Peptides Selected for the Protein Nanocage Pores Change the Rate of Iron Recovery from the Ferritin Mineral

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Pores regulate access between ferric-oxy biominal inside and reductants/chelators outside the ferritin protein nanocage to control iron demineralization rates. The pore helix/loop/helix motifs that are contributed by three subunits unfold independently of the protein cage, as observed by crystallography, Fe removal rates, and CD spectroscopy. Pore unfolding is induced in wild type ferritin by increased temperature or urea (1–10 mM), a physiological urea range, 0.1 mM guanidine, or mutation of conserved pore amino acids. A peptide selected for ferritin pore binding from a combinatorial, heptapeptide library increased the rate of Fe demineralization 3-fold (p < 0.001), similarly to a mutation that unfolded the pores. Conjugating the peptide to Desferal® (desferrioxamine B mesylate), a chelator in therapeutic use, increased the rates to 8-fold (p < 0.001). A second pore binding peptide had the opposite effect and decreased the rate of Fe demineralization 60% (p < 0.001). The peptides could have pharmacological uses and may model regulators of ferritin demineralization rates in vivo or peptide regulators of gated pores in membranes. The results emphasize that small peptides can exploit the structural plasticity of protein pores to modulate function.

Pores in ferritin protein cages are an example of pores in molecular or ionic barriers; other examples are pores in membranes. In ferritin, pores control reactions between reductants outside the protein and the ferric mineral inside. As with many gated pores, ferritin pores are formed by α-helices in multiple subunits that surround the iron path and are modulated by unfolding (1, 2) (see Fig. 1). Ferritin pores are arranged symmetrically around the protein cage, eight for 24 subunit maxi-ferritins and four for 12 subunit maxi-ferritins, also called Dps proteins (2, 3).

Ferritins concentrate Fe for biological use and also detoxify Fe/O2 or H2O2 in the ferric/oxy biominal inside the proteins of Archaea, bacteria, and Eukaryota, including higher plants and animals. Fe ions destined to enter the mineral appear to reach the ferrooxidase coupling site through the pores for the first step in biominalization (2, 4–7). The critical roles of ferritins are illustrated by lethality of deletions in mice, neurological effects of mutations in humans, and pathogen responses to host-released oxidants (3, 8, 9). In addition, dual genetic regulatory systems with DNA (antioxidant-response elements) enhances linking ferritin regulation to antioxidant response proteins (10, 11) and mRNA “promoters” (iron-response element) linking ferritin regulation to Fe trafficking proteins emphasizes the central role of ferritin in Fe and oxygen metabolism. Finally, the ferritin Fe reactions with O2 or H2O2 (2, 4–7) and the presence of ferritin in anaerobic archaea (12) suggest an ancient role for ferritins in the transition to aerobic metabolism.

The rates of Fe transport from the ferritin mineral through the nanocage channels and pores to chelators on the outside of the nanocage are initiated by external reductant and increase under several conditions. (i) Highly conserved, ferritin pore residues are changed. (ii) The temperature is increased. (iii) Urea is added at physiological concentrations (1–10 mM). The increased rate of Fe2+ exit coincides with localized unfolding of ferritin pore helices (crystallography) and a decrease in the melting transition for 10% of the total α-helix content, TM = 56 °C; TM = 43 °C in 1 mM urea (CD spectroscopy) (2, 13–15). One mM urea is in the physiological range of urea concentrations. In contrast to the pores, global unfolding of the protein nanocage requires high temperatures (>80 °C) or high concentrations of urea (6 M) plus an acidic pH (pH = 4.5). Folding/unfolding of protein subdomains, such as the ferritin pores, occurs in other ion channel proteins, e.g. Refs. 16 and 17.

Removal of Fe from ferritin in vivo occurs during Fe deficiency, during hemoglobin synthesis, or after Fe loss (e.g. hemorrhage) (19–21). In cell cultures, Fe removal from ferritin occurs in the cytoplasm when Fe deficiency is induced by increased expression of the Fe efflux protein, ferroportin (20, 22). During Fe overload, ferritin in lysosomes increases and lysosomal Fe contributes to cytoplasmic Fe traffic (23). In general, the mechanisms of Fe transport from the biominal are less studied than the mechanisms for oxidizing and mineralizing Fe in ferritin (2, 18).

Residues that alter ferritin pore structure and function are Leu-110/Leu-134 and Arg-72/Asp-122 conserved in the Archaea, bacteria, and Eukaryota (13–15). The ferric-oxy biominal inside the ferritin protein nanocage is stable in solu-

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§ Residue numbering is based on the first ferritin sequence published, that for horse spleen ferritin (43). A sequence numbering system based on recombinant human H ferritin, differs from the one used here, by four numbers, due to the longer N-terminal sequence in that polypeptide.
tion without added reductant (24). A current, albeit incomplete model for Fe traffic out of the ferritin ferric/oxy biominal is as follows. (i) Fe³⁺ is reduced and hydrated to convert mineralized Fe³⁺ to mononuclear Fe²⁺. (ii) Fe²⁺ moves through the protein channels to and through the pores. (iii) Fe²⁺ exiting the protein cage is complexed to a chelator or putative protein chaperone. Complexation of the exiting Fe²⁺ with a chelator can be monitored by the visible absorption of Fe²⁺, bipyridyl, or Fe³⁺, desferroxamine B, a therapeutic chelator, formed outside the protein (13, 15, 25).

We hypothesized that if very low (0.1 mM) concentrations of guanidine or urea unfolded/opened ferritin gated pores (15), peptides would also selectively recognize the ferritin protein nanocage pores and that such peptides could be identified in a combinatorial peptide library by selection with two ferritins, one with folded pores (wild type) and one with pores unfolded by substitution of a conserved amino acid in the channel (L134P) (15). Our hypothesis was supported by finding, among five peptides that selectively bound to ferritin, one peptide that increased pore unfolding in wild type ferritin (peptide 1) (reductant-dependent increase in Fe²⁺ chelator complex formation rate) with no effect on Fe²⁺ entry/mineralization and no effect on the L134P protein with fully open pores. Another peptide (peptide 4) decreased rates of Fe chelation. In addition, increasing DFO⁴ concentration near the pore by conjugation to peptide 1 via a succinyl-serine link overcame the rate difference between wild type protein + peptide and L134P that may reflect competing reactions during oxidation to stable Fe³⁺ -DFO. The peptides identified could model peptides or proteins that regulate ferritin pores and may have pharmaceutical uses. The peptides also provide a model for other protein pores, such as toxin peptides or voltage-gated Ca²⁺ and K⁺ channels (26–28) or peptides that participate in regulating fusion pores important in secretion (29).

**EXPERIMENTAL PROCEDURES**

**Protein Isolation and Mineralization and Analysis of Fe Chelation Rates**—Recombinant ferritin apoprotein nanocages were purified, and the Fe/oxy mineral was formed (480 Fe/protein nanocage) in solutions of ferritin nanocages (2.06 μM protein, 100 mM MOPS, and 100 mM NaCl, pH 7.0), as described previously (13, 14). Formation of Fe²⁺-bipyridyl was monitored at 522 nm, and formation of Fe³⁺-desferrioxamine B was monitored at 430 nm (15); the Fe chelator complexes are outside the protein based on ultrafiltration (13, 14). Initial rates of iron release were calculated from the progress curves for the formation of Fe chelator complexes as described previously (15) using 0–1 min (>40 data points) for fast reactions or 0–2 min (>80 data points) for slower reactions. The goodness of fit parameter for linear fits (χ²/degrees of freedom in the model) was set to <6. All nonlinear models tested were rejected by the program; for linear fits, p = 0.04. Data from 3 independent measurements/protein preparations were averaged for each time point, and the average was used for fitting. The mineral formed inside of ferritin in vitro has the spectroscopic properties of the natural biominal (18, 30).

**Selective Peptide Binding to Ferritin Using Phage Display**—Ferritin binding peptides were identified using phage display and a combinatorial library (2.8 × 10⁵) of random heptapeptides fused to a minor coat protein (pIII) of M13 phage (Ph.D.TM-7, from New England Biolabs, Ipswich, MA) (31, 32). At the time the experiments were designed, selection of the heptapeptide combinatorial library was based on costs of the only other commercial phage display library (a dodecapeptide) or synthesis of such a library. Ferritin H (frog) was used as the adsorbent for the peptides. After four rounds of selection, no further decrease in phage number occurred. Phage-displaying Group A peptides (n = 428) were eluted from WT ferritin with the mutant L134P that had unfolded pores (13–15). Phage in Group B (n = 600) were eluted with WT ferritin after elution with L134P protein. After translating in silico the phage DNA that encoded the two groups of binding peptides, five consensus peptide sequences were obtained. Group A had two consensus peptide sequences, 1 and 2, and Group B had three consensus peptide sequences, 3, 4, and 5.

**Effects of Peptides on Ferritin Pore Function**—Peptides, predicted from the DNA identified by phage display, were synthesized as undecapeptides; the cost/benefit of determining binding constants with radioactive peptides is beyond the scope of this report and the hypotheses tested. Heptapeptides were synthesized with two phage coat protein residues at the N terminus (HS) and two at the C terminus (GG) to facilitate synthesis of some of the sequences and to enhance solubility. Peptides were obtained from AnaSpec, Inc. (San Jose, CA). Effects of the five peptides on ferritin demineralization rates were analyzed by mixing mineralized ferritin with the peptide for 1 h, at 25 °C, before initiating demineralization by adding reductant, 2.5 mM FMN, 2.5 mM NADH (24). Initial rates of Fe removal from the mineral and protein nanocage were measured as initial rates of formation of Fe²⁺-bipyridyl or air-oxidized Fe³⁺-desferrioxamine B, as described in earlier studies (14, 15).

**Coupling of Peptide 1 to a Chelator**—Peptide 1 was derivatized and conjugated to DFO (1) via a DFO-succinyl-serine-peptide 1 link. (Note that the numbers in bold indicate the intermediates in the synthesis in Scheme 1, supplemental materials.) The approach of using carboxyl activation of a DFO derivative avoided the necessity for extensive protecting group chemistry on the two primary amino groups of the peptide. The addition of serine to the N terminus of peptide 1 created Hser-His-Ser-Asn-Thr-Tyr-Phe-Pro-Lys-Gly-Gly-COOH. Desferrioxamine B was reacted with succinic anhydride (33–35) to form succinyl desferrioxamine B (2) (Scheme 1, supplemental materials) that contained both a linker and a free carboxyl group for conjugation to the peptide. To provide temporary protection of the hydroxamate components of the succinyl-DFO (2) (Scheme 1, supplemental materials), and to facilitate active ester formation, the succinyl ferrioxamine (SFO) (3) (Scheme 1, supplemental materials) was formed (33, 34, 36, 37). Coupling of Ser-pep-
tide 1 with SFO occurred upon reaction of the N-hydroxysuccinimide active ester of SFO in dimethylformamide with an aqueous solution of the peptide at pH 8; careful control of the pH enhanced coupling of the N-terminal serine to SFO and minimized reactions with the more basic epsilon amino group of the internal lysine. The major isolable product of the reaction, identified as the peptide 1-SFO conjugate by LC/MS/MS (Scheme 1, supplemental materials), was coupled via the terminal serine of the peptide. Minor products were also analyzed by LC/MS/MS. A product ion scan of the precursor m/z 1028 (SFO-SHNTYYFPKGG+H)+ yielded m/z 505 (SFO-SHNTYYFPKGG+2H-(NTTYFPKGG))2+, which corresponded to SFO coupled to the terminal serine amine and fragmented between the internal serine and asparagine. Another product ion scan of the precursor m/z 1027 [SHSNTYYFPK(SFO)GG+H]+ yielded m/z 922 (SHSNTYYFPK-(SFO)GG+H-(GG, SHSNTYYF))2+, which corresponded to SFO attached to lysine with fragmentation between lysine and glycine and between phenylalanine and proline. Aqueous Na2EDTA and phenol/chloroform extraction (38) yielded the desferri-conjugate (5) (Scheme 1, supplemental materials) as a white powder; the molecular ion peak corresponded to the predicted molecular mass for the desferri-conjugate.

![Graph showing A_222 nm vs Reaction Time (min)]

FIGURE 1. Peptides, selected for ferritin pore binding from a combinatorial peptide library, change rates of removing Fe from the ferritin mineral inside the protein nanocage. Top left, progress curve for demineralization of recombinant, frog ferritin H protein nanocages. The solution contained: protein, 2.06 μM; Fe, 0.99 mM; MOPS, 100 mM; NaCl, 100 mM, at pH 7.0, which yields mineralized ferritin with 480 Fe/nanocage. The addition of reductant, 2.5 mM NADH and 2.5 mM FMN, was required to detect Fe2+-bipyridyl, forming on the outside of the protein nanocage (13). Peptides, recognizing the ferritin pore, identified in a combinatorial, heptapeptide, phage-display library (2.8 x 10^9 individual amino acid sequences, New England Biolabs), were mixed with mineralized ferritin protein nanocages for 1 h before initiating demineralization. Calculation of initial reaction rates is described in Ref. 15 and under “Experimental Procedures.” The data are averages from triplicates in 2–5 independent experiments with two different protein preparations; the error is the standard deviation. Progress curves for ferritin WT protein + peptide 1 (Δ-), no peptide control (○-○), ferritin WT protein + peptide 4 (●-●). Top right, a view of the ferritin protein nanocage down the 3-fold axis; the gated pore helices are either folded (gold) or unfolded (red ribbons), and the remainder of the four α-helix bundle subunits are light blue. Pore unfolding/folding is independent of the nanocage structure and is more sensitive to temperatures, to urea concentrations (1 mM), and to amino acid substitution of pore gates (13–15); redrawn from Protein Data Bank file 1MFR. Bottom, comparisons of Fe exit rates and effects of temperature for ferritins ± peptide or with pores opened by mutation.

| Protein                  | Initial rate of Fe2+-bipyridyl formation, pH=7.0 (mMol) x 10^−1 | Binding Peptide Sequence | Rate Change with Temperature (Arrhenius equation) F_L (kJ/mole) |
|-------------------------|---------------------------------------------------------------|--------------------------|-------------------------------------------------------------|
| Wild Type H (H-wt)      | 1.10 ± 0.13                                                   | Not applicable            | 88.5 ± 2.3                                                  |
| H-wt + Pep1             | 6.80 ± 0.37                                                   | HSNTYYFPKGG              | 79.8 ± 2.6                                                  |
| H-wt + Pep4             | 0.41 ± 0.05                                                   | HSHHALDGSSG              | Not determined                                              |
| H-L134P, Pore Gate Mutant | 4.03 ± 0.22                                                 | Not applicable            | 77.4 ± 2.1                                                  |

| RESULTS AND DISCUSSION |

The strategy for identifying peptides that modulate ferritin pore function was as follows. (i) Search a combinatorial peptide library for ferritin binding peptides. (ii) Analyze the effects of the identified peptides on rates of transferring Fe from the ferritin mineral inside the protein nanocage to a chelator outside the nanocage by monitoring the formation of the colored, chelator complexes outside the protein cage, Fe2+-bipyridyl or Fe3+-DFO, as described previously (13–15). Properties of the reactions include multiphasic kinetics (Fig. 1) and redundant dependence (24).

Five consensus peptide sequences were obtained from the peptides selected for WT ferritin binding. Each of the five peptide sequences represented convergence among the in silico translation products of multiple DNA sequences obtained after four rounds of selection and phage amplification; there was no change in the numbers of phage selected between rounds three and four. The peptides identified represent two groups obtained by elution of phage-displaying peptides bound to WT ferritin (Fig. 1). Group A phage were eluted with unfolded pore protein, ferritin L134P (13). Group B phage peptide elution used WT ferritin, after removing Group A peptides. The five consensus peptide sequences identified by in silico translation of the DNA sequences obtained correspond to those in a large number of open reading frames from the human genome. However, examining the possible interactions of such a large number of proteins with ferritin is currently impractical. Group A sequences (n = 600) encoded two consensus sequences: peptides 1-NTYYFPK and 2-SHTSSPL. Group B sequences (n = 400) encoded three consensus sequences: peptides 3-GDW-600, 4-HHALDGS, and 5-HHALGGS.

Peptide 1 increased the rate of WT ferritin demineralization 3.3-fold (Fig. 1), suggesting that the pores were unfolded as they were with 1 mM urea or by 0.1 mM guanidine (15) or by replacement of conserved, amino acids around the pores that influenced pore gating (14). The peptide had no effect on Fe mineralization rates when added to the unmineralized protein cage. Three of the peptides, peptide 2 in Group A and peptides 3 and 5 in Group B, had no effect on rates of formation of Fe2+-bipyridyl, indicating that binding to ferritin did not disrupt normal pore structure. Peptide 4 in Group B, in contrast to peptide 1 in Group A, decreased the rate significantly, by 63% (p < 0.001). Interaction of peptide 4 with ferritin might influence
pore unfolding and/or reductant access to the ferric mineral or might induce conformational changes elsewhere.

Peptide 1, NTYYFPK, is very hydrophobic and could, by altering native pore structure, model a regulatory protein in vivo that functions during Fe deficiency. Peptide 4, HHALDGS, could bind to negatively charged Glu and Asp residues around the pore (2, 39) and could effectively clamp the pore shut and be a model for cellular proteins that lead to the observed retention of mineralized Fe in ferritin during inflammation. By diminishing the Fe available for reactions with oxidants, such a peptide would have anti-inflammatory properties in vivo.

Effects of temperature on the rate of Fe demineralization depend on protein structure; they are independent of the mineral/solution transition or the chelator. For example, lowering the temperature decreased the rate for both the WT type ferritin and the pore mutant protein, L134P, at any temperature. However, when WT and L134P proteins were compared under the same conditions of mineralization and mineral dissolution, the Fe exit rate was always faster for ferritin with unfolded pores than the wild type. For the Arrhenius plot, over the temperatures 10–45 °C, $E^\ddagger = 88.5 \pm 2.3$ kJ/mole for WT protein and $77.4 \pm 2.1$ kJ/mole for H-L134P. (Note that no ferritin pore mutant studied to date altered the melting temperature of the protein nanocages (15).) When peptide 1 was complexed with WT ferritin, $E^\ddagger = 79.8 \pm 2.6$ kJ/mole, a value comparable with H-L134P that indicates induced pore unfolding by peptide 1 equivalent to the pore gate mutation. Such data and the composition of peptide 1 (Fig. 1) suggest disruption of helix-helix interactions around the pore, similar to the disruption of the Leu-110-Leu-134 interaction caused by L134P substitution. In analogy to antisense RNA, which alters the structure of RNA, peptide 1 is an "antisense peptide."

To determine the effect of a different chelator on Fe demineralization rates, we chose DFO, an Fe$^{3+}$ chelator in common use in vivo (40). When the rates for Fe$^{2+}$-bipyridyl and Fe$^{3+}$-DFO reporters were compared (Figs. 1 and 2), peptide 1 increased the Fe exit rate from WT ferritin significantly ($p < 0.001$) with either chelator. Moreover, peptide 1 had no effect on L134P (Fig. 2), where the pores were already unfolded, eliminating the possibility of chelator side reactions. However, the effect of peptide 1 was smaller with Fe$^{3+}$-DFO than with Fe$^{2+}$-bipyridyl for WT ferritin, where peptide 1 induced rates that were comparable with L134P (Figs. 1 and 2). We reasoned that competition for Fe$^{2+}$ during oxidation from Fe$^{2+}$-DFO to Fe$^{3+}$-DFO could explain the difference in effects of peptide 1 with the two reporters. Such a competing reaction is Fe$^{2+}$ entry into the protein cage and binding at the ferroxidase site. (The stability constants for Fe$^{3+}$-DFO differ from Fe$^{2+}$-bipyridyl and Fe$^{3+}$-DFO more than $10^7$ (41).) Fe$^{2+}$ reentry could also contribute to the very slow removal rates of Fe observed with DFO in vivo.

We then determined the effect of increasing local DFO concentrations around the pore by tethering DFO to peptide 1; the strategy should minimize competing reactions. To conjugate DFO to peptide 1, we used a succinyl-serine link (Fig. 2) (see “Experimental Procedures” and Scheme 1 in the supplemental materials for synthesis steps). Availability of the conjugate, pc-1, required use at 0.11 mM to permit examination of experimental variability. Since the mineralized Fe concentration was 0.99 mM and since the reactions with and without free peptide used 2.5 mM DFO, the same concentration of DFO was included in the reaction mixture with pc-1 to trap Fe released from the pc1-DFO conjugate before oxidation. The rates for WT, WT + peptide 1, WT pc-1, L134P, L134P + peptide 1, L134P + pc-1, respectively, were $0.28 \pm 0.04, 0.93 \pm 0.26, 2.33 \pm 0.17, 2.49 \pm 0.11, 2.22 \pm 0.31$, and $2.24 \pm 0.19$ (Fig. 2). Such data show that increasing the local concentration of chelator around the ferritin pores facilitates formation of the Fe$^{3+}$-DFO complex with a rate equivalent to L134P, for which the rate was unaltered by added peptide or peptide-DFO conjugate. The complexity of the Fe exit pathway in ferritin revealed here indicates either that Fe reentry occurs regularly for a small frac-

$^5$ Bipyridyl toxicity precludes the use of bipyridyl in vivo.
tation of Fe ions leaving the mineral or that in vivo, Fe chaperones stably complex the Fe$^{2+}$ ions exiting from ferritin, preventing reentry.

The two peptides that had opposite functional effects (Fig. 1) on rates of removing Fe from the mineral inside the ferritin nanocage to chelators on the outside, identified from a combinatorial, heptapeptide library, have pharmacological potential either as an antioxidant (peptide 4) or, when conjugated to DFO (peptide 1), as an improved chelator in sickle cell disease or thalassemia. The peptides might also model in vivo regulation of ferritin pore function. For example, holding the ferritin pores closed (peptide 4, Fig. 1) when little or no Fe is needed to synthesize Fe proteins would minimize consumption of cellular reductants and escape of redox active Fe. On the other hand, opening the ferritin pores (peptide 1, Fig. 1) when Fe is needed to synthesize Fe proteins would increase mineral reduction and transport of Fe from the mineral through the ferritin nanocage to sites of protein synthesis. Finally, since ferritin pores are studied in situ, i.e. in the protein nanocage that is stable in solution, they contrast with other pores that are studied in synthetic bilayers where conformational modification can occur (42). Thus, the peptide-pore interactions in ferritin can model the function of other protein pores in ion channel proteins.

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