

# “Opening” the Ferritin Pore for Iron Release by Mutation of Conserved Amino Acids at Interhelix and Loop Sites<sup>†</sup>

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**ABSTRACT:** Ferritin concentrates, stores, and detoxifies iron in most organisms. The iron is a solid, ferric oxide mineral ( $\leq 4500$  Fe) inside the protein shell. Eight pores are formed by subunit trimers of the 24 subunit protein. A role for the protein in controlling reduction and dissolution of the iron mineral was suggested in preliminary experiments [Takagi et al. (1998) *J. Biol. Chem.* 273, 18685–18688] with a proline/leucine substitution near the pore. Localized pore disorder in frog L134P crystals coincided with enhanced iron exit, triggered by reduction. In this report, nine additional substitutions of conserved amino acids near L134 were studied for effects on iron release. Alterations of a conserved hydrophobic pair, a conserved ion pair, and a loop at the ferritin pores all increased iron exit (3–30-fold). Protein assembly was unchanged, except for a slight decrease in volume (measured by gel filtration); ferroxidase activity was still in the millisecond range, but a small decrease indicates slight alteration of the channel from the pore to the oxidation site. The sensitivity of reductive iron exit rates to changes in conserved residues near the ferritin pores, associated with localized unfolding, suggests that the structure around the ferritin pores is a target for regulated protein unfolding and iron release.

Ferritin is found universally in microorganisms, plants, and animals. The major function is concentrating iron as a solid mineral inside the protein for storage, until needed, to compensate for the  $\sim 10^{11}$ -fold difference in iron solubility and the effective concentration of iron in cells (1). Identification of ferritin in strict anaerobes and protection of cells from oxygen toxicity (2–4) suggest an additional role for ferritin in oxygen detoxification related to the consumption of oxygen associated with the conversion of Fe(II) to ferric oxide mineral during the mineral formation process. As many as 4500 Fe atoms can be packed into the cavity ( $2.56 \times 10^5$  Å<sup>3</sup> or 256 nm<sup>3</sup>) of the ferritin protein. All cells use ferritin early in maturation with some specialized cells maintaining organismal iron reservoirs, such as hepatocytes, red cells of vertebrate embryos, and seed endosperm (1). The dual levels of gene expression (DNA and mRNA) emphasize the importance of ferritin in bacteria and animals; ferritin expression is coordinated with several other proteins of iron and oxygen homeostasis (5–9). Many studies have focused on Fe(II) entry and mineral formation in ferritin (10). Recent emphasis has been on the characterization of the first oxidation product, a blue diferric peroxo complex (11–14). Few studies have focused on how the iron is released from ferritin.

Exploring how iron might be released from the solid ferric oxide, buried 20 Å beneath the outer ferritin protein surface, has been daunting. Ferritin is a remarkably stable protein assembly, resisting 6 M urea at neutral pH and temperatures of  $>70$  °C. The unusually high stability of ferritin and observations that ferritin appears in lysosomes during iron overload, with identification of partly degraded ferritin protein in lysosomes (15), have led to the suggestion that iron can only be released from degraded, lysosomal ferritin. Recently, however, brief study of a recombinant frog H-ferritin, with spontaneous mutation (L134P),<sup>1</sup> revealed a greatly accelerated rate of formation of Fe(II) bipyridyl after the reduction of the stable ferritin iron mineral (16). Analysis of the protein crystals showed that the ferritin was a normal protein assembly with conserved contacts except at the junction of three subunits. Here, disorder in the ends of the C and D helices and the connecting C/D loop created eight areas of localized unfolding in the spherical protein. Using the physiological reductant (FMN/NADH) first developed for ferritin in 1978 (17), the bipyridyl chelator removed all the iron from the L134P ferritin in 5 min compared to 2 h for the wild-type ferritin (16). The region of protein unfolding in the L134P ferritin affected the site where iron is thought to cross the boundary between protein and solution/cytoplasm during iron entry or exit, based on the effects observed in protein crystals and iron exit (16) and by site-directed mutagenesis (e.g., 18, 19). In addition, the disordered region appeared to affect the channel or path of Fe(II) to the ferroxidation site ( $\sim 15$  Å), since the L134P

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<sup>1</sup> The numbering system used is that of the original sequence of horse spleen L ferritin, which is still commonly used. Another numbering system, also frequently used, is based on the human H sequence, which has a four amino extension at the N-terminus. The numbering adds 4 to the numbering system for horse spleen ferritin, making L134 into L138 in the alternative numbering.

substitution decreased the rate of iron oxidation slightly (16); the decay of the ferroxidation complex was also slowed slightly coincident with the appearance of a new tyrosine–Fe bond (20).

Several conserved, hydrophobic, and ionic interactions were disrupted in the L134P protein. Normal protein assembly in the absence of the conserved interactions indicates that the conserved amino acids have a function beyond assembly. The coincidence of folding/packing disorder at the 3-fold channel/pore with enhanced iron release focused attention on a new target to explore in solving the problem of iron exit from ferritin. Since proline, in the first mutant studied, is a particularly nonconservative replacement, the need for further study of more conservative substitutions was indicated (Figure 1). In addition, since L134 forms part of a hydrophobic core in the bundle that includes L110 in the C helix opposite L134 in the D helix (Figure 1 and references 21–25), changes in L134 and L110 should be studied to determine if the effects are similar. There are several other structurally conserved features at or near the ferritin pore that have not yet been examined for functional importance. For example, the ionic interactions of R72 and D122, between helix B and the C/D loop,<sup>2</sup> are conserved in four different ferritin structures (24, 25). In addition, the C/D loop itself is a very sharp turn of five amino acids, between helices C and D in each subunit, around the pores. Proteins containing a series of changed ion pairs, conservative and nonconservative amino acid replacements (residues 72, 110, 122, and 134), or longer C/D loops (inserting five amino acids between residues 124 and 125) were compared under identical conditions, to test the contribution of the ion pair (R72/D122), the hydrophobic pair (L110/L134), and the C/D turn to iron exit. In contrast to previous studies which measured events at sites inside altered protein (iron oxidation, mineral formation) (e.g., 1, 10, 19, 21), the assay used measures rates of iron reaching the outside after passing through the pore (16). The results show that iron exit is sensitive to even small changes at several sites around the pore, suggesting that ferritin pores could be a target for regulated unfolding or opening side to increase iron removal in response to cell need.

## EXPERIMENTAL PROCEDURES

**Cloning.** Site-directed mutagenesis of ferritin was carried out using the coding region from the H-frog ferritin, wild-type cDNA (5F12L) (27) in PTZ 19U. Mutations were produced using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The oligonucleotide primers were 5F12L134P (5'-CACCTGTTCTCAGGTATTCAGTCTCC-3'), 5F12L134A (5'-CACCTGTTCTCAGCGCTTCAGTCTCC-3'), 5F12L134G (5'-CACCTGTTCTCAGCCCCCTCAGTCTCC-3'), 5F12L134E (5'-CACCTGTTCTCAGTCTCTCAGTCTCC-3'), 5F12L134V (5'-CACCTGTTCTCAGCACTTCAGTCTCC-3'), 5F12L110A (5'-GTGCAGGTCCAGAGCGGCTGATTCACA-3'), 5F12L110V (5'-GTGCAGGTCCAGCAAGGCTGATTCACA-3'), 5F12LR72D (5'-CACCTGTTCTCAGGTATTCAGTCTCC-3'), and 5F12LR72DD122R (5'-CCAAGAAGTCACACAGATGGGGCGAACCTTGTCAGATCC-3').

An extension of the C/D loop was created by inserting five amino acids (KVDPH) between positions 124 and 125; the sequence is a repeat of residue positions between 120 and 124. The oligonucleotide 5F12 insertion (5'-ATGGGGGTCACCTTATGGGGGTCACCTTGTCAGATCC-3') was used as the primer. After confirming the sequence, altered coding regions were subcloned in the *NdeI* and *EcoRI* sites of pET-9a and expressed in *E. coli* BL21(DE3) cells. The frog H ferritin wild-type sequence (5F12) is in pET3a (16).

**Protein Expression and Purification.** All the recombinant proteins were purified as previously described (22). The proteins were expressed in *E. coli* as stable assembled proteins, purified by normal procedures that included the use of heating to selectively coagulate nonferritin proteins by heating (70 °C, 10 min). Dialysis tubing was boiled with a solution of Na<sub>2</sub>CO<sub>3</sub> (10 mM) and EDTA (10 mM), and acid-washed glassware was used to minimize the introduction of adventitious iron during purification (28).

**Kinetic Studies of Iron Release.** Mineral was formed in the recombinant apoferritins (2.08 μM), as previously described (16), by adding a solution of ferrous sulfate in 0.001 M HCl (480 Fe/assembled ferritin molecule) to apoferritin in 0.1 M MOPS and 0.2 M NaCl, pH 7.0, incubating 2 h at room temperature and then overnight at 4 °C. Previous studies showed that when the ferritin mineral was formed by adding an acidic solution of ferrous sulfate to the protein in buffer (0.1 M), the ferric iron was inside the protein and was inaccessible to chelators (29, 30). In contrast, the addition of NADH/FMN and bipyridyl to solutions of ferric citrate produces 100% conversion to Fe(II) bipyridyl immediately, faster than a measurement can be made and much, much faster than when the ferric iron is inside ferritin as described here. Iron release was triggered in ferritin, with mineral formed in vitro, by adding 2.5 mM bipyridyl, 2.5 mM FMN, and 2.5 mM NADH to the ferritin in 0.1 M MOPS, 0.2 M NaCl (pH 7.0 or 6.0), and monitoring the absorbance of the Fe(II) bipyridyl complex at 522 nm. The initial rates were determined by a linear fit of the data points from 0 to 1 min after mixing, which represents the first phase of iron release. The multiphase nature of both iron entry/oxidation and iron reduction/exit in ferritin has recently been reviewed (10).

## RESULTS

**Effect of Substitutions at Leucine 134 and Leucine 110, between the Subunit C and D Helices, on Reductive Iron Release and Chelation.** Initial studies of iron release from altered ferritin revealed the effect of the nonconservative replacement of leucine 134 by proline (16). The amide bond of leucine 134 is hydrogen bonded both to helix C in the same subunit and to a helix in the neighboring subunit (24, 25). A proline substitution at any site is particularly disruptive. The goals of the several amino acid substitutions designed were to test the following: (1) effects of amino acids less disruptive than proline at position 134; (2) the effect of changing the hydrophobic partner of L134 with substitutions at position 110 (see Figure 1). Accordingly, rates of iron release were studied in L134A, L134G, L134E, L134V, L110A, and L110V.

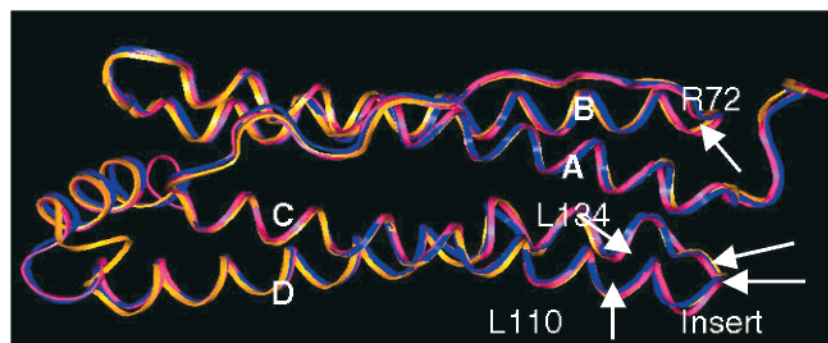
Recombinant protein of each type was purified from *E. coli* with little (<10/24 subunits) iron by taking precautions

<sup>2</sup> The shortest distance between the original side chain at position 72 and the carboxylate side chain at position 122 is 3.8–4.6 Å.

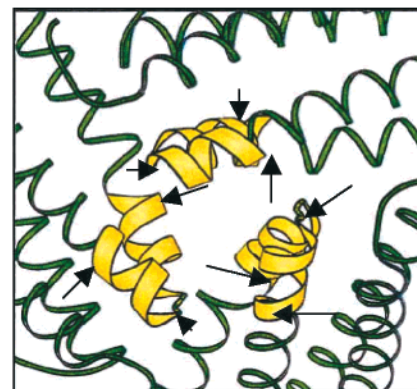
# A Sequence of ferritins in the C and D helices near the 3-subunit junction at the pore

	70	80	90	100	110	120	130
						<b>KVDPH</b> -insertion ↓	
Frog H	NKRGGRIVLQ	DVKKPERDEW	GNTLEAMQAA	LQLEKTVNQA	LLDLNKLASD	KVDPHLCDFL	ESEYLEEQVKS
Human H	NQRGGRIFLQ	DIKKPDCDDW	ESGLNAMECA	LHLEKVNQNS	LLELHKLATD	KNDPHLCDFI	ETHYLNEQVKA
Rat H	NQRGGRIFLQ	DIKKPDRDDW	ESGLNAMRCA	LHLEKSVNQS	LLELHKLATD	KNDPHLCDEI	ETHYLNEQVKS
Mouse H	NQRGGRIFLQ	DIKKPDRDDW	ESGLNAMECA	LHLEKSVNQS	LLELHKLATD	KNDPHLCDEI	ETYYLSEQVKS
Chick H	NQRGGRIFLQ	DIKKPDRDDW	ENGLTAMECA	LHLEKVNQNS	LLELHKLATE	KNDPHLCDFI	ETHYLDEQVKA
Frog M	NKRGGRVVLQ	DIKKPERDEW	GNTLEAMQAA	LQLEKTVNQA	LLDLHKLATD	KVDPHLCDFL	ESEYLEEQVKD
Frog L	NQRGGRVFLQ	SVEKPERDDW	ANGLEALQTA	LKLQKSVNQA	LLDLHAVAAD	KSDPHMTDFL	ESPYLSESJET
Human L	NQRGGRIFLQ	DIKKPDRDDW	GKTPDAMKAA	MALEKKLNQA	LLDLHALGSA	QADPHLCDFI	ETHFLDEEVKL
Rat L	NERGGRALFQ	DVDKPSQDEW	GKTLEAMKAA	LALEKLNQQA	LLDLHALGSA	QADPHLCDFL	ESHFLDKEVKL
Mouse L	NQRGGRALFQ	DVQKPSQDEM	GKTQEAMEAA	LAMEKLNQQA	LLDLHALGSA	RTDPHLCDFL	ESHYLDKEVKL
Soybean	NTRGGRVVLH	PIKNAPSEFE	HVEKGDALYA	MELALSLEKL	VNEKLLNVHS	VADRNNDPQM	ADFIIESEFLSE
Maize FM1	NKRGGRVRLQ	SIVTPLTEFD	HPEKGDALYA	MELALALEKL	VNEKLHNLHG	VATRCNDPQL	TDFIIESEFLEE
MaizeFM1	NKRGGRVRLQ	SIVTPLTEFD	HPEKGDATYA	MELALALEKL	VNEKLHSLHG	<u>VATRCNDPQL</u>	IDFIIESEFLEE
						C/D Turn	

B



D



C

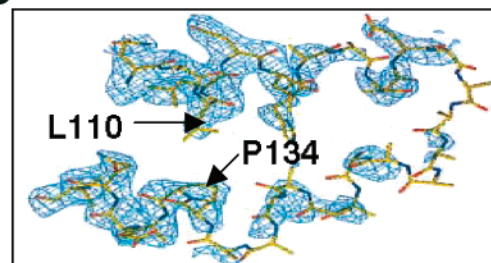


FIGURE 1: Sites of mutation which enhance iron exit from ferritin. (A) Primary structure in the region where 3 subunits assemble; 8 pores are formed at the 3 subunit junctions of the 24 subunit ferritin molecule. Sequence is compared for ferritins among animals and in plants. The amino acid numbering system used is based on the sequence of horse and other vertebrates for both H and L (1, 24); another numbering system for H subunits adds +4 to the residue numbers (25). Residues substituted by site-directed mutagenesis are shown in boldface type, the five amino acids in the C/D turn are underlined, and the site of insertion used to extend the C/D turn is indicated by an arrow. (B) Ferritin subunit backbone shows the four helix bundle (A–D) and the sites of mutation at conserved residues (arrows) near the C/D turn; structures from three different ferritins (frog, horse, human) are shown in three different colors, superimposed to show the high conservation of secondary structure [modified from reference (22); the file was provided by H. Oberer]. Note: The ferritin pore is on the right in the figure. (C) Ferritin subunit backbone in frog L134P crystals showing the disorder around the C/D turn at the pore of the assembled ferritin. (D) Unfolding of L134P displayed in the assembled protein at the pore where 3 subunits interact; all other regions of the protein were folded as in (B) in a protein which assembled normally (16). Arrows show sites of mutation that were studied here. The C and D helix ends and the C/D turn are shown as broad “ribbons” and the ordered regions as canonical polypeptide backbone as in (B) and in ref 16.

to minimize the binding of adventitious iron during purification. The heat stabilities of the recombinant proteins were comparable to wild type, since the yields were similar and purification involved heating protein extracts to coagulate unrelated proteins. Wild-type and altered proteins were mineralized to the same degree (480 Fe atoms/assembled

protein) by adding ferrous sulfate as previously described and aging the mineral at least 17 h at 4 °C before use (16).

Both conservative and nonconservative substitutions of hydrophobic residues for leucine 134 enhanced iron release (Figure 2). Moreover, similar results were obtained whether the substitutions were in the C (L110) or D (L134) helices.



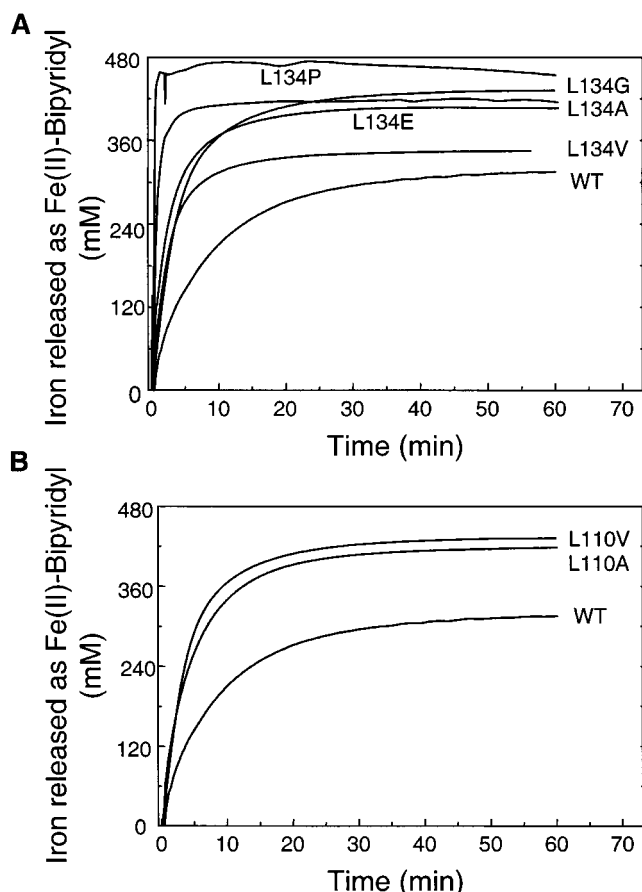


FIGURE 2: Effect of substitutions for hydrophobic residues of helix C and helix D on iron release from ferritin. Recombinant frog H apoferritin ferritins (2.08  $\mu$ M) were mineralized by mixing with aqueous solutions of ferrous sulfate in 0.001 N HCl, at a ratio of 480 iron atoms/ferritin molecule in 0.1 M MOPS (pH 7), 0.2 M NaCl; mixtures were incubated at room temperature (2 h), then at 4  $^{\circ}$ C overnight. Iron exit was triggered by the addition of 2.5 mM FMN, and 2.5 mM NADH for reduction, with 2.5 mM bipyridyl to chelate Fe(II). Absorbance changes at 522 nm monitored both Fe(II) formation/chelation and exit from the protein–mineral complex as previously described (16). The results presented are representative of 3–5 experiments with 2–3 independent preparations of each protein. (A) Progress curves of iron release from H-WT (wild-type) ferritin and various substitutions at L134. (B) Progress curves of iron release from H-WT ferritin and various substitutions at L110, which is opposite L134 in the assembled molecule. Note that changing the side chains of the C/D hydrophobic core in either the C or the D helix had similar effects.

Initial rates of iron release/chelation were increased 1.5–5-fold compared to wild-type ferritin, and the time required to chelate the entire core was 3–30 times faster than wild type. The similarity of the effects of replacing L134 and L110 emphasizes the importance of intrasubunit, helix–helix interactions on subunit packing at the 3-fold ferritin pore in controlling iron release rates.

The remarkably high sensitivity of iron release rates to any changes in the structure of the 3-fold pore is indicated by the similarity of effects of both conservative and non-conservative amino acid substitutions (Table 1). Sixty percent mineral dissolution occurred within 2–4 min for all the amino acid substitutions, compared to 30 min for the wild type (Table 1), whether the leucine side chain was shortened by one carbon (Leu  $\rightarrow$  Val) or eliminated (Leu  $\rightarrow$  Gly). The multiphase nature of the iron release reaction is well-known [reviewed in (10)]. In the later phase of mineral core

dissolution (from 60 to 80%), properties of iron release from the altered proteins fell into two groups with one having much faster release (2–7 min) than the other (14–24 min), and both faster than the  $\geq 60$  min for the wild type (Table 1). In the second phase of mineral dissolution, when the remaining mineral was small ( $\sim 200$  Fe atoms or  $\sim 16$  Fe/nucleation site), proteins with the more conservative amino acid substitutions (Leu  $\rightarrow$  Ala, Leu  $\rightarrow$  Val) had slower release rates than those with nonconservative substitutions (such as Leu  $\rightarrow$  Gly and Leu  $\rightarrow$  Pro). However, L134E was unexpectedly sluggish in releasing iron from small iron minerals (Table 1), suggesting that the carboxylate side chain might provide a transient iron binding site. Why the range of effects of protein structure was wider at the end of the mineral dissolution is not clear. Possibilities include secondary effects of the protein structure at the pore, which alter mineral or more limited access of reductant chelator to mineral with conservative amino acid substitutions (partially vs fully unfolded pore).

**Effect of Lengthening the C/D Loop in Ferritin (Decreasing C and D Helix Interactions) on Reductive Iron Release and Chelation.** The ferritin subunit is a bundle of four  $\alpha$ -helices (A, B, C, D), a folding motif found in a number of other proteins. However, the CD loop or turn in the four helix bundle is extremely short (five amino acids), making the end of the subunit pinched (22–25), as if to pack more effectively at the junction of three subunits. Lengthening the CD loop will change the orientation of amino acids in the C/D turn, such as D122, which forms an ion pair with R72, and between helices (L134/L110) (Figure 1). To examine the effect of lengthening the CD on triggered iron release, a pentapeptide sequence duplicating the native turn sequence, KVDPH, was added between sequence 124 and 125 (Figure 1C). Recombinant protein, expressed and purified as previously described (16), had yields and heat stabilities comparable to wild type. After mineralizing the proteins in parallel with the wild-type protein (480 Fe/protein), iron release was triggered by reduction with NADH and FMN and measured as the bipyridyl complex.

The effect of increasing the length of the C/D turn was comparable to that of the most nonconservative amino acid substitutions at L134 (Table 1). Since the number of histidine residues was doubled in the protein with the longer C/D turn, the change in the protein function could relate to charge repulsion. But decreasing the pH, which would diminish the ionization of histidine in the C/D loop, actually increased the rate of iron release (Figure 3B). Thus, it is more likely that lengthening the C/D turn by inserting KVDPH changes/decreases the helix D/helix C interactions between hydrophobic amino acids, such as L134/L110.

**Effect of Changing the Conserved Ion Pair R72/D122 between the C/D and B/C Loops in Ferritin on Reductive Iron Release and Chelation.** Ferritin has a number of conserved ion pairs that include R72 and D122 at the N-terminal end of the long BC loop and D122 in the C/D turn (24, 25). The ion pair is near the 3-fold pore in the assembled 24-mer. To examine the importance of the putative salt bridge on iron release, two proteins were created by site-directed mutagenesis to disrupt the ion pair: R72D and a double substitution, R72D/D122R “rescue” protein.

The data show (Figure 4) that disrupting the R72/D122 ion pair by the R72D substitution enhanced iron release, and

Table 1: Sensitivity of Ferritin Mineral Dissolution [Reduction/Fe(II) Chelation] to Substitution of Conserved Amino Acids Near the Junction of Three Subunits, the Ferritin "Pore"

protein <sup>a</sup>	initial rate <sup>b</sup> of Fe exit [(mmol/s) × 10 <sup>3</sup> ] at		mineral dissolution time (min)	
			pH 7.0	
	pH 7.0	pH 6.0	60%	80%
H-WT	0.93 ± 0.10	2.48 ± 0.56	30.35 ± 0.54	≥ 60
C Helix H-L110				
H-L110V	3.48 ± 0.47		3.14 ± 1.01	14.33 ± 1.79
H-L110A	2.63 ± 0.41	5.52 ± 0.49	3.81 ± 0.41	23.02 ± 3.08
D Helix H-L134				
H-L134V	2.52 ± 0.64		3.91 ± 1.63	20.03 ± 8.32
H-L134A	2.93 ± 0.66	5.71 ± 0.76	3.61 ± 0.71	22.47 ± 1.57
H-L134E	3.03 ± 0.31		2.78 ± 1.09	24.06 ± 1.94
H-L134G	3.68 ± 0.39		2.65 ± 0.92	7.94 ± 1.13
L134P	4.01 ± 0.88	6.63 ± 1.02	1.53 ± 0.69	1.78 ± 0.64
Lengthening the C/D Turn (Duplicating Turn Sequence KVDPH)				
insertion of KVDPH	3.78 ± 0.34	5.48 ± 1.01	2.68 ± 0.93	6.57 ± 0.89
Ion Pair from B/C (R72) and C/D (D122) Loops				
R72D	3.09 ± 0.86		1.81 ± 0.55	22.03 ± 2.43
R72D, D122R	4.76 ± 0.92		3.05 ± 0.91	4.69 ± 0.86

<sup>a</sup> Recombinant frog proteins, produced by site-directed mutagenesis, expressed in *E. coli* as apoproteins (≤2 Fe/molecule), were prepared in 0.1 M MOPS, 0.2 M NaCl. Mineral was formed in vitro with ferrous sulfate in 0.001 N HCl added at 480 Fe/assembled protein (see Experimental Procedures). The amino acid substitutions caused no detectable change in the temperature stability of the proteins at and above the temperature of the iron release studies. <sup>b</sup> Reduction and chelation were triggered by adding 2.5 mM each of FMN, NADH, and bipyridyl to solutions of protein with mineral (1.05 μM protein and 0.5 mM Fe). Measurement of the change in absorbance, at 522 nm/s [the absorbance maximum for the Fe(II)–bipyridyl complex], reflected the conversion of ferric mineral to Fe(II) in solution in the bipyridyl complex. An absorbance change of 0.8/s equals 0.5 mM or 100% of the Fe reduced and complexed per second. Note that the significant differences seen in the first phase of the reaction are exaggerated in the later phases; here, differences in pore size, or the fraction of the mineral surface accessible to solvent, may make a larger contribution to the rate. The initial rates were determined by a linear fit of the data points from 0 to 1 min after mixing, which represents the first phase of iron release; the data are averages of 3–5 measurements using 3 independent protein preparations for each amino substitution. The error is presented as the standard deviation. Note that the mineral in ferritin was stable until the reductant was added, and the bipyridyl complex was recoverable in ultrafiltrates which excluded the protein (16). Comparison of wild-type to mutant proteins, each with 8 pores, indicates that in H-L110V or H-L134A the pores are ~3× more "open" than in the wild-type ferritin.

the double substitution, rather than rescuing the altered protein, made the difference even greater. After the addition of reductant, the initial rates of mineral dissolution of the altered proteins were increased 3–5-fold compared to the parent protein. Dissolution of 60% of the mineral was more than 10–20 times faster in the altered protein than for the "rescue" mutant (R72D, D122R). For the slower phase of iron release/chelation, 80% mineral dissolution was 4 times longer in the protein with single amino acid substitution than in the double "rescue" substitution, and 12 times longer in the wild-type protein. Comparison of the ion pair substitutions with the C/D turn insert showed similar changes in reductant-triggered iron release (Figure 4).

Melting studies showed that ferritin with the disrupted or altered ion pair in the proteins was stable in the physiological range of temperatures (and up to 70 °C) (Jin and Theil, unpublished experiments). Gel filtration showed that both altered proteins were assembled normally. However, the alterations at the 3-fold pore increased the retention time slightly but significantly (Pancorbo and Theil, unpublished experiments), suggesting that the disorder in subunit folding at the C/D turn and the 3-fold pore, observed in the L134P crystals (16), caused localized collapse of the regions in the supramolecular structure at the junction of subunits and decreased the effective volume of the protein.

## DISCUSSION

Determining the role of the protein component of ferritin for the controlled formation, maintenance, and dissolution

of the ferric mineral is a problem without precedent in biology. Understanding mechanisms that control calcium phosphate mineral dissolution in the physiology of bone and teeth is analogous to the ferritin problem, but the large number of molecules, cells, and signals required (31) makes the complexity much greater than in ferritin. More recent progress has been made in understanding formation of the ferritin iron mineral than for the dissolution of the mineral. Progress was easier, in part because the protein-catalyzed oxidation of ferrous iron and the transfer of ions through a protein channel, that are the first steps of ferritin mineral formation, have mechanistic analogies to other proteins [reviewed in (1)]. Formation of the solid ferric mineral in ferritin has been envisaged to be largely inorganic (32), dependent on the protein for providing chelation/oxidation/numeral/mineral nucleation surface facing a large cavity. Many types of iron–protein interactions can be defined in ferritin such as binding to the pore, moving through the channels between the pore and the ferroxidase site, translocation from the catalytic site to the nucleation site on the inner cavity, translocation from the mineral, and moving to and through the exit pore. Of these, it is release from the mineral and moving to and through the exit pore which have few, if any, analogies to well-understood biological phenomena.

Evidence for the role of ferritin protein in iron release was obscured until recently (16) for a number of reasons. First, the ferritin multisubunit assembly is very stable and often considered to be rigid, noted by terms such as "protein shell".

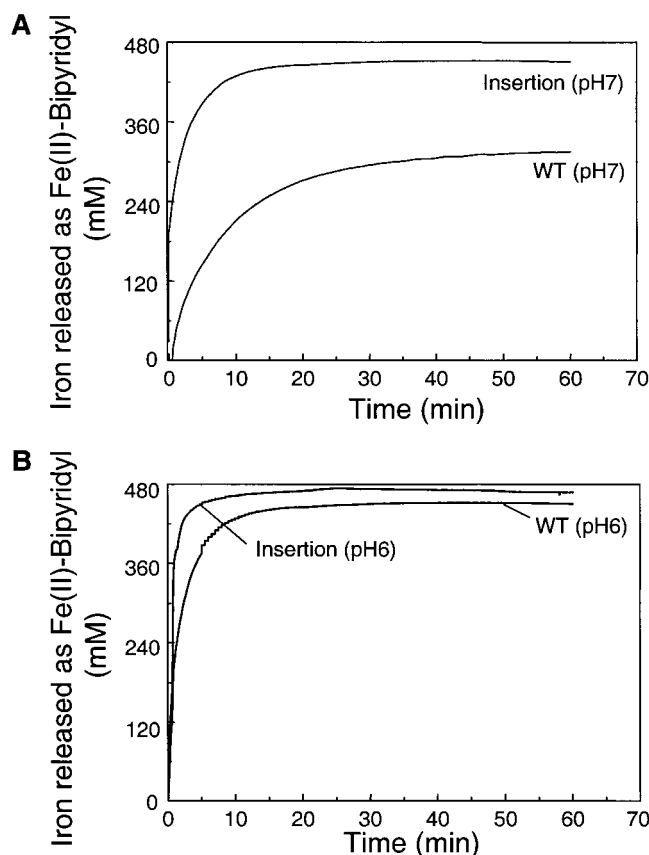


FIGURE 3: Effect of increasing the turn distance between helix C and helix D on iron release. Recombinant frog H apoferritins (2.08  $\mu$ M) were mineralized by mixing with aqueous solutions of ferrous sulfate in 0.001 N HCl at a ratio of 480 iron atoms/ferritin molecule in 0.1 M MOPS (pH 7), 0.2 M NaCl; mixtures were incubated at room temperature (2 h) and then at 4 °C overnight. Iron exit was triggered by the addition of 2.5 mM FMN, and 2.5 mM NADH for reduction, with 2.5 mM bipyridyl to chelate Fe(II). Absorbance changes at 522 nm monitored both Fe(II) formation/chelation and exit from the protein–mineral complex as previously described (16). The results are representative of 3–5 experiments using 3 independent preparations of each ferritin. (A) Progress curves of iron release, at pH 7, from H-WT ferritin and H-ferritin “insertion”, in which 5 amino acids (KVDPH) that duplicate residues 120–125 were inserted into the C/D loop between residues 124 and 125 (Figure 1). (B) The progress curve of iron release at pH 6, from wild-type and “insertion” ferritins. Note that pH increases the rate of Fe(II) exit similarly for both proteins, suggesting that reductant/chelator access to the mineral is the rate-limiting step.

Second, slow chelation rates for iron in ferritin made differences among natural ferritins difficult to detect (16, 30). Third, the high conservation of primary, secondary, tertiary, and quaternary structure among ferritins from diverse organisms suggested a strong relationship between subunit sequence and ferritin protein assembly (1). Finally, cells with excess iron, to the point of toxicity, have degraded ferritin in lysosomes, suggesting that, at least in some cases, the ferritin protein itself had no direct role in iron release but was degraded to release the iron mineral (e.g., 15). Given the extremely complex regulatory system for ferritin production (5–8, 33), protein degradation seems likely to be only one of several mechanisms for releasing iron from the ferritin mineral. In the past, conserved amino acids in ferritin were attributed to requirements for protein folding/assembly or mineral formation. However, new information (16, Figures

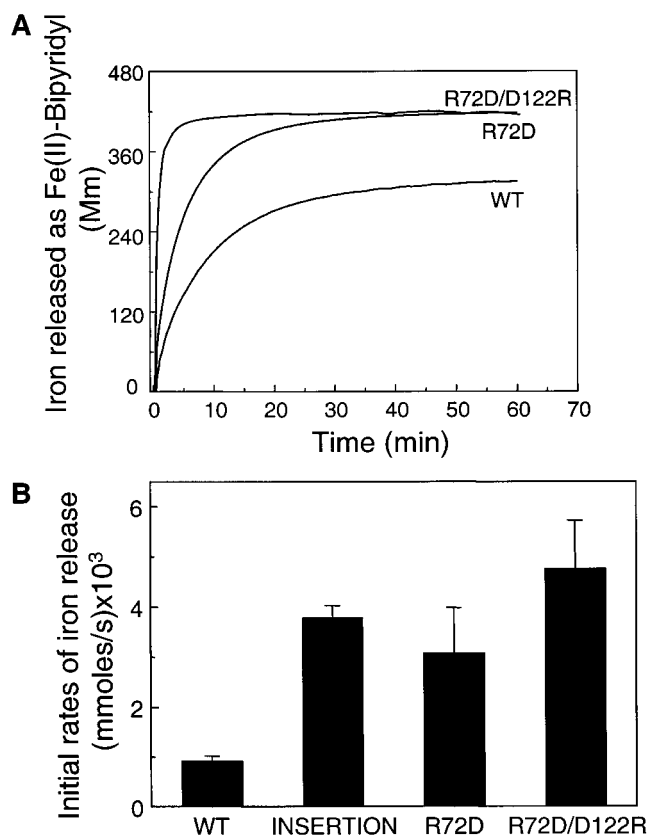


FIGURE 4: Effect of disrupting the ionic interactions between the B/C and C/D loops by substitutions at R72 and D122 on iron release from ferritin. Recombinant frog H apoferritin ferritins (1.05  $\mu$ M) were mineralized by mixing with aqueous solutions of ferrous sulfate in 0.001 N HCl at a ratio of 480 iron atoms/ferritin molecule in 0.1 M MOPS (pH 7), 0.2 M NaCl; mixtures were incubated at room temperature (2 h) and then at 4 °C overnight. Iron exit was triggered by the addition of 2.5 mM FMN, and 2.5 mM NADH for reduction, with 2.5 mM bipyridyl to chelate Fe(II). Absorbance changes at 522 nm monitored both Fe(II) formation and exit from the protein–mineral complex as previously described (16). (A) Progress curves of iron release from the recombinant proteins. (B) Comparison of the effect of two types of changes in the C/D loop. The error, the standard deviation of the mean of the initial rate of Fe, is indicated by a line above each box. Note the similarity of effects either changing ionic interactions or changing the length of the C/D turn on mineral dissolution of the assembled protein.

2–4) now suggests that some of the amino acids around the ferritin pores have been conserved for pore function (opening/closing) rather than global protein folding/assembly or mineral formation.

Ferritin subunits have a short turn between helices C and D (Figure 1B) that is highly conserved. In and around the turns are conserved amino acids for which no previous role had been assigned, other than folding and assembly. Substitutions of leucine by proline originally studied (16) [leucine 134 (aspartic, glycine, alanine) and at leucine 110 (alanine)] had effects comparable to the proline substitution (Table 1), showing the importance of the hydrophobic interactions in iron release. Fe(II) bipyridyl was released as much as 12 times faster in altered proteins compared to wild-type protein, after the addition of reductant and chelator. Particularly striking is the observation that deletion of a single carbon from the leucine side chain (valine substitution) whether at position 110 or at position 134 increased reductive release of iron significantly (Figure 2).



A contribution of the C/D turn to the exit of chelated iron from ferritin was emphasized by the increased rates of reductive iron release observed from ferritin in the protein with an insertion of an extra five amino acids in the C/D turn (Figures 1 and 3). Attempts to shorten the C/D turn (less than five amino acids) failed because no protein could be recovered. Additional evidence for the importance of the C/D loop structure in controlling rates of iron release is the effect of substitutions that change a conserved ion pair, aspartic acid (D122) in the C/D turn and an arginine in the BC loop (R72) (Figures 1 and 4). Replacing the ion pair (D122/R72) with another ion pair (R122/D72) was insufficient to restore wild-type function, indicating that charge repulsion alone did not explain the effect of changing the environment of D122. Further evidence that the critical structural requirement is more than the ionization of amino acid side chains is that the relative effect of changing the pH, which would change the charge on the insertion protein more than on the wild type due to the extra histidine in the insertion, had comparable effects on iron exit for both proteins (Table 1). Thus, three different parameters: inserting additional charged amino acids into the loop, reversing the ion pair in the C/D loop, and decreasing the pH, all indicate that geometry rather than charge in the C/D loop is the dominant factor in the contribution of the ion pair to rates of iron exit from ferritin.

Native ferritin structure at or near the 3-fold junction of subunits depends on multiple, conserved contributions, exemplified by the L134/L110 hydrophobic and R72/D122 ion pairs, and by the sharp C/D turn. Change in any of the conserved features near the pores, small or large, is sufficient to give the maximum effect on triggered dissolution of the mineral (Figures 2–4). [Note that triggered dissolution of mineral, measured in this study as the rate of conversion of ferric iron in the solid mineral inside the protein to ferrous bipyridyl in solution outside the protein, is the result of many reactions. Include are: electron transfer to the mineral, ligand exchange, and phase transition of mineral Fe–O to soluble Fe(II) hydrated or chelated to bipyridyl before, during, or after exit from the protein. Any or all or a combination of the steps could be altered by the amino acid substitutions and the associated unfolding around the pores.] Based on the crystal structure of the L134P protein (Figure 1B) (16), changes in the amino acid sequence open the ferritin pores by disordering the helix packing between residues 110 and 135. In the assembled ferritin molecule of 24 subunits, there will be 8 regions of disordered or collapsed structure distributed symmetrically around the spherical protein (16). Such a change in the spherical ferritin structure likely relates to the decrease in protein volume (increased retention time in gel filtration) observed in the R72D and D122R proteins (Pancorbo, B. M., and Theil, E. C., unpublished results). Similar changes in gel filtration have been reported for recombinant ferritins with amino acid substitutions at other residues around the 3-fold pores (H114, C126, D127, and E130) (19). Iron entry and translocation to the ferroxidase sites ( $\geq 13$  Å), which is thought to include the region of the ferritin pores, are slightly inhibited by the L134P mutation (16), suggesting that the localized unfolding has partially disrupted the channels to the catalytic sites as well as opening the pores.

The structure at the 3-fold pores of assembled ferritin appears to be delicately poised to open and close with few or no effects on the assembly of the rest of the molecule, based on the results in Figures 2–4 and Table 1. A similar concept had been developed for the triggered opening and closing of the syntaxin structure during SNARE assembly that influences intracellular trafficking (34). Thus, regulated unfolding may be a more widespread feature of protein evolution. Inherent in the ferritin supramolecular structure is the potential for localized responses to environmental perturbations for opening the protein pores to increase reaction rates between the iron mineral, reductant, and chelator and to control iron utilization. In addition to future searches for cellular factors that can “open” ferritin, targeting ferritin pores can lead to strategies for more effective chelators to remove iron from ferritin and the body more effectively in iron overload. We have now shown (16, Figures 2–4) that some of the amino acids around the ferritin pores have been conserved for pore function (opening/closing), rather than global protein folding/assembly or metal binding and mineral formation.

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