus, along the fourfold axis, have been modified. In addition, the carboxy groups were modified by chemical reaction on the wild-type and two mutants.

Preliminary analysis of the mutants showed (1) that interactions along the twofold axis and at the end of D α -helix are important for ferritin assembly, (2) that deletion or misplacing of the E α -helix strongly decreases ferritin stability and inhibits formation of the iron core, (3) that mutations that are expected to modify the properties of the two channels do not alter ferritin iron incorporation, and (4) that the ferro-oxidase site is not located at the C-terminus, around the loop or on the hydrophilic channel.

EXPERIMENTAL

Ferritins and mutants

Human liver ferritin and recombinant ferritin H-chain were purified as described by Levi *et al.* (1988), and recombinant ferritin L-chain was purified as described by Levi *et al.* (1989). Mutants were obtained by oligonucleotide-directed mutagenesis (Sollazzo *et al.*, 1985) of the plasmid pEMBLex2HFT (Levi *et al.*, 1987) by inserting at the proper position amber codons and by substituting or deleting codons. Mutant colonies were screened by hybridization to mutagenic oligonucleotide, and the sequence of positive clones was confirmed by the chain-termination method (Sanger *et al.*, 1977). One mutant (M1) originated from a cloning artifact, and its sequence was determined by DNA sequencing of the plasmid. *E. coli* strain GC382 cells transformed with the plasmids were induced as described by Levi *et al.* (1987). The thermostable mutants were purified by the same procedure as that used for the wild-type, by heating the cellular homogenate at 75°C for 5 min, $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on a Sepharose 6B column. The non-thermostable 91 and 92 mutants were

purified, with omission of the heat step, as described by Levi *et al.* (1988), by $(\text{NH}_4)_2\text{SO}_4$ precipitation, gel filtration on Sepharose 6B and preparative electrophoresis. Purity was assessed by SDS/polyacrylamide-gel electrophoresis (Levi *et al.*, 1988). Iron was removed from the ferritins and muteins as described by Macara *et al.* (1972), by incubation for 18 h in 1% (w/v) sodium thioglycollate, pH 5.5, in a stoppered test tube with excess 2,2'-bipyridyl and extensive dialysis against 0.1 M-Hepes/NaOH buffer, pH 7.

Analytical methods

Protein concentration was determined by using the BCA assay (Pierce Chemical Co.) calibrated with bovine serum albumin. In the presence of Hepes buffer, the Bio-Rad Laboratories protein assay was used. Native electrophoresis in 7.5% polyacrylamide gels and electrophoresis in the presence of SDS in polyacrylamide gradient gels were performed as described by Levi *et al.* (1988). The molecular masses of purified recombinant proteins were evaluated by loading 50–100 μg samples on Superose 6 f.p.l.c. columns (Pharmacia) equilibrated in 20 mM-Tris/HCl buffer, pH 7.4, at a flow rate of 0.5 ml/min. The column was calibrated with recombinant ferritin H-chain, recombinant ferritin L-chain, their dimers, thyroglobulin and catalase.

Iron incorporation

Soluble extracts of the strains induced in LB medium containing 1 mM-ferric nitrilotriacetate were subjected to non-denaturing polyacrylamide-gel electrophoresis and stained with Prussian Blue. In addition, solutions of the purified mutants (0.5–1 mg/ml in 0.1 M-Hepes/NaOH buffer, pH 7.0) were made 1 mM with respect to freshly prepared $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ and incubated for 1–2 h at room temperature. After centrifugation (at 10000 *g* for 5 min) they were run on non-denaturing electrophoresis and stained with Prussian Blue as described by Levi *et al.* (1988).

Iron kinetics

Iron-uptake reactions were performed as described by Levi *et al.* (1988), at a molar ratio of 1000 Fe(II) atoms/apo-ferritin molecule, and monitored at 310 nm. Ferroxidase activities were performed as described by Bakker & Boyer (1986), in 0.2 M-sodium acetate buffer, pH 6.0, containing 4 mg of apo-ovotransferrin (Diaconal, Recordati, Italy)/ml, 0.2 μM -apoprotein and 0.1 mM freshly prepared $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, and the absorbance was monitored at 470 nm in an Aminco dual-wavelength spectrophotometer, with reference at 600 nm. Kinetic rates were calculated by absorbance variation after 3 min of reaction.

Chemical modifications

The solvent-accessible carboxy groups were modified as described in Wetz & Crichton (1976). Glycine ethyl ester was added to a solution of apo-(ferritin H-chain) or apo-muteins (1 mg/ml) to a final concentration of 0.84 M. The pH was adjusted and maintained at 4.9 during the reaction. The reagent 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide was added to a final 0.1% concentration, and the mixture was incubated at room temperature for 2 h. The proteins were then extensively dialysed against 0.1 M-Hepes/NaOH buffer, pH 7.0. Modification of the carboxy groups was confirmed by

Fig. 1. Schematic representation of the mutations on the ferritin H-chain sequence

Shaded boxes indicate the A–E α -helices; the vertical arrows point to the sites of mutagenesis. The insert is a magnification of the C-terminal sequence. The names of the mutants and the types of mutation (in parentheses) are shown. The single substitutions are indicated by horizontal arrows and the stretch substitutions by the replaced sequences; **bold** lines show the deleted regions, and Δ is for residue deletions.

Table 1. List of recombinant ferritin H-chain mutants

Abbreviation: am, amber mutation. (a) Mutant described also in Treffry *et al.* (1989); (b) mutant described also in Luzzago & Cesareni (1989); (c) mutant described also in Levi *et al.* (1988). * Mutations into the equivalent sequence of L-chain.

Region	Name	Mutation
Loop	117	Deletion L ⁸²
	118	Deletion I ⁸⁵
	(a)* 9Cd	K ⁸⁶ → Q
	119	Deletion P ⁸⁸
	M1	Duplication P ⁸⁸ –L ¹⁰⁶ + DD ^{91,92} → NV
Threefold axis	(a)* 203	K ⁸⁶ → Q + ATDKN ^{121–125} → GSRAT
	(a) 205	K ⁸⁶ → Q + D ¹³¹ → A
	(a) 174	K ⁸⁶ → Q + D ¹³¹ → H
	(a) 204	K ⁸⁶ → Q + E ¹³⁴ → A
	(a) 173	K ⁸⁶ → Q + E ¹³⁴ → H
	(a) 173 + 174	K ⁸⁶ → Q + D ¹³¹ → H + E ¹³⁴ → H
C-Terminal	(b) 116	L ¹⁵⁵ –am
	(b, c) 91	P ¹⁶¹ –am
	(b) R1	Y ¹⁶⁸ → D
	(b) R2	L ¹⁶⁹ → R
	(b) R3	D ¹⁷¹ → Y
	(b) R4	N ¹⁵⁴ → I + M ¹⁵⁸ → L
	(b) R5	A ¹⁶⁶ → D
	(b) R6	FD ^{170,171} → CN
	(b) 103	G ¹⁷⁶ –am
	* 150 + 102	H ¹⁷³ → L + GDS ^{176–178} → KHD–am
	(b) 92	Fusion C-terminus with β -galactosidase α -peptide

Fig. 2. Expression and purification of ferritin mutants

Cellular soluble homogenates after induction (a) and purified proteins (b) were analysed by SDS/polyacrylamide-gel electrophoresis followed by staining with Coomassie Blue. The arrows indicate the positions of the peptides whose synthesis is induced by heat shock. Mutants are indicated by the numbers above the gel slots; rHF, recombinant ferritin H-chain control; w.t., wild-type; 382, untransformed *E. coli* strain GC382. Protein markers (Std) and their molecular masses are indicated.

anodal electrophoretic mobility in 1% agarose gels at pH 8.8, and quantified by h.p.l.c. amino acid analysis of the hydrolysed samples.

RESULTS

The C-terminal sequences, the loop and the threefold axis areas of the ferritin H-chain were altered by site-directed mutagenesis to obtain information on the relevance of these portions on ferritin assembly and functional activity. The mutants were analysed for recovery in *E. coli* extracts, preliminary biochemical characteristics and reactivity with iron.

Mutants

The mutants are represented in Fig. 1 and listed in Table 1, grouped on the basis of the regions that have been altered: loop, threefold axis and C-terminus.

Mutant 102 + 150 consisted of the replacement of the last ten amino acid residues by the C-terminal amino acid residues of the L-chain; the remaining C-terminus mutations, already described elsewhere, were deletions of the last seven, 22 and 28 amino acid residues (mutants 103, 91 and 116 respectively), the fusion with the α -peptide of β -galactosidase (mutant 92) and random mutants (mutants R1–R6), which were found to affect folding of the E α -helix (Luzzago & Cesareni, 1989). The loop mutants 117, 118 and 119 consisted of deletions of single amino acid residues in the loop sequence, and mutant M1 of a duplication, with two point mutations, of an 18-amino acid-residue stretch. The mutants along the threefold axis have been already described (Treffry *et al.*, 1989) and consisted of the replacement of aspartic acid 131 and glutamic acid-134, which line the channel, by histidine and alanine, and of the replacement of a five-amino acid-residue stretch at the mouth of the channel

Fig. 3. Native polyacrylamide-gel electrophoresis of (a) soluble cellular extracts after induction and (b) purified mutein 92

(a) Electrophoresis of soluble cellular extracts after induction followed by staining with Coomassie Blue. Abbreviation: w.t., wild-type. (b) Electrophoresis of purified mutein 92 and recombinant ferritin H-chain (rHF) control, after 1 h incubation with $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, pH 7.0 [at a molar ratio of 1000 Fe(II) atoms/molecule of ferritin], followed by staining with Coomassie Blue (C.B.) and Prussian Blue (P.B.). The position of ferritin monomer is indicated by the arrows.

by the equivalent five-amino acid-residue stretch present in the L-chain (mutant 203).

Mutein expression

The same *E. coli* strain was transformed with the various plasmids, and mutein synthesis was induced by the same heat-shock procedure. After induction the cellular soluble extracts were analysed by SDS/polyacrylamide-gel electrophoresis to evaluate accumulation of the mutant peptides. All the plasmids except 117 and 118 induced the accumulation of the peptides of the expected molecular mass, and the extents of accumulation were apparently analogous to the wild-type (Fig. 2a). The plasmids 117 and 118, with deletions of single residues involved in subunit dimer formation, did not induce the accumulation of detectable amounts of recombinant peptides. When used in a translation system *in vitro*, however, they were as active as the wild-type plasmid (results not shown), suggesting that the lack of accumulation was mainly caused by proteolytic degradation during expression in *E. coli*.

Native polyacrylamide-gel electrophoresis of the cellular extracts of the induced bacteria showed that all mutant peptides assemble into proteins with electrophoretic mobility analogous to that of the wild-type, the only exception being mutein 116, with the last 28 amino acid residues deleted, which did not appear to assemble into any detectable structured proteins (Fig. 3a).

Fig. 4. Progression plots of the iron-uptake reactions of the apo-muteins with changes in the C-terminal sequence

Experiments were performed in 0.1 M-Hepes buffer, pH 7.0, containing 0.1 μM -apo-H-chain and 0.1 mM- $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$. Mutants are indicated by their names; rHF, recombinant ferritin H-chain; rLF₀, recombinant ferritin L-chain.

Mutein molecular size

The soluble cellular extracts of the assembled mutants were heated for 5 min at 75 °C, clarified and analysed by non-denaturing polyacrylamide-gel electrophoresis. Only muteins 91 and 92, with extensive alterations at the C-terminal sequence, did not resist the treatment. The thermal stability of the other muteins was exploited for their purification (Fig. 2b). The purified muteins were analysed by gel-filtration runs on a Superose-6 f.p.l.c. column, which separates ferritin monomer clearly from oligomers. The elution profiles were analogous for all mutants, with the presence of a minor proportion of oligomers, similarly to the wild-type. Estimates of apparent molecular masses are referred to the monomer peaks. Wild-type H-chains and muteins R2 and 102 + 150 were co-eluted at 13.86 ± 0.01 ml, the muteins with deleted sequences were eluted with smaller molecular mass (e.g. mutant 91 at 14.44 ml) and the ones with inserted sequences were eluted with larger molecular mass (e.g. mutants M1 and 92 at 13.29 and 13.43 ml respectively). Interestingly, all the muteins with changes along the threefold axis were consistently eluted before the wild-type H-chain at 13.62–13.71 ml, with estimated molecular mass 50–100 kDa higher than that of recombinant ferritin H-chain.

Iron incorporation

When induction was performed in the presence of 1 mM-ferric nitrilotriacetate in the *E. coli* medium, the wild-type H-chain and all the assembled muteins were purified as amber-coloured proteins, and after electrophoresis could be stained by Prussian Blue, but muteins 91 and 92 could not (results not shown). The capacity to in-

Fig. 5. Progression plots of the ferro-oxidase reactions of the muteins with changes in the C-terminal (a) and hydrophilic-channel (b) sequences

Experiments were performed in 0.2 M-acetate buffer, pH 6.0, containing 0.1 mM- $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 4 mg of apo-ovotransferrin/ml and 0.2 μM -apo-H-chain. Mutants are indicated by their names; rHF, recombinant ferritin H-chain; rLF₀, recombinant ferritin L-chain; OT, control without ferritin added.

corporate iron was also assessed by incubation of the apoproteins *in vitro* for 2 h with a 1000-fold molar excess of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ at pH 7.0 in the presence of O_2 and Prussian Blue staining of the electrophoresed samples. Again muteins 91 and 92 could not be stained, whereas all the other ones could [Fig. 3(b) and Fig. 7 of Levi *et al.* (1988)].

Reactions with Fe(II)

The purified apoproteins were analysed for activity in the ferro-oxidase reaction, which uses transferrin as final Fe(III) acceptor (Bakker & Boyer, 1986), and in the iron-uptake reaction, at pH 7.0, at a molar ratio of 1000 Fe(II) atoms/molecule of apo-ferritin. All the muteins showed detectable activity in both reactions, with rates higher than with the ferritin L-chain used as negative control (Levi *et al.*, 1989). Thus none of the mutations removed or completely altered the catalytic site(s). Mutein activities were never higher than that of wild-type H-chain, and we found a significant correlation between ferro-oxidase and iron-uptake activities ($r = 0.89$, $P > 0.001$).

The 9Cd and M1 muteins, with alterations of the loop sequences, had activities 80–100% of the wild-type H-chain, i.e. within the experimental variability (results not

Fig. 6. Progression plots of iron-uptake (a) and ferro-oxidase (b) reactions of apo-H-chains and their derivatives after reaction with carbodi-imide and glycine ethyl ester (dc)

Conditions were as indicated in the legends to Figs. 4 and 5.

shown). Similarly the muteins with the minor modifications of the C-terminal sequence (muteins 103, 102+150 and R2) had activities similar to that of the wild-type H-chain, whereas muteins 91 and 92 showed a decrease of about 50% (Figs. 4 and 5a). A similar decrease in activity to about 50% of that of the wild-type H-chain was found in all the muteins with changes around the hydrophilic channel (Fig. 5b), in agreement with previous findings (Treffry *et al.*, 1989).

Apo-(wild-type H-chains) and apo-muteins 173 and 174 were treated with the water-soluble carbodi-imide and glycine ethyl ester to obtain a modification of 7 ± 1 carboxy groups/molecule. The modified proteins lost iron-uptake activity and the ferro-oxidase activity was decreased to about 20% (Fig. 6).

DISCUSSION

Only three of the 22 mutations that have been analysed prevent ferritin assembly. One of these, previously reported (Luzzago & Cesareni, 1989), leads to the synthesis of a protein in which the last 28 residues have been removed (mutein 116). The other two each have one amino acid residue deleted, leucine-82 (mutein 117) and isoleucine-85 (mutein 118).

Leucine-82 and isoleucine-85 are located in a region of hydrophobic interactions between two subunits with alternating hydrophobic and hydrophilic residues (Ford *et al.*, 1984). Thus the deletion of a single amino acid residue is expected to alter these interactions by shifting polar residues into the position of apolar ones. This does not seem to occur in the case of deletion of proline-87 (mutein 119), which is also part of the hydrophobic patch, but being located at the end of the patch it probably does not shift the frame of the interaction.

Interestingly the mutein 116 peptide could be recovered in detectable amounts after most induction experiments, whereas the mutein 117 and 118 peptides could never be identified, probably because of rapid degradation after synthesis. The former, involving the deletion of the C-terminus, is not expected to inhibit subunit dimer formation, but the two latter mutations should, as they involve alterations at the relevant sequence. Dimer formation appears to be the first step in ferritin assembly (Stefanini *et al.*, 1987; Gerl & Jaenicke, 1987), and the present findings suggest that it may provide some degree of stability to ferritin subunits. This, together with the finding that none of the other muteins, including the less stable muteins 91 and 92, showed any detectable subunit-ferritin equilibrium, strongly suggest that free H-chains are not stable entities (Linder *et al.*, 1989).

All the other 19 muteins assemble into ferritin-like proteins, with electrophoretic mobility and apparent molecular mass similar to those of wild-type ferritin. Interestingly the typical ferritin 24-mer structure and stability were conserved even after extensive modifications such as the 18-amino acid-residue duplication in the loop sequence. These results show that this protein is extremely tolerant and that extensive alterations, including deletions and insertions, can be accommodated without disturbing the general assembly/folding pathway.

Among the ferritin-like proteins assembled from muteins that were analysed only the two with extensive alterations at the C-terminus (muteins 91 and 92) had lost the typical ferritin stability at 75 °C. In both of them

the interactions around the E α -helix were eliminated, either by its deletion or by its flipping outside the molecule (Luzzago & Cesareni, 1989). It is suggested that such interactions contribute to the exceptional thermostability, but are not essential in the pattern of ferritin assembly (Luzzago & Cesareni, 1989).

Iron interactions

The E α -helix forms the hydrophobic channel, and muteins 91 and 92 are expected to have this channel improperly formed: they have lost the major ferritin function to store iron. In the attempt to localize in more detail the residues involved in such function, we deleted the last seven residues pointing into the inner cavity, and replaced the last ten residues, including histidine-173, by the corresponding C-terminal amino acid residues present in the L-chain. We also analysed some random mutations that were found to effect E α -helix folding, including the dramatic substitution leucine $\rightarrow$ arginine at residue 169 lining the channel. None of these alterations produced major effects on formation of the iron core.

Similarly no major effect was observed as a result of the mutations along the threefold channel: replacement of the residues that make it hydrophilic, and replacement of the five-amino acid-residue stretch at the mouth of the channel by the equivalent but completely different five-amino acid-sequence stretch of the L-chain.

In conclusion, this preliminary mutational analysis of the two channels indicates (1) that the presence of E α -helix is essential for core formation, probably because it provides the chemical properties of the cavity that may drive in iron and retain it (Levi *et al.*, 1988), and (2) that the functional roles of the hydrophobic and hydrophilic environments of the two channels for iron incorporation remain unclear. More extensive studies of the effects of substitutions are needed to clarify the roles of the two channels.

The functional activities of the muteins were also monitored with respect to the kinetics of iron uptake and ferro-oxidase activity. The two activities were correlated, suggesting that they involve the same or close site(s), or alternatively that all the mutations affect the ferro-oxidase activity and, if this is the kinetically limiting process, iron uptake would be proportionally altered. The largest decrease in the activities (to about 50% of that of the wild-type) was found for the extensive alterations of the E α -helix (muteins 91 and 92) and for all the substitutions around the hydrophilic channel. The former two have altered structures, as shown also by their diminished stability. All the muteins with changes around the threefold axis were found to have apparent molecular masses 50–100 kDa larger than that of the wild-type H-chain. These alterations are located on the C and D α -helices and they may induce the assembly into a less compact structure. These data are more suggestive of a long-distance conformational effect on protein structure/function than on specific alterations of iron-binding sites.

As an alternative approach to localize the catalytic site we performed chemical modification of the accessible carboxy groups. About seven residues were blocked by the reaction, and the modified proteins, wild-type and mutants, had greatly diminished iron-uptake and ferro-oxidase activities. This finding confirms that in the H-chain the carboxy groups are directly involved in ferritin-iron interactions (Wetz & Crichton, 1976). We have

previously suggested that the carboxy groups on the outer protein surface, consistently substituted in all the sequenced H- and L-chains, may be responsible for the specific H-chain activity (Levi *et al.*, 1988). In the present work they were either replaced (aspartic acid-123 in munein 203) or duplicated (glutamic acid-94 and glutamic acid-101 in munein M1) without any major functional effect being observed.

This work was carried out in the framework of the Contract nos. BAP-0246 to P. A. and BAP-0252 to G. C. of the Commission of the European Communities. A. L. was supported by an E.C.-B.A.P. short-term Fellowship. We are grateful to Dr. R. Paroni and Dr. I. Fermo for amino acid analysis.

REFERENCES

- Arosio, P., Adelman, T. G. & Drysdale, J. W. (1978) *J. Biol. Chem.* **253**, 4451-4458
- Bakker, G. R. & Boyer, R. F. (1986) *J. Biol. Chem.* **261**, 13182-13185
- Boyd, D., Vecoli, C., Belcher, D. M., Jain, S. K. & Drysdale, J. W. (1985) *J. Biol. Chem.* **260**, 11755-11761
- Chasteen, N. D. & Theil, E. C. (1982) *J. Biol. Chem.* **257**, 7672-7677
- Costanzo, F., Colombo, M., Staempfli, S., Santoro, C., Marone, M., Frank, R., Delius, H. & Cortese, R. (1986) *Nucleic Acids Res.* **14**, 721-736
- Ford, G. C., Harrison, P. M., Rice, D. W., Smith, J. M. A., Treffry, A., White, J. L. & Yariv, J. (1984) *Philos. Trans. R. Soc. London B* **304**, 551-565
- Gerl, M. & Jaenicke, R. (1987) *Eur. Biophys. J.* **15**, 103-109
- Harrison, P. M., Andrews, S. C., Ford, G. C., Smith, J. M. A., Treffry, A. & White, J. L. (1987a) in *Iron Transport in Microbes, Plants and Animals* (Winkelman, G., van der Helm, D. & Neiland, J. B., eds.), pp. 445-475, VCH Verlagsgesellschaft, Weinheim
- Harrison, P. M., Ford, G. C., Rice, D. W., Smith, J. M. A., Treffry, A. & White, J. L. (1987b) *Biochem. Soc. Trans.* **15**, 744-748
- Heusterspreute, M. & Crichton, R. R. (1981) *FEBS Lett.* **129**, 322-327
- Leibold, E. A., Aziz, N. N., Brown, A. J. & Munro, H. N. (1984) *J. Biol. Chem.* **259**, 4327-4334
- Levi, S., Cesareni, G., Arosio, P., Lorenzetti, R., Sollazzo, M. & Cortese, R. (1987) *Gene* **51**, 267-272
- Levi, L., Luzzago, A., Cesareni, G., Cozzi, A., Franceschinelli, F., Albertini, A. & Arosio, P. (1988) *J. Biol. Chem.* **263**, 18086-18092
- Levi, S., Salfeld, J., Franceschinelli, F., Cozzi, A., Dorner, M. & Arosio, P. (1989) *Biochemistry* **28**, 5173-5184
- Linder, M. C., Kakavandi, H. R., Miller, P., Wirth, P. L. & Nagel, G. M. (1989) *Arch. Biochem. Biophys.* **269**, 485-496
- Luzzago, A. & Cesareni, G. (1989) *EMBO J.* **8**, 569-576
- Macara, I. G., Hoy, T. G. & Harrison, P. M. (1972) *Biochem. J.* **126**, 151-162
- Munro, H. N. & Linder, M. (1978) *Physiol. Rev.* **58**, 317-396
- Murray, M. T., White, K. & Munro, H. N. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7438-7442
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463-5467
- Santoro, C., Marone, M., Ferrone, M., Costanzo, F., Colombo, M., Minganti, C., Cortese, R. & Silengo, L. (1986) *Nucleic Acids Res.* **14**, 2863-2876
- Sollazzo, M., Frank, R. & Cesareni, G. (1985) *Gene* **37**, 199-206
- Stefanini, S., Vecchini, P. & Chiancone, E. (1987) *Biochemistry* **26**, 1831-1837
- Stefanini, S., Desideri, A., Vecchini, P., Drakenberg, T. & Chiancone, E. (1989) *Biochemistry* **28**, 378-382
- Stevens, P. W., Dodgson, J. B. & Engel, J. D. (1987) *Mol. Cell. Biol.* **7**, 1751-1758
- Theil, E. C. (1987) *Annu. Rev. Biochem.* **56**, 289-316
- Torti, S. V., Kwak, E. L., Miller, S. C., Miller, L. L., Ringold, G. M., Myambo, K. B., Young, A. P. & Torti, F. M. (1988) *J. Biol. Chem.* **262**, 12638-12644
- Treffry, A. & Harrison, P. M. (1984) *J. Inorg. Biochem.* **21**, 9-20
- Treffry, A., Harrison, P. M., Luzzago, A. & Cesareni, G. (1989) *FEBS Lett.* **247**, 268-272
- Wetz, K. & Crichton, R. R. (1976) *Eur. J. Biochem.* **61**, 545-550

Received 18 April 1989/12 June 1989; accepted 10 July 1989