Ferritin Protein Nanocage Ion Channels
GATING BY N-TERMINAL EXTENSIONS

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Background: Ferritins, cytoplasmic protein nanocages, with internal and cytoplasmic pores terminating trans-cage ion channels, reversibly concentrate iron and scavange oxidants.

Results: Changing ferritin conserved channel residues altered Fe2+ exit, channel flexibility, protein-crowding sensitivity, ion binding, and N-terminal folding.

Conclusion: Eukaryotic ferritin N termini form cytoplasmic gates stabilized by hydrogen bonds and ionic bonds.

Significance: Shared structure and function of ferritin with membrane ion channels includes cytoplasmic, N-terminal gates.

Ferritin protein nanocages, self-assembled from four-α-helix bundle subunits, use Fe2+ and oxygen to synthesize encapsulated ferric oxide minerals. Ferritin minerals are iron concentrators stored for cell growth. Ferritins are also antioxidants, scavenging Fenton chemistry reactants. Channels for iron entry and exit consist of helical hairpin segments surrounding the 3-fold symmetry axes of the ferritin nanocages. We now report structural differences caused by amino acid substitutions in the Fe2+ ion entry and exit channels and at the cytoplasmic pores, from high resolution (1.3–1.8 Å) protein crystal structures of the eukaryotic model ferritin, frog M. Mutations that eliminate conserved ionic or hydrophobic interactions between Arg-72 and Asp-122 and between Leu-110 and Leu-134 increase flexibility in the ion channels, cytoplasmic pores, and/or the N-terminal extensions of the helix bundles. Decreased ion binding in the channels and changes in ordered water are also observed. Protein structural changes coincide with increased Fe2+ exit from dissolved, ferric minerals inside ferritin protein cages; Fe2+ exit from ferritin cages depends on a complex, surface-limited process to reduce and dissolve the ferric mineral. High concentrations of bovine serum albumin or lysozyme (protein crowders) to mimic the cytoplasm restored Fe2+ exit in the variants to wild type. The data suggest that fluctuations in pore structure control gating. The newly identified role of the ferritin subunit N-terminal extensions in gating Fe2+ exit from the cytoplasmic pores strengthens the structural and functional analogies between ferritin ion channels in the water-soluble protein assembly and membrane protein ion channels gated by cytoplasmic N-terminal peptides.

Membrane channel proteins and ferritin nanocages move ions across organic barriers. In ferritins, ferrous ions move through the protein cage to and from the cytoplasm and destinations within the cage (1, 2). After entering the protein cage through the ion channels (Fig. 1) (3, 4), the Fe2+ ions are guided to iron and oxygen oxidoreductase sites that are buried in the four-helix bundle subunits of eukaryotic ferritins. The diferric oxo or hydroxo products of catalytic coupling navigate through the protein cage, forming mineral nuclei (5). Nanomineral nuclei grow in the large, central ferritin cavity that is 60% of the cage volume. Only a few of the thousands of iron atoms in ferritin are bound directly to the protein itself at any one time. Thousands of other iron atoms are in a solid, Fe2O3·H2O mineral sequestered in the capacious central cavity. Iron is recovered from ferritin in vivo in response to signals of iron requirements for cell division, repair of oxidative damage, or specialized functions, such as hemoglobin or nitrogenase synthesis. The phase transitions that form the solid, ferric oxide mineral inside soluble, ferritin protein cages and the regulated reduction and dissolution of the caged ferric oxide mineral that provides iron ions for cytoplasmic metabolism are not well understood. Ferritin is central to biology, however. Illustrations of the importance of ferritins include presence in all forms of life, lethality of gene deletion in mouse embryos (6), coordinated regulation of DNA with other antioxidant response proteins (1, 7, 8), and, in animals, iron-regulated noncoding mRNA structures that coordinate the synthesis of iron trafficking and storing proteins (9, 10).

Flexible pores (11, 12) at the ferritin cytoplasmic surface and at the entry to the ferritin mineral growth cavity, connected by

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§ The atomic coordinates and structure factors (codes 3SE1, 3SH6, and 3SHX) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Fe^{2+} entry and exit channels, separate the reducing environment of the cell and the caged, ferric mineral (Fig. 1). Fe^{2+} entry and exit channels in eukaryotic ferritins are ~15 Å long (3, 5). They are composed of helices and loops from three subunits juxtaposed around the 3-fold axes of ferritin cage structures (Fig. 1). There are eight such channels in 24-subunit ferritins and four in 12-subunit miniferritins (also called Dps proteins). Ion channels in membranes, by comparison, range in length from 10 to 90 Å. The quaternary structure and function of ferritins are remarkably conserved, despite variations in sequence (up to 80%), in the size of the subunit N- and C-terminal extensions and in oxidoreductase mechanisms (1, 2, 13).

To minimize leaking of Fe^{2+} from reactions of the ferric mineral with cytoplasmic reductants, ferritin protein pore gates are largely closed until iron is required by the cells. The cytoplasmic signals and carrier proteins for Fe^{2+} release from ferritin minerals are not known, but physiological “gating” of ferritin is observed in vivo. An illustration is the recovery of iron from tissue ferritin to synthesize new red blood cells after hemorrhage (14, 15). In solution, opening and closing ferritin pore gates are observed as changes in the reduction and dissolution of ferritin minerals and Fe^{2+} exit from the protein cage; Fe^{2+} exit is triggered by natural reductants NADH plus FMN (16). Chaotropes, low heat, substitution of specific conserved, channel amino acids, and tight binding peptides, selected from combinatorial libraries, all alter ferritin mineral reduction and dissolution and Fe^{2+} exit (12, 17, 18). Selectivity of ferritin channel residue function is indicated by the absence of an effect of M-E130A and M-D127A channel residue substitutions on Fe^{2+} exit but large (>97%) losses of oxidoreductase activity (4). In contrast, the H-R72D substitution causes large effects on Fe^{2+} exit with no detectable effect on oxidoreductase activity (18).

To identify the cytoplasmic gates that control mineral dissolution and Fe^{2+} exit from ferritin, we compared the effects in high resolution protein crystal structures of amino acid substitutions close to the cytoplasmic pores (M-R72D, and M-D122R) deep inside the Fe^{2+} iron entry and exit channels (M-L134P). A previous, lower resolution protein crystal structure of frog H-L134P ferritin (11) indicated extensive helix unfolding in the ion channels. However, despite the 85% sequence identity between the H and M ferritin subunits, wild-type H ferritin from frogs is difficult to express. Suitable crystals for high resolution x-ray diffraction have never been obtained, and there is much less mechanistic information than for frog M (1).

Our results reveal the structural and functional importance of the interaction network between the subunit bundle, N-terminal extension residues Gln-6 and Asn-7, Arg-72 (helix 2), and Asp-122 (helix 3–4 loop) for gating Fe^{2+} exit. Studies of M-R72D, M-D122R, and M-L134P, each in different parts of ferritin ion channels, showed distinct differences in localized channel flexibility (crystallographic B’ values), channel metal ion binding, and Fe^{2+} exit. Protein crowders restored normal Fe^{2+} exit in the variants. The significance of identifying the N-terminal extensions of the subunits as the Fe^{2+} exit gates on the cytoplasmic surface of ferritin ion channels is 2-fold. First, the gates are potential sites for regulating iron release from ferritin. Second, our results emphasize mechanistic similarities between ion channels in the water-soluble ferritin cage and N-terminal-gated ion channels in membranes (19–22).

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—The frog M ferritin Fe^{2+} ion channel mutants, M-R72D, M-D122R, and M-L134P, were generated by the QuikChange method (Stratagene). The template was a pET-3a vector encoding wild-type frog M ferritin (12). The sequences of all expression vectors were confirmed by DNA sequencing. Recombinant proteins were expressed in Escherichia coli BL21(DE3) pLysS (Stratagene) (12). For R72D and D122R, betaine and sorbitol were used in the culture medium to minimize incorrect folding and to increase yields (23). Expressed proteins were purified as described (12, 24). The final buffer for the proteins was 100 mM MOPS (pH 7.0) and 100 mM NaCl. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay kit) with bovine serum albumin as a standard.

**Iron Release**—Recombinant ferritins, isolated as the self-assembled cages with <4 iron per cage, were mineralized by adding solutions of freshly prepared 50 mM FeSO_{4} in 1 mM HCl to the buffered protein solutions. The final protein concentrations were 1.0 μg per protein cages and 480 μM Fe, except in the protein crowding experiments, where the protein cages were 2.08 μM, and iron was 1.0 mM. After mixing, the solutions were incubated for 2 h at room temperature and then overnight at 4 °C to complete the iron mineralization reaction (11).

Fe^{2+} exit from caged ferritin minerals was initiated by reducing the ferritin mineral with added NADH and FMN and trapping the reduced and dissolved Fe^{2+} as the pink Fe^{2+}-(2,2'-bipyridyl)_{3} complex outside the protein cage (16). The intermediate steps in ferric reduction on the mineral surface and Fe^{2+} transit from the mineral surface through the ion channels to the outside of the cage are not characterized currently. Fe^{2+} exit from the protein cage was measured as the absorbance of Fe^{2+}-(2,2'-bipyridyl)_{3} at the λ_{max} of A_{522 nm} as described previously (11). Briefly, to 500 μl of mineralized ferritin, 25 °C, 500 μl of a mixture of 2.5 mM FMN, 2.5 mM NADH, and 2.5 mM bipyridyl, all in the final protein buffer, was added to the protein solution. Initial rates, which can only be computed for the linear phase that accounts for only 7% of the iron in ferritin, used the extinction coefficient at 522 nm of 8,340 M⁻¹ cm⁻¹ for the Fe^{2+}-(2,2'-bipyridyl)_{3} complex. The time (min) required for 25 and 50% of the mineralized ferric iron (t_{25} and t_{50}) to be dissolved and exit the cage as Fe^{2+} was also determined. Data analyses used Igor Pro software, version 5.0.5.A (WaveMetrics). Averaged data were from 5–8 independent experiments, using at least two different preparations of each protein. Errors are the S.D. Statistical analyses used Student’s t test.

**Protein Crystallization**—Crystals of wild-type M ferritin and the modified ferritins (M-R72D, M-D122R, and M-L134P) were obtained by mixing 2 μl of protein solution (5–20 mg/ml) with an equal volume of the precipitant solution, 2.0 M MgCl_{2} and 100 mM Bicine⁶ (pH 9.0) (3). Cubic crystals formed within

⁶The abbreviations used are: Bicine, N,N-bis(2-hydroxyethyl)glycine; PDB, Protein Data Bank.
Ferritin Ion Channels and Pore Gates Control Fe\(^{2+}\) Exit

### TABLE 1

| Data collection          | Frog M ferritin variant | R72D       | D122R       | L134P       |
|--------------------------|-------------------------|------------|-------------|-------------|
| Wavelength (Å)           | 1.11587                 | 1.11587    | 1.11587     |             |
| Space group              | F432                    | F432       | F432        |             |
| Unit cell edge (a - b - c) (Å) | 183.560                | 183.434    | 183.775     |             |
| Resolution range (Å)     | 1.65-106.00             | 1.45-91.67 | 1.35-64.96  |             |
| No. of unique reflections| 20.53                   | 17.3       | 13.1        |             |
| Completeness (%)         | 100 (100)               | 100 (100)  | 100 (100)   |             |
| Multiplicity             | 0.102 (1.187)           | 0.088 (1.006) | 0.071 (0.807) |             |
| I/σ                      | 22.6 (2.2)              | 20.4 (2.2) | 19.5 (2.3)  |             |

Refinement parameters:
- $R$: 0.165
- $R_{\text{free}}$: 0.195
- Root mean square deviation from ideal values:
  - Bond lengths (Å): 0.034
  - Bond angles (degrees): 2.484
  - No. of atoms: 271
  - Mg(H\(_2\))\(O\): 8
  - Cl\(^-\): 9
- Average B values (Å\(^2\))\(^b\): 20.4

*\(^a\) Values in parentheses are for the highest resolution shell.
*\(^b\) For all protein atoms. The average B value for wild-type protein (PDB code 3KA3) is 14.6 Å\(^2\).

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1–2 days at 4 °C. The crystals were transferred to the precipitant solution containing 20% (v/v) ethylene glycol as a cryoprotectant for at least 5 min before flash-freezing in liquid nitrogen.

X-ray Data Collection and Structure Refinement—X-ray diffraction data were collected at 11,111 eV on Beamline 8.3.1 at the Advanced Light Source at the Lawrence Berkeley National Laboratory. The crystals were maintained at 100 K during data collection. Data were processed using the programs ELVES, MOSFLM, and SCALA (25–27). Data collection statistics are summarized in Table 1.

Initial models were obtained by molecular replacement with EPMR (28). The search model was a single subunit of wild-type frog M ferritin (PDB code 1MFR) (29), excluding Mg\(^{2+}\) ions and water molecules. The structures were refined using the program REFMAC5 (30). Structural models were visualized and manually modified based on the 2m$F_o$ - m$F_c$ and m$F_o$ - D$F_c$ electron density maps by using the program COOT (31, 32). Water molecules were positioned initially using ARP/wARP (33). Positions of Mg\(^{2+}\) ions were deduced from relatively strong electron density and octahedral coordination. Atomic temperature factors were refined isotropically for R72D ferritin and anisotropically for the D122R and L134P variants.

Model quality was evaluated using the WHAT IF web server (34). Structure figures were prepared using PyMOL (35).

The degree of protein flexibility was assessed using the B values of the Ca atoms. Because B values are influenced by parameters that differ between structures, the B values were normalized for each model. The normalized B value (B’ value) of each Ca atom was calculated using the equation, B’ = (B - B\(\langle B\rangle\))/σ(B), where (B) and σ(B) are the mean and the S.D. of the B values for the Ca atoms of the protein, respectively (36).

### RESULTS

Amino Acid Substitution Selectively Alters Localized Channel Flexibility—Ferritin protein cages, which are 85% α-helical, are unusually stable, resisting 6 m urea or temperatures of 80 °C. Thus, the localized unfolding of regions of the protein cages, when first observed, was unexpected (11, 12, 39). The effects of localized unfolding of ferritin protein on Fe\(^{2+}\) exit identified the channels around the 3-fold axes as the sites of Fe\(^{2+}\) exit. In wild-type ferritin, the exit pores are unusually sensitive to small changes in the aqueous solvent, such as physiological urea concentrations (1 mM), and temperatures far below the protein cage $T_m$ (12).

To obtain structural information on the effects of changing channel amino acids that regulate Fe\(^{2+}\) exit, we used the frog M ferritin model for several reasons. First, we obtained high resolution (1.3–1.7 Å) protein crystal structures not available for frog H ferritins. Second, mechanistic information of oxidative and mineral growth for frog M is much more extensive than for frog H or human H ferritin, in part because of the spectroscopically favorable kinetics of the different peroxo intermediate (1). Finally, the amino acid substitutions made in the frog M protein are at residues conserved in eukaryotic ferritins and in many bacterial ferritins.

Comparisons of the crystal structures of wild-type frog M ferritin and the M-R72D, M-D122R, and M-L134P variants reveal that localized structural changes are associated with each of the amino acid substitutions (Fig. 2). In addition, we calculated the normalized B values (B’ values) of the models to correct for the effects of differences in crystallographic resolution (36) and to assess changes in main-chain flexibility. As judged by increases in the normalized B values of the Ca atoms, the M-D122R and M-L134P substitutions increased the disorder of the main chain around the 3-fold pore, including the ion channel residues 110–134 (Fig. 2E).

M-D122R is a substitution of the aspartate residue in the short loop that links helices 3 and 4 of each of the three subunits that compose a single ferritin Fe\(^{2+}\) channel (Fig. 1). When the ion pair between Asp-122 and Arg-72 is disrupted in M-D122R, the atomic coordinates for the ion channel and N terminus are almost identical to that of wild type. However, the electron density in and around the channel (residues 102–138) is lower than in wild-type or in M-L134P. B’ values for the backbone Ca atoms in the M-D122R structure are higher throughout the entire channel, including the N terminus (residues 5–13) and around the entrances to the external pores (Fig. 2, C and E).

The M-R72D substitution disrupts the conserved ion pair between Arg-72, near the end of helix 2, and Asp-122 in the helix 3–4 loop (Fig. 1B). The B’ values throughout the four-helix bundle of the M-R72D model are similar to those in the wild-type ferritin structure (Fig. 2B). Mg\(^{2+}\) from the crystallization solution (2.0 M MgCl\(_2\)) mediates an interaction between Asp-72 and Asp-122 (supplemental Fig. S1) and probably compensates for the negative charge of the carboxylate group introduced at Asp-72. However, the N-terminal residues of M-R72D are extensively disordered, unique among the protein struc-
Disorder in N-terminal Extensions of Ferritin Subunit Helix Bundles in M-R72D Ferritin Increases Fe\(^{2+}\) Exit and Identifies Cytoplasmic Gates—Fe\(^{2+}\) exit from ferritins is the result of a complex, surface-limited process where electrons originating from reduced flavin in solution outside the protein cage convert Fe\(^{3+}\) ions on the surface of the protein-caged mineral to Fe\(^{2+}\). Reduction is followed by hydration of the oxo bridges between each iron atom in the mineral and migration of dissolved Fe\(^{2+}\) from the mineral surface to and through the ion channels to the outside of the protein cage. Where and how the electrons are transferred from the flavin to the ferric ions in the mineral, what is the transit path of the Fe\(^{2+}\) ions to the inner pore of the ion channels, and where the Fe\(^{2+}\) reacts with chelators, such as bipyridyl, are unknown. Although important questions, they have been experimentally intractable to date. However, monitoring formation of the Fe\(^{2+}\)-bipyridyl complex outside the ferritin protein cage (11, 12, 39) has been a useful way to understand the role of the protein cage in regulating the stability of the ferric mineral and preventing the leakage of Fe\(^{2+}\) from ferritin minerals that could occur from reactions with cytoplasmic reductants (1, 2).

When the N-terminal sequence is disordered by loss of interactions with the conserved Leu-110 in helix 3 (18), the M-L134P substitution in helix 4 disrupts the interactions of the M-L134P variant compared with wild-type M (Fig. 2, D and E). Similarly, the 3-fold pore residues 114–133 are extensively disordered in the L134P mutant of frog H ferritin, which also shows disorder in the N-terminal extension residues 1–12 (Fig. 2E).

The M-L134P substitution in helix 4 disrupts the interactions with the conserved Leu-110 in helix 3 (18). Main-chain fluctuations increase in ion channel residues 125–135 of the M-L134P variant compared with wild-type M (Fig. 2, D and E). Similarly, the 3-fold pore residues 114–133 are extensively disordered in the L134P mutant of frog H ferritin, which also shows disorder in the N-terminal extension residues 1–12 (Fig. 2E).
Ferritin Ion Channels and Pore Gates Control Fe\textsuperscript{2+} Exit

A. Wild-type M  
B. M-R72D  
C. M-D122R  
D. M-L134P  

F: Ferritin iron entry/exit channel residues

\begin{verbatim}
Frog M  70-QNKRG-75---110-LDDLHKLATDKVDPHLCDFLESEYLL-134  
Frog H  70-QNKRG-75---110-LDDLHKVGSDKVDPHLCDFLETEYLL-134
\end{verbatim}

FIGURE 2. Changing ion channel residues selectively affects main-chain flexibility. A–D, view from the internal surface of ferritin protein cages around the ion channel exits. The same sections of three helices form the ion channels around the 3-fold symmetry axes of the cage. The colors indicate the distribution of normalized B value (B' value) for Cα atoms: white, B' < 0.5; green, 0.5 < B' < 1.0; yellow, 1.0 < B' < 1.5; magenta, 1.5 < B' < 2.0, red, 2.0 < B'. A, wild-type frog M (PDB code 3KA3); B, frog M-L134P (PDB 3SHX); C, frog M-D122R (PDB code 3SH6); D, frog M-R72D (PDB code 3SE1). E, B' values of the Cα atoms for frog M ferritins and frog H-L134P (PDB code 1BG7) from (11); wild-type H and M ferritin isoforms share 85% sequence identity. F, amino acid sequence comparisons.

Changes in External Environment Can Rescue Ferritins with Altered Ion Channels or Pore Gates—The sensitivity of Fe\textsuperscript{2+} exit in wild-type ferritin proteins to small molecules added to the buffer (e.g. physiological urea (1–10 mM) (12, 17, 18) or binding peptides (12, 17, 18)) suggested that ferritin pores and channels might be sensitive to large macromolecules in solution that mimic cytoplasmic crowding. Two globular proteins with opposite overall charge, bovine serum albumin and egg white lysozyme, are frequently used at high concentrations to create protein crowding (37, 38). Could they decrease ion channel fluctuations in ferritin (Fig. 2)?

Fe\textsuperscript{2+} exit was monitored for solutions of ferritin M-R72D, M-D122R, and M-L134P and wild type after incubation of the mineralized ferritins with 3.8 mM BSA (250 mg/ml) or 8.7 mM lysozyme (125 mg/ml). The excluded volumes, calculated using the radii in solution (40), were 1.6 or 0.33 \times 10^{-25} m^3/protein molecule, respectively, which increases the solvent excluded by 37% (BSA) or 17% (lysozyme).

Wild-type M ferritin was relatively resistant to the high concentrations of BSA or lysozyme (Fig. 5). In contrast, Fe\textsuperscript{2+} exit from the variant ferritins, M-D122R, M-R72D, and M-L134P, was returned to wild-type values (Fig. 5) by protein crowding. To eliminate the possibility that NADH or FMN binding to BSA decreased available reductant, we determined the effect of doubling the reductant concentration. If NADH or FMN had become limiting in the presence of BSA, adding excess reduc-
tant should increase Fe\(^{2+}\) exit. However, when the concentration of NADH and FMN was doubled, Fe\(^{2+}\) exit from variant M-R72D was still like wild-type protein (supplemental Fig. S2). Lysozyme, used at only 125 mg/ml because of solubility, had a smaller effect on the variant proteins than BSA at 250 mg/ml. (Fig. 5). The differences could be due to difference in viscosity because the viscosity of the lysozyme solution was about one-third that of BSA solutions, or the smaller effect of lysozyme could relate to the smaller amount of solvent excluded: 17% for lysozyme compared with 37% for BSA. Finally, the smaller effect of lysozyme could be due to specific BSA or lysozyme interactions with ferritin or to a combination of specific binding effects, viscosity, and excluded solvent. When the selective effects of heptapeptides (17) or stoichiometric concentrations of urea (12) on ferritin pores are considered with the quantitatively different effects of lysozyme and BSA, the existence of specific cytoplasmic regulators or chaperones that regulate ferritin Fe\(^{2+}\) exit is tenable.

**Mutations Alter Metal Ion Binding in Ferritin Ion Channels**—The highest resolution crystals in a screen with multiple conditions were grown in the presence of 2 M MgCl\(_2\) (3). Three hydrated Mg\(^{2+}\) ions are aligned in the crystals in a row inside wild-type ferritin ion channels (Fig. 6A). Mg\(^{2+}\) is also a convenient divalent cation model for air-sensitive Fe\(^{2+}\) ions (1). Mg\(^{2+}\) binds at a number of ferritin sites known to be Fe\(^{2+}\) sites, but the affinity is relatively low because Mg\(^{2+}\) is displaced by Co\(^{2+}\) and does not inhibit Fe\(^{2+}\) and O\(_2\) reactions (3, 42).

The Mg\(^{2+}\) ions in the ferritin channels span the distance from the cytoplasmic pore to the channel exits into the central cavity. This arrangement of Mg\(^{2+}\) ions in ferritin ion channels (3) is reminiscent of K\(^{+}\) ions in a voltage-dependent K\(^{+}\) channel crystallized at high [K\(^{+}\)] (41). However, K\(^{+}\) channels differ in containing only protein loops contrasting with ferritin channel helices and loops, by using backbone carbonyls to bind metal ions, contrasting with ferritin side-chain carboxylates, and in being anhydrous, contrasting with ordered water in ferritin. One of the Mg\(^{2+}\) ions is bound by a cluster of three Glu-130 residues at the channel constriction (Fig. 1C). Glu-130 is required for diferric peroxo formation during normal catalysis (4). Three Mg\(^{2+}\) ions cluster at the ferritin ion channel exits linked through coordinated H\(_2\)O to three conserved Asp-127 residues. Asp-127 is also required for normal diferric peroxo formation.


**Ferritin Ion Channels and Pore Gates Control Fe^{2+} Exit**

![Image](image_url)

**FIGURE 4.** Effects of changing ferritin ion exit and entry channel residues on Fe^{2+} exit from eukaryotic ferritin protein cages. Progress curves for the surface-limited reduction and dissolution of protein-caged ferric minerals and Fe^{3+} exit from the protein cages of wild-type M-R72D, M-R122D, and M-L134P ferritins. The Fe^{3+} release reaction was initiated by mixing solutions of mineralized ferritin with a solution of 2.5 mM FMN, NADH, and bipyridyl and monitoring Fe^{3+}-(bipyridyl) outside the protein cages. (Numerical rates are shown in [supplemental Table S2](#)). Data are representative of two independent protein preparations, with 3–4 repetitions for each. Black, M-WT; green, M-L134P; red, M-R72D; blue, M-D122R.

**TABLE 2** Ion channel residues that influence ferritin ion mineral dissolution and Fe^{2+} exit

| Protein     | \( \frac{V^2}{r} \) (7% of mineral) | Time of 25% mineral dissociation | Time of 50% mineral dissociation |
|-------------|------------------------------------|---------------------------------|--------------------------------|
| Wild type   | 78 ± 8.2                          | 43.0 ± 7.1                      | >60.0                      |
| R72D        | 77 ± 7.8                          | 7.4 ± 1.0                      | 17 ± 3.5                  |
| L134P       | 75 ± 7.3                          | 7.0 ± 1.2                      | 19 ± 3.8                  |
| D122R       | 63 ± 6.6                          | 5.8 ± 0.8                      | 12 ± 2.4                  |

* Initial rates of Fe^{2+} exit can only be computed for the first 30 s, when the progress curve is linear; only 7% of the Fe^{2+} ions exit during this period. Changing ferritin cage ion channel structure has no effect on the initial rate; such iron probably represents iron bound in the protein cage rather than dissolved from the bulk mineral. Mineral dissolution, a surface-limited process, and Fe^{2+} exit continue for minutes to hours. Note that substitutions at other conserved ion channel residues, exemplified by Glu-130 and Asp-127, have no effect on Fe^{2+} exit (4).

* Significantly slower \( (p < 0.01) \) than any of the ferritins with substitutions at conserved ion channel residues.

* Fe^{2+} exit is significantly faster in M-D122R at 60 min than for M-R72D \( (p < 0.05) \) and M-L134P.

formulation, but D127A can be partly rescued with increased [Fe^{2+}] \( (4) \). The Mg^{2+} sites in the ion channels are fully occupied in the crystals of wild-type M ferritin (3), ([supplemental Table S1](#)).

The number of Mg^{2+} binding sites and the site occupancies decrease in the ferritin variants compared with wild-type ferritin (Fig. 6 and [supplemental Table S1](#)). Effects are smallest in M-R72D followed by M-L134P and M-D122R. The R72D substitution, which destabilizes the cytoplasmic N-terminal peptide gates (Figs. 2 and 3), results in replacement of Mg^{2+} at Thr-118 (site A, [supplemental Table S1](#)) by Mg^{2+} at Asp-72. There is no metal ion binding at channel site C, Asp-127, Glu-130, and Ser-131 (Fig. 6 and [supplemental Fig. S1](#)). The differences in metal binding may reflect changes in electrostatics (43) caused by the negatively charged Asp in place of positively charged Arg at position 72.

Disrupting ion channel structure by the M-L134P or M-D122R substitutions (Fig. 2) decreases metal occupancies in the channel more than in the M-R72D protein. In M-L134P, site occupancy decreased at two of three Mg^{2+} positions in the ion channel and eliminated the cluster of metal ion binding sites around the three Asp-127 residues in the ion channel exits. When refined with full occupancy, the two sites in L134P resulted in excess electron difference density. Maps calculated with occupancy manually set to 50% no longer showed excess electron density and gave reasonable \( B \) values; occupancy refinement is not reliable at this resolution due to correlation between \( B \) values. Mg^{2+} binding at the channel constriction was unchanged in M-L134P or M-D122R. Ordered water in the channels decreased in both M-L134P and M-D122R, especially at the cytoplasmic pores (Fig. 6). The largest effect on ion metal binding in the ferritin ion channels occurs in the M-D122R variant. Changes in \( B \) values occur at both the N terminus and the ion channel (Fig. 2E). Mg^{2+} only binds at one site in the channel, the channel constriction (Fig. 6D). The largest effects of the amino acid substitutions that change main-chain structure are on Fe^{2+} exit (Fig. 4) in contrast to ferritins with substitutions at channel residues Glu-130 and Asp-127, which alter Fe^{2+} entry and oxidoreduction with no effect on Fe^{2+} exit (4).

**DISCUSSION**

Ion entry and exit channels in ferritin protein cages share structural and functional features with membrane ion channels: the use of charged residues on surfaces of protein helices to move metal ions from one environment to another across a largely hydrophobic barrier. The lines of metal ions in the channels detected in protein crystals of both a K^+ channel (41) and
ferritin protein cages (3) (Fig. 6A) are a visual demonstration of the similarities between ion channels in soluble ferritin protein cages and ion channels in membrane proteins. In integral membrane ion channels, the cell membrane creates the barrier to ion flow. In ferritin, the protein cage itself separates cytoplasmic iron and iron in the mineral growth cavity. The complexity of the ferritin protein cage, which was long thought to be just a “shell” around the mineral, continues to emerge (1, 2, 5, 13) as does the complexity of membranes and membrane channels (44–46). Three main conclusions from the ferritin structure and function studies reported here are as follows: 1) the N-terminal segment is a critical part of the cytoplasmic gate for Fe

2+
 exit; 2) flexible ion channel regions are stabilized by specific interactions and by external crowding effects of proteins like BSA; and 3) decreased binding of metal ions and increased flexibility at specific channel sites increases reduction and dissolution of the caged ferritin mineral, a surface-limited process, and Fe

2+
 exit from the protein cage.

The N termini in eukaryotic ferritins protrude from the four-α-helix subunit bundles and interact with residues around the ion channel cytoplasmic pores (3, 47, 48) (Figs. 2 and 4). Proximity of the N termini to the cytoplasmic pores was observed in ferritin protein structures as long ago as 1978 (49), but the functional significance remained unknown until now. The correlation of N-terminal structural changes in the M-R72D variant with increased Fe

2+
 exit makes clear the gating function of the N-terminal segment. Conserved residues Gln-6 and Asn-7 form hydrogen bonds to Arg-72 in helix 2 (Fig. 3). Arg-72 in turn is linked through a salt bridge to Asp-122 in the loop between helices 3 and 4, around the pores. This network of interactions stabilizes the channel and pores in a closed position. Disruption of the network unfolds the pore gates (the N-terminal subunit extension) (Figs. 2 and 3), and Fe

2+
 exit increases (Fig. 4).

The network residues Gln-6, Asn-7, Arg-72, and Asp-122 are present in all eukaryotic ferritins. They are absent in the 12 subunit miniferritins (Dps proteins) of archaea and bacteria, where the N-terminal subunit extensions vary widely in sequence and in some cases form DNA binding sites (13). The same residues control both Fe

2+
 entry and Fe

2+
 exit in the smaller ferritins (13). Miniferritins are selectively regulated by stress and expressed under different physiological conditions than 24-subunit ferritins present in the same bacteria (2, 50). H

2
O

2
 is often the preferred oxidant in miniferritin mineral synthesis. The multipurpose activity of eukaryotic ferritins, both in cellular iron nutrition and recovery from oxidant damage, requires responses to multiple signals, which may explain the complex ion channel and pore structures.

Ion channel structure, channel metal ion binding, and Fe

2+
 exit are differentially changed by each of the amino acid substitutions that we characterized here. Because the channels and pores are composed of three subunits, each substitution changes three amino acids in close proximity. Asp-122 in the short loop between helices 3 and 4 plays a major role in directing Fe

2+
 out of the protein cage. Not only does Asp-122 minimize fluctuations of the cytoplasmic gate, indicated by decreased Fe

2+
 exit (Fig. 4); it increases metal ion binding in the channel (Fig. 6). M-D122R is catalytically active despite the altered metal binding in the channel. Mg

2+
 occupancy at the channel constriction (site B, supplemental Table S1) is unchanged by the M-D122R substitution (Fig. 6 and supplemental Table S1).

The hydrophobic properties of Leu-134, deep inside the Fe

2+
 ions, also influence metal ion binding. Without Leu-134, no metal ions bind at the interior channel exits around the three Asp-127 residues. In contrast, three metal ions bind at this site in wild type in crystals of the wild-type protein (Fig. 6). The M-L134P substitution also decreases the occupancies of sites A and C (supplemental Table S1). Substitution of the master residue, Arg-72, in the cytoplasmic gate stabilizing network (Fig. 2) has relatively small effects on metal ion distribution in the channel. The different contributions of Arg-72, Asp-122, and Leu-134 to ferritin ion channel function are reflected in the difference among ion channel structural fluctuations, metal ion binding, and Fe

2+
 exit. The ability of high concentrations of external proteins in solutions to restore normal channel Fe

2+
 exit in variant M ferritins emphasizes the sensitivity of ferritin ion channels to the external environment.

The increase in Fe

2+
 exit when metal occupancy in the channel decreases illustrates the complexity of the production and exit of Fe

2+
 from ferritin. If Fe

2+
 exit simply depended on elec-
Ferritin Ion Channels and Pore Gates Control Fe\textsuperscript{2+} Exit

trostatics and ion binding in the channels, decreased ion binding in M-L134P, for example, would not increase Fe\textsuperscript{2+} exit. However, Fe\textsuperscript{2+} exit also depends on electrons flowing from the external reductant to the protein-caged mineral. Changes in channel structure that facilitate electron flow and mineral reduction will increase Fe\textsuperscript{2+} exit. Such changes could involve direct contact of reductant with the mineral or enhanced electron transfer. As a result of changed channel structure, hydrophobic or positively charged amino acids can influence Fe\textsuperscript{2+} exit even if metal ion binding in the channel decreases. Contrasting with the complex channels in eukaryotic ferritins, substitution of channel carboxylate residues in miniferritins affects both Fe\textsuperscript{3+} entry and Fe\textsuperscript{2+} exit, suggesting that in these smaller ferritins, channel electrostatics dominate control of both Fe\textsuperscript{2+} entry and exit (13).

Ferritin expression in animals, where there are no separate H\textsubscript{2}O\textsubscript{2}-consuming miniferritin genes known, is part of a metabolic feedback loop. The ferritin substrates, iron and oxidants, activate ferritin gene expression, both DNA and mRNA, to increase ferritin protein synthesis. Ferritin protein, by consuming iron and oxidant to synthesize iron mineral, shuts down the feedback loop (51). The complex network of intracage protein–protein interactions in ferritin that stabilize the cytoplasmic pore gates suggests that the gates may be targets for cytoplasmic ligands that control ferritin pore function and iron utilization. The specific features of genetic regulation and structure in eukaryotic ferritins may reflect the relatively long life spans and/or high dioxygen gradients of many animal cells.

Local, structural fluctuations in eukaryotic ferritin iron ion channels, including unfolding of the N-terminal cytoplasmic pore gates observed here in high resolution protein crystal structures, have little impact on overall ferritin cage structure. Complementary solution data on the differential stability of ferritin pores and ion channels and the cage include exquisite channel sensitivity to urea (>6,000 times more sensitive than the cage) and heat (channel \(T_m\) > 28 °C lower than the cage) (12). Even short peptides (e.g. tight binding heptapeptides selected from a large combinatorial library) change Fe\textsuperscript{2+} exit, possibly mimicking cytoplasmic gating ligands (17, 39). The biological ligands that control ferritin cytoplasmic gating and Fe\textsuperscript{2+} exit in vivo, which have eluded identification efforts to date, will have a large impact on understanding iron protein synthesis and on chelation therapies for treating hypertransfusion iron overload. Even now, however, the emerging structural and functional analogies between the cytoplasmic gates and ion channels in membrane channel proteins (19–22) and in water-soluble ferritin protein cage, provide novel opportunities for iron channel exploration.

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