Moving Iron through Ferritin Protein Nanocages Depends on Residues throughout Each Four α-Helix Bundle Subunit

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Eukaryotic H ferritins move iron through protein cages to form biologically required, iron mineral concentrates. The biomimetics are synthesized during protein-based Fe²⁺/O² oxidoreduction and formation of [Fe³⁺O]₄ multimers within the protein cage, on route to the cavity, at sites distributed over ~50 Å. Recent NMR and Co²⁺-protein x-ray diffraction (XRD) studies identified the entire iron path and new metal-protein interactions: (i) lines of metal ions in 8 Fe²⁺ ion entry channels with three-way metal distribution points at channel exits and (ii) interior Fe³⁺O nucleation channels. To obtain functional information on the newly identified metal-protein interactions, we analyzed effects of amino acid substitution on formation of the earliest catalytic intermediate (diferroxeroxinate-A₆50 nm) and on mineral growth (Fe²⁺O-A₃50 nm), in A26S, V42G, D127A, E130A, and T149C. The results show that all of the residues influenced catalysis significantly (p < 0.01), with effects on four functions: (i) Fe²⁺ access/selectivity to the active sites (Glu130), (ii) distribution of Fe²⁺ to each of the three active sites near each ion channel (Asp127), (iii) product (diferroxo) release into the Fe³⁺O nucleation channels (Ala26), and (iv) [Fe³⁺O]₄ transit through subunits (Val142, Thr149). Synthesis of ferritin biomimetics depends on residues along the entire length of H subunits from Fe²⁺ substrate entry at 3-fold cage axes at one subunit end through active sites and nucleation channels, at the other subunit end, inside the cage at 4-fold cage axes. Ferritin subunit-subunit geometry contributes to mineral order and explains the physiological impact of ferritin H and L subunits.

Ferritins are an ancient superfamily of protein nanocages that synthesize, reversibly, iron concentrates for cellular use, heme, FeS cluster, and Fe-protein synthesis and provide oxidant protection by consumption of dioxygen or hydrogen peroxide and ferrous iron during stress; the protein cavities containing the minerals are ~60% of the cage volume (1–4). Ferritin differ in cage size, location, and mechanism of catalytic sites, mineral size, and mineral crystalinity; the Fe²⁺/O² oxidoreductase sites are also called ferroxidase sites or FC (ferroxidase centers) sites (1–5). In eukaryotic H ferritins, Fe³⁺ and dioxygen are substrates for oxidoreductase sites and are catalytically coupled at multiple protein sites to synthesize differic o xo mineral precursors (Fig. 1). Consumption of both iron and oxygen by ferritins accounts for the antioxidant response and iron-controlled gene regulation of ferritins in eukaryotes (6). Many catalytic proteins use iron and oxygen to produce a variety of organic products. Such products include unsaturated fatty acids such as oleate stearoyl-CoA desaturase-1 (7), deoxyribose (ribonucleotide reductase), and polyhydroxyl modification of oxygen-sensing DNA transcription factors, e.g. hypoxia-inducible factor-α (8). Only in ferritins are both substrates inorganic. The exclusive use of inorganic substrates may relate to the ancient origins of the ferritins, which are distributed in all kingdoms and in both anaerobes and aerobes; ferritin gene deletion is lethal early in mammalian embryogenesis (9).

There are the two types of protein channels that move iron into and through the 24 subunit cages during synthesis of [Fe³⁺O]₄ in eukaryotic ferritins. The two functional types of ferritin channels are: (i) ion entry channels around the 3-fold axes, for Fe²⁺ substrate access to oxidoreductase sites (Asp127, Glu130) and (ii) Fe³⁺O nucleation channels, distal to the active sites on the long axes of each subunit bundle (Ala26, Val142, Thr149), where differic o xo mineral precursors produced by oxidoreductase fuse to tetramers and larger multimers before exiting from the protein cage for mineral growth.

Recent high resolution structural studies show that ferritin is a soluble ion channel protein with lines of multiple metal ions (10), much like K⁺ and other membrane ion channel proteins. The eight Fe²⁺ entry channels (Fig. 1) suggest roles beyond just electrostatics for the conserved carboxylates, such as ion selectivity at the channel constriction or directing Fe²⁺ substrate ions to active sites in three subunits that also form the entry channels (10). The Fe³⁺O nucleation channels were recently identified using ¹³C-¹³C solution NMR spectroscopy in the presence and absence of Fe³⁺, by the disappearance of resonances within 5 Å of Fe³⁺O moving away from the active sites (11). Nucleation channel exits into the cavity are clustered around the 4-fold axes of the cage, which facilitates ordered mineral growth. Functional studies of carboxylate residues in the Fe²⁺ ion entry channels have been mostly limited to later stages after differic peroxo (DFP) decay (12–14) except (15), and none has examined the function of residues in the Fe³⁺O...
Moving Iron through Ferritin Nanocages

nucleation channel. Several recent reviews addressing the general topic of ferroxidation and catalysis in ferritin proteins include Refs. 1–4.

We now report the functional effects of amino acid substitutions on the initial Fe2+/O2 reaction intermediate, diferric peroxo (Fe3+O−O−Fe3+), and mineral precursors (Fe3+O)n (Fig. 1C), during multiple catalytic turnovers, for residues in the Fe3+ entry channels, identified by Co2+, Mg2+, protein co-crystalllography (10) and earlier studies (16, 17), and on residues in the Fe3+ postoxidation/nucleation channel, identified by NMR spectroscopy (11). Our results show that conserved carboxylates in the Fe2+ ion entry channels, connecting the external cage surface to the central, mineralization cavity (Fig. 1, A and B), have two different functions, i.e. Fe2+ entry and Fe3+ distribution among multiple active sites. In addition, we show that amino acid substitutions in the Fe3+ postoxidation/nucleation channels affect oxidation and decay of the diferric peroxo intermediate. Concepts of ferritin function have usually considered that movement of iron through the protein cage depended on residues in localized regions within each 4-helix bundle subunit. The results of this study, by contrast, reveal functional residues spanning the length of each 4-helix bundle subunit, from Ala26 to Thr149, in the assembled ferritin protein cage.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis**—Frog-M ferritin was used as the template because Fe-protein interactions have been studied extensively by Mössbauer, resonance Raman, EXAFS, UV-visible spectroscopy and x-ray crystallography as reviewed in Ref. 5, and more recently by active site amino acid substitutions (18). MCD/CD (19) and very high resolution x-ray crystallography (10). Site-directed amino acid substitutions in frog-M ferritin protein cages were generated by PCR on the expression plasmid pET-3a frog-M, using the QuikChange® II site-directed mutagenesis kit (Stratagene). Coding regions of all protein expression vectors were analyzed for DNA sequence confirmation (UC Berkeley DNA sequencing facility). The forward primers that were used are listed in the supplemental text, with bold nucleotides representing the mutated codon; reverse primers were complementary to the forward primers.

### Protein Expression—pET-3a constructs encoding wild-type frog-M ferritin and mutants were transformed into *Escherichia coli* BL21(DE3)pLysS cells and subsequently cultured in LB medium containing ampicillin (0.1 mg/ml). Cells were grown at 37 °C, until A600 nm reached 0.6–0.8. Induction, at 30 °C with isopropyl thio-β-D-galactopyranoside (0.5 mM final concentration), was 4 h, when cells were harvested (14, 20). Recombinant ferritins were purified as described previously (14, 20–22). In short, cells were broken by sonication, and the cell free extract obtained after centrifugation (2 h, 18,000 rpm, 4 °C) was incubated for 15 min at 65 °C as first purification step. After removal of the aggregated proteins (30 min, 18,000 rpm, 4 °C), ferritin was precipitated with 65% ammonium sulfate, resolubilized in 25 mM bis-Tris-propane, pH 7.5, and dialyzed against the same buffer. Next, the sample was loaded onto a Q-Sepharose column (Vc = 200 ml) and eluted with a linear NaCl gradient of 0–1 M in bis-tris-propane, pH 7.5. Fractions containing ferritin (monitored by SDS-PAGE) were combined, precipitated with ammonium sulfate (65%), resolubilized in volumes of 100 mM MOPS, pH 7.0, containing 100 mM NaCl, and dialyzed against the same buffer. Protein concentration was determined with a Bradford assay, and iron content was analyzed after boiling in 1 N HCl, as the Fe2+–1,10-phenanthroline complex (23, 24).

### Fe2+/O2 Catalysis and Fe3+O Mineralization—Single turnover iron oxidation (uptake of 48 Fe2+/ferritin cage, 2 Fe2+/subunit), in frog-M ferritin, wild type or with amino acid substitutions, was monitored as the change in A230 nm (diferferic peroxo or DFP) (12, 25) or A350 nm (Fe3+O) after rapidly mixing (<5 ms) equal volumes of 100 μM protein subunits (4.16 μM protein cages) in 200 mM MOPS, pH 7.0, containing 200 mM NaCl with a freshly prepared 200 μM ferrous sulfate in 1 mM HCl in a UV/visible, stopped-flow spectrophotometer (18). Generally, 2000 data points were collected during 10 s. The incubation time between subsequent additions of Fe2+ (80 mM) (48 Fe2+/nanocage) was 4–16 h (12, 25). The progress of oxidation (DFP + diferric o xo + Fe3+O tetrads + mineral nuclei + mineral) was monitored using the nonspecific absorbance between 300 and 450 nm, at A350. Absorbance curves for Fe3+O between 300 and 450 nm were also collected for solutions of 240 μM protein subunits (10 μM ferritin cages) with an iron concentration of 480 μM (2 Fe2+/subunit; 48 Fe2+/protein cage).

Initial rates of DFP and [Fe3+O]n species formation were determined from the linear fitting of the initial phases of 650 nm and 350 nm trace (0.01–0.03 s). The rate of decay of DFP complex was determined by exponential fitting of the decreasing part of the progress curve at 650 nm (0.1–1.5 s). The data presented are averages from a minimum of 4–6 experiments each using a minimum of two or more independent protein preparations, and the error is presented as the S.D.

**FIGURE 1. Ferritin nanocage structure.** A, frog-M ferritin nanocage structure, viewed from the outside with the 3-fold axis near the center, shown with one subunit highlighted in blue (Protein Data Bank ID code 1MFR). Eight Fe2+ ion channels (10), each formed by residues from three subunits around the 3-fold axes, lead through the protein cage from the outside of the cage to the large (8-nm diameter) central cavity, near several residues of the active sites; B, a single subunit of the frog-M ferritin showing all of the residues studied by site-directed mutagenesis; C, steps in ferritin mineral synthesis.

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Moving Iron through Ferritin Nanocages

**Fe³⁺ O Mineral Dissolution/Chelation**—Empty ferritin protein cages were mineralized with ferrous sulfate (480 Fe²⁺ /cage), in 100 mM MOPS, pH 7.0, containing 100 mM NaCl, as described previously (20). Mineral dissolution was monitored as formation of Fe²⁻ -bipyridyl, at A₃₂₂ nm, outside of the ferritin nanocavity after reduction of Fe³⁺ as described previously (20, 21, 26). A mixture of reductant (NADH and FMN) was added to a solution of 0.5 μM mineralized ferritin (0.25 mM Fe) containing 2.5 mM bipyridyl at 25 °C; final concentrations of NADH and FMN were 2.5 mM. Initial rates of iron release were calculated from the linear part of the Fe²⁻ -bipyridyl formation (522 nm trace). The data were averaged from a minimum of four experiments using a minimum of two independent protein preparations.

**RESULTS**

**Fe²⁺ Enters Ferritin Ion Channels Controlled by Glu¹³⁰ and Is Directed to Active Sites by Asp¹²⁷**—Negatively charged ferritin residues Glu¹³⁰ and Asp¹²⁷ were originally identified by sequence conservation and location in 3-fold pores (16, 17). The three Glu¹³⁰ residues are in the middle of the Fe³⁺ ion channels, one from each of the three subunits that form the channels, at a constriction (Fig. 1B); the 8 channels are formed by the juxtaposition of the helix₃-loop helix₄ regions of three subunits around the 3-fold axes of the protein cage. Three Asp¹²⁷ residues are at the end of the channels at the entries to the large central cavity (8-nm diameter) where mineral forms. The influence of Glu¹³⁰ and Asp¹²⁷ on catalysis at the Fe²⁺ /O₂ oxidoreductase sites, i.e. on d ferric peroxo formation, has never been studied, although substitutions at Glu¹³⁰ and Asp¹²⁷ are known to inhibit formation of the multiple Fe³⁺ O species measured in the range A₃⁰₅–₄⁵⁰ nm (27, 28), where Asp¹²⁷ and Glu¹³⁰ are equivalent to Asp¹³⁴ and Glu¹³⁴ in the human ferritin studied. The three Glu¹³⁰ residues and the three Asp¹²⁷ residues from each of the three subunits that form the eight Fe²⁺ -entry channels in the 24-subunit cage structure, all participate in protein-metal ion interactions in the channels, based on crystal structures (10, 29–31). We predict that E130A and D127A will have different effects during multiple catalytic turnovers.

Ferritin protein nanocages with E130A or D127A substitutions in each subunit were analyzed for differences in d ferric peroxo formation, compared with wild type. DFP has a λ_max at 650 nm (12, 33, 34). Fe²⁺ was added in multiple aliquots, each of which saturated the active sites, i.e. 48 Fe²⁺/ferritin nanocage (2 Fe²⁺/active site) to achieve distinguishable turnovers of the active site. Based on the NMR experiments (11), which determined where Fe³⁺O is, in the protein cage, four turnovers were required for [Fe³⁺O]₆ complexes (see Fig. 1C) to complete the transit through the nucleation channels to the cavity. In addition to the DFP complex, we also measured absorbance changes at 350 nm. Many Fe³⁺ O species contribute to the absorbance in 300–450 nm range, which monotonically decreases with increasing wavelength (27, 28, 35, 36). We monitored changes in A₃₅₀ nm because they can be observed for long time periods, during mineral growth, after DFP has decayed (Fig. 2B). During ferritin mineral formation, the many Fe³⁺ O species absorbing in the 300–450 nm range (Figs. 1C and 2D) include DFP at the active sites (12, 13, 37), diferric o xo product (12, 25), and mineral nuclei that form as Fe³⁺O multimers moving through the channels to the cavity (11) (reaction scheme shown in Fig. 1C). Fig. 2D shows the absorbance versus wavelength spectrum of WT ferritin (48 Fe²⁺ /cage) immediately after the addition of Fe²⁺ or 60 min later. Mineral growing in the protein cavity also contributes to the absorbance in this range. Assignment of specific spectral properties to each of the Fe³⁺O species that occur during iron oxidation and mineral formation is not possible without additional different spectroscopic information analyses related to kinetics of mineral formation; currently the mixed spectra of the Fe³⁺O species are unresolved. We use changes in A₃₅₀ nm to monitor mineral formation because it is far enough away from spectral contributions due to aromatic amino acids and still high enough to provide sensitive measurements.

Glu¹³⁰ is absolutely required for formation of the DFP catalytic intermediate of ferritin (Fe²⁺ /O₂ oxidoreductase activity) because insignificant amounts of DFP formed in E130A under any conditions tested (up to 8 Fe²⁺/active site or 192 Fe²⁺ /cage) in stepwise Fe²⁺ addition experiments (Fig. 2A). In fact, the relative rates of Fe²⁺ oxidation are comparable with those for the animal-specific, L-type ferritin subunit that lacks a catalytic site (14) and where Fe²⁺ oxidation is thought to be facilitated by iron chelation at residues on the protein or on the caged, inorganic mineral (38). The initial rate of oxidation measured at 350 nm (ΔA₃₅₀ nm/s) was ~1% wild type.

Asp¹²⁷ is required for DFP formation when the amount of Fe²⁺ added is low (Fig. 2, A and C); partial rescue occurs when six or more Fe²⁺ are added/active site (Fig. 2, A and C), likely due to saturation of competing, weaker Fe²⁺ sites that are inactive when Asp¹²⁷ is present. Rates of DFP formation in D127A ferritin are 45% of WT (Fig. 2C) when an equivalent of six or more Fe²⁺/active site were added. When changes in A₃₅₀ nm were analyzed at six or more Fe²⁺/active site, the initial rates were only 20% of the WT (Fig. 2, A and C). Such results emphasize contributions of Asp¹²⁷ carboxylates around the Fe²⁺ channel exits (Fig. 3), to directing Fe²⁺ substrate to each of the three catalytic sites in the middle of the helix bundles that form the ion channels. With the addition of six or more Fe²⁺/active site, the inhibitory effect of D127A is partly reversed; DFP formation is restored and by 10 s after adding Fe²⁺, long after DFP decay, the mineral growth (A₃₅₀ nm) reaches WT values (Fig. 2B). Such results suggest that nonspecific Fe²⁺ binding sites, compete with the active sites for binding in the absence of Asp¹²⁷, but are saturated when enough Fe²⁺ is added. Apparently, Asp¹²⁷ is too far away to alter reactions after oxidation and during mineral growth.

To show that the effects of altering pore residues Glu¹³⁰ and Asp¹²⁷ were selective for Fe²⁺ catalysis, we studied rates of mineral dissolution (NADH/FMN reduction + bipyridyl chelation of Fe²⁺ ions after dissolution and exit from the cage), because other conserved Fe²⁺ ion channel residues, e.g. Arg²⁷⁷, Leu¹¹⁰, Asp¹²², and Leu¹³⁴, near the outside end of the channel, altered mineral reduction (20, 21). Once the hydrated ferric oxide mineral was formed in WT, D127A and E130A ferritins, the caged mineral was dissolved, and Fe²⁺ released at the same rates (supplemental Fig. S2) among all of the proteins. Such results emphasize the selectivity of Glu¹³⁰ and
Moving Iron through Ferritin Nanocages

FIGURE 2. Negatively charged residues in the 3-fold pore region are required for iron entry and active site access. For single turnover iron oxidation (uptake of 48 Fe$^{2+}$/ferritin cage) (A–C), monitored by the change in $A_{550\text{ nm}}$ (DFP) or $A_{550\text{ nm}}$ (all Fe$^{3+}$) rapid mixing (<5 ms) protein concentrations were 2.08 $\mu$m ferritin cages (50 $\mu$m ferritin subunits) 100 $\mu$m ferrous sulfate solution in 100 mM MOPS, pH 7.0, 100 mM NaCl. Subsequent additions Fe$^{3+}$ were 48 Fe nanocage (2/diiron active site) (see “Experimental Procedures”). For the absorbance spectra of Fe$^{3+}$ O in ferritin (D), the final protein concentration was 10 $\mu$m ferritin cages and 480 $\mu$m Fe$^{2+}$ in 100 mM MOPS, pH 7.0, 100 mM NaCl, A, initial rates of DFP formation ($\Delta A_{550\text{ nm}}$/s) for the four catalytic cycles. Inset, DFP absorbance spectrum in WT frog-M ferritin, modified from Ref. 29. Progress curves at 650 nm, after each addition of iron, are illustrated in supplemental Fig. S1. B, Progress curves of $A_{550\text{ nm}}$ for the fourth (192 Fe$^{2+}$/cage) catalytic cycle: wild type, solid line; D127A, D; and E130A, * C, initial rates of single turnover for the formation of DFP ($\Delta A_{550\text{ nm}}$/s) and iron oxidation ($\Delta A_{550\text{ nm}}$/s) for the first (48 Fe$^{2+}$/cage) and fourth (192 Fe$^{2+}$/cage) catalytic cycles. The numbers are averages from at least four to six experiments, each performed on at least two independent preparations of protein. D, spectra of Fe$^{3+}$O species between 300 and 450 nm measured immediately (manual mixing) after Fe$^{2+}$ addition and after 60 min.

Asp$^{127}$ contributions to Fe$^{2+}$ active site access and the reaction with dioxygen.

Conserved Residues in the Fe$^{3+}$ Nucleation Channel of Ferritin, Identified by NMR Spectroscopy, Affect Ferritin Function—Residues in the postoxidation channels, identified by NMR in the presence and absence of Fe$^{3+}$ (11), influence Fe$^{2+}$/O$_2$ reaction rates at the active sites (Fig. 4, A and B). The nucleation channels, also called post-oxidation channels, are distal to the active sites, on the long axis of each subunit bundle; they open around the 4-fold axes on the inner surface of the cage. The Fe$^{3+}O$ paramagnetic effects indicated that the residues were $\sim$5 Å away from Fe$^{2+}$ oxidized in situ (11). Note that the apparent absence of carboxylate residues in the Fe$^{3+}O$ channels relates in part to incomplete site-specific NMR assignments that underrepresented carboxylates due to large size and number of residues (480 kDa, 175 residues/subunit, 24 subunits), an unusually large number of carboxylate residues, the small $^{13}$C chemical shifts for Glu and Asp, the narrow dispersion between Asn/Asp or Gln/Glu, and the effects of $\alpha$-helix bundle structure (low chemical shift dispersion). Thus, carboxylate ligands in the channels may simply remain among those residues for which assignments remain to be made. Alternatively, when Fe$^{3+}O$ moves through the channels, charge balance could be maintained, e.g. by proton loss from water coordinated to Fe$^{3+}$ in the Fe$^{3+}O$ species.

Residue Ala$^{26}$ was identified as a part of the iron nucleation channel in the ferritin solution NMR study with and without Fe$^{3+}O$ species (11). In addition, we used covariation analysis, with the methods in Ref. 39 and analyzing $\sim$350 sequences of ferritins that were either catalytically active (His) or inactive (Leu). Residue 26 is part of a covariation network of 15 residues e.g. Ser$^{26}$ occurs in wild-type, catalytically inactive, frog L ferritin, emphasizing the functional importance of Ala$^{26}$.
in ferroxidation and iron nucleation. For this reason and because of increased hydrophilicity and similar size of serine to alanine, the A26S substitution was studied in the well characterized frog-M, an H-type ferritin.

The position and orientation of Ala26 in the 4-helix bundle close to the active site at the entry of the nucleation channel suggest a role in catalysis and product release (Fig. 4D). However, when more Fe2+/H2O was added, inhibition of oxidation was significant (p < 0.0001) (Fig. 4A). In contrast to oxidation, DFP decay was sensitive to inhibition (p < 0.0001) even in the first catalytic turnover (Fig. 4B). Such observations show that Ala26 participates in product (Fe3+/H2O) release, which explains the stabilization of the DFP intermediate in A26S, even during the first catalytic cycle (Fig. 4B). Fe2+/H2O formation was inhibited 25% during the first catalytic turnover (supplemental Table S1), contrasting with DFP formation, which was only affected after the second addition of substrate (96 Fe2+/cage). The A350 nm was always lower in A26S than WT (Fig. 5) and even after 1 h had not reached the levels in WT protein, suggesting different properties of the Fe3+/O2 nuclei. Thus, in addition to a role for Ala26 in the rates of product release from the Fe2+/O2 oxidoreductase center, the change in the entry to the nucleation channel changes the spectral properties of the growing mineral nuclei (11), possibly by altering hydration of the hydroxo bridges.

Thr149 in helix 4, and nearby Val152 in the loop connecting helices 1 and 2 of a neighboring subunit, are 15–20 Å from the

FIGURE 4. Ala26 near the active site destabilizes the DFP catalytic intermediates in ferritin. Single turnovers (48 Fe2+/nanocage; 2 Fe2+/oxidoreductase site) by wild-type frog-M ferritin (WT) and site-directed amino acid substitutions variants were monitored, \( \Delta A_{350} \) (DFP) and \( \Delta A_{450} \) (Fe3+/O, which includes DFP, diferric oxo, Fe3+/O4 tetramers, and mineral), after rapidly mixing equal volumes ferritin cage protein with ferrous sulfate solutions, described in Fig. 2 and under “Experimental Procedures.” The results are averages from two to three preparations of protein analyzed four to six times each, and the error is the S.D. Significantly different from WT, *, p < 0.0001 or **, p < 0.006 (t test). A, initial rates of DFP formation (\( \Delta A_{350} \)). B, initial rates of DFP decay. C, frog-M ferritin nanocage structure viewed from the outside, with the 4-fold axis near the center (Protein Data Bank ID code 1MFR). The residues near the 4-fold pores are shown in red or blue spheres. D, part of a single subunit of the frog-M ferritin showing the ferroxidase center and the position of residue Ala26 nearby.

FIGURE 5. Ala26, a residue near the entry of the Fe3+/O2 nucleation channel, influences properties of growing ferritin mineral nuclei. Fe2+/H2O was added in aliquot of 48 Fe2+/nanocage, under the conditions described in Fig. 2 and under “Experimental Procedures.” The absorbance between 300 and 450 nm (Fig. 2D), which increases during and after DFP decay (>0.1 s), is a combination of DFP, diferric oxo, and other ferric oxo multimers. Many studies analyze ferritin activity/mineralization at 305 or 320 nm (19, 20, 27, 28); we use A350 nm to avoid contributions from protein side chains. When DFP dominates the Fe3+/O spectrum, Ala26 and WT are the same; only after DFP decays, when diferric oxo/hydroxo and larger multinuclear species begin to form, is the A350 nm absorbance difference detectable. The results are averages from two to three preparations of protein analyzed four to six times each, and the error is the S.D. Significantly different from WT, *, p < 0.0001 (Student’s t test).
active site residues Asp\textsuperscript{127} or Glu\textsuperscript{130} and near the Fe\textsuperscript{3+}O channel exits into the cavity, around the 4-fold axes (Figs. 1B and 4C). Note that in the NMR experiment (11), Thr\textsuperscript{149} and Val\textsuperscript{52} were not affected by Fe\textsuperscript{3+} oxidized \textit{in situ} until 144 Fe\textsuperscript{3+} were added (6 Fe\textsuperscript{2+}/active site) with additional broadening of NMR signal when 192 Fe\textsuperscript{2+} were added (8 Fe\textsuperscript{2+}/active site). Cysteine was chosen as the substitution for threonine because a Fe\textsuperscript{3+}-O interaction, if it existed, would be changed by the substitution of sulfur for oxygen. Glycine was chosen as the substitution for valine to change the hydrophobicity in the loop around Val\textsuperscript{52}. Compared with WT ferritin, rates of Fe\textsuperscript{2+} oxidation, monitored as \textDelta A\textsubscript{455 nm}, or \textDelta A\textsubscript{350 nm} decreased significantly (p < 0.001) in V42G and T149C ferritins at all of the catalytic cycles analyzed (cycles 1–4), (Fig. 4A and supplemental Table S2); DFP stability also decreased compared with WT (p < 0.001 for V42G and p < 0.006 for T149C) (Fig. 4B). In addition, in V42G the development of the broad absorbance band for Fe\textsuperscript{3+}O (\Delta A\textsubscript{350 nm}/s) lagged behind WT after mixing (supplemental Table S2). The threonine/cysteine substitution and valine/glycine substitution likely cause sufficient conformational changes throughout the channel from the active site to the channel exits to alter Fe\textsuperscript{3+}O passage through the channel.

None of the amino acid substitutions studied, A26S, T149C, and V42G, like D127A and E130A, had any effect on mineral dissolution after adding reductant (FMN/NADH) with bipyridyl as an Fe\textsuperscript{2+} reporter. Such results indicate the separation of residue-specific protein-Fe interactions during iron entry and exit.

**DISCUSSION**

Ferritin ferric oxo minerals are built up from the diferric oxo products of catalysis in a multistep process that depends in part on the protein cage itself (Fig. 1C). The role of the protein in mineral buildup, beyond the catalytic reactions at the catalytic (oxidoreductase or ferroxidase) centers, has only recently been suggested by structural studies (11), which show that nucleation occurs inside the long (\textapprox 20 Å) Fe\textsuperscript{3+}O nucleation channels (Fig. 1, A–C). However, negatively charged residues in the ion entry channels form an electrostatic environment favorable to moving Fe\textsuperscript{2+} ions to active sites (2–4). The carboxylate residues appear to have additional functions. For example, the three Asp\textsuperscript{127} residues from each subunit appear to also distribute Fe\textsuperscript{2+} substrate to each of the three active sites in the subunits that form the entry channels around the 3-fold axes (Fig. 3). Moreover, the three Glu\textsuperscript{130} residues, which form a constriction in the iron entry channel, may control not only metal ion access but also metal ion selectivity, blocking entry of large, divalent cations. Together, the structural results identified new potential functions or new amino acid-iron interactions with potential contributions to ferritin protein function (10, 11), that we studied here by making amino acid substitutions at conserved positions (Ala\textsuperscript{26}, Val\textsuperscript{42}, Asp\textsuperscript{127}, Glu\textsuperscript{130}, and Thr\textsuperscript{149}) and by covariation between catalytically active (H) and inactive (L) ferritins (39).

Substitutions of Ala\textsuperscript{26}, Val\textsuperscript{42}, Asp\textsuperscript{127}, Glu\textsuperscript{130}, and Thr\textsuperscript{149} all changed ferritin function during the first four catalytic cycles when mineral nuclei are still in the protein cage and before mineral growth; in the case of Glu\textsuperscript{130} and Asp\textsuperscript{127}, the effects were different (Fig. 2) as predicted using the new structural data (10). The functional effects fell into four categories: (i) Glu\textsuperscript{130}, where all three carboxylates are close together and the channel is constricted, controls Fe\textsuperscript{2+} access to the active sites (DFP formation); (ii) three proximal carboxylate residues, Asp\textsuperscript{127}, one from each subunit, regulate Fe\textsuperscript{2+} distribution to three active sites; (iii) Ala\textsuperscript{26} near the active site at the entry to the Fe\textsuperscript{3+} nucleation channel, regulates release of product, diferric oxo, and, thus, formation of the Fe\textsuperscript{3+}O tetramer between two Fe\textsuperscript{3+}O dimers; (iv) hydrophilic and hydrophobic residues in and around the Fe\textsuperscript{3+} nucleation channel influence Fe\textsuperscript{2+} oxidation and turnover. The specificity of the residues for mineral formation was shown by the absence of any effects on mineral dissolution in the ferritins with amino acid substitutions at Ala\textsuperscript{26}, Val\textsuperscript{42}, Asp\textsuperscript{127}, Glu\textsuperscript{130}, or Thr\textsuperscript{149}.

Three Glu\textsuperscript{130} carboxylates form a constriction in the middle of the eight Fe\textsuperscript{2+} entry channels, one from each subunit that creates each channel and controls Fe\textsuperscript{2+} access to the active sites for DFP formation. Substitution of the negatively charged glutamate carboxylates with neutral alanine created a protein with no significant DFP formation (Fig. 2, A and C), as if the catalytic sites were inactive. E130A behaved like L ferritin, which has no catalytic sites and where mineral formation is attributed to chelating effects of conserved, clusters of carboxylate ligands on the inner surface of the protein (27, 28, 40). The diameter of the Fe\textsuperscript{2+} channels at the Glu\textsuperscript{130} constriction is \textapprox 5.4 Å, Fe\textsuperscript{2+} hexahydrate has a diameter of \textapprox 4.5 Å. Multiple small ions such as Mg\textsuperscript{2+} line up in the channels at and below the Glu\textsuperscript{130} constriction whereas a single, larger Co\textsuperscript{2+} ion is “stuck” at Glu\textsuperscript{130} in protein cocryystals (10). In addition to attracting the ions into the channel by the high concentration of negative charge, the close fit of Fe\textsuperscript{2+} at the Glu\textsuperscript{130} channel constriction suggests that Glu\textsuperscript{130} also exerts some metal ion selectivity.

The Asp\textsuperscript{127} residues in the Fe\textsuperscript{2+} entry channels, one from each ferritin subunit that form the channels, surround the channel exits into the mineral cavity (Figs. 1B and 3). Asp\textsuperscript{127} influences Fe\textsuperscript{2+} access to the three active sites because substitution with alanine prevented formation of the DFP intermediate, when only small numbers of Fe\textsuperscript{2+} were added. The amino acid substitutions D127A and E130A had no detectable effect on Fe release (supplemental Fig. S2). However, when more Fe\textsuperscript{2+} was added (6 Fe\textsuperscript{2+}/active site) DFP was detected (Fig. 2, A and C). The rescue of catalytic activity in D127A, by adding more Fe\textsuperscript{2+} substrate, contrasts with E130A and has several possible explanations, although in each case the electrostatic potential in the channels decreases with the substitution of alanine for each of the carboxylates. First, it may take at least 6 Fe\textsuperscript{2+} to eliminate competition from the carboxylate chelating residues on the inner surface (40). Second, with such a large number of Fe\textsuperscript{2+} ions, the dependence on subunit-subunit cooperativity around the channel may be overcome (Fe\textsuperscript{2+} binding at the active site, monitored by MCD/CD had a Hill coefficient of 3 (19) that could only be explained by subunit-subunit interactions). Third, in a recent structure of Mg\textsuperscript{2+}-ferritin cocystal, metal ions were bound to the three Asp\textsuperscript{127} carboxylates around each channel exit on the inner surface of the cage (Fig. 3), pointing toward the three active sites nearest each channel (10). A reasonable model that
Moving Iron through Ferritin Nanocages

fits the available data is that Fe$^{2+}$ after passing the Glu$^{30}$ “filter” is directed by Asp$^{127}$ to each of the three active sites in the subunits that make up each pore. Binding of Fe$^{3+}$ at the active sites, thus, depends on: (i) the orientation of Fe$^{3+}$ by Asp$^{127}$ toward each of the three active sites in the subunits that form each pore, indicating the functional significance of cage assembly geometry (Fig. 3); (ii) conformational changes that enhance the coordinated filling of three active sites around each entry pore; and (iii) tight binding of Fe$^{2+}$ at the active sites that outcompetes binding to clusters of carboxylate residues on the inner surface of the cavity (40).

Ala$^{26}$, close to the active site at the beginning of the Fe$^{3+}$ nucleation channel (Fig. 4D), enhances reaction/product release because rates of oxidation and decay were decreased in A26S ferritin cages, except for the first catalytic cycle when DFP formation was normal but decay was still slower. The importance of conserved Ala$^{26}$ was indicated by proximity to the diferric oxo dimer (<5 Å) in the NMR experiment when 48 Fe$^{2+}$/cage were added (2 Fe$^{2+}$/active site) (11), as well as co-variance analysis (39) of catalytically active (H-type) and inactive (L-type) ferritin subunits. In addition, when the A$^{350 nm}$ is measured after the DFP intermediate has been converted to the diferric oxo precursor (<1 s), contributions of A26S to slower reactions such as mineral growth itself are observed (Fig. 5).

A26S may also influence Fe$^{3+}$O tetramer formation in the channels, observed by magnetic susceptibility (11), because the full effect of A26S is not observed until 48 Fe$^{2+}$/cage are present (data not shown). Possibly the steric resistance or potential hydrogen bond interaction of the serine, which replaces alanine in the A26S ferritin, not only inhibits diferric oxo product release from the catalytic sites but also inhibits the reaction of two Fe$^{3+}$O dimers to form Fe$^{3+}$O tetramer in the nucleation channel, possibly by interfering with the correct orientation of Fe$^{3+}$O dimers, after they leave the active sites.

Val$^{12}$ and Thr$^{149}$, situated near each other and near the exits of the Fe$^{3+}$ nucleation channels, at the cavity entrance, are ~25 Å from Fe$^{2+}$ entry channel exits into the cavity. They most likely influence Fe$^{2+}$/O$_2$ oxidoreductase catalysis and Fe$^{3+}$O mineral nucleation through effects on subunit conformations along the long axes of each subunit bundle and at subunit-subunit interfaces, as well as another subunit adjacent to the one with Thr$^{149}$ (Fig. 1B). A role for uncharged residues in moving ions through channel proteins has recently been demonstrated in a Kv2.1 channel (41). The Val$^{12}$ and Thr$^{149}$ are in different subunits (11), interactions between pairs of subunits may be key in moving Fe$^{3+}$O through ferritin nucleation channels. Such an idea is supported by the inhibitory effects of subunit cross-links between pairs of ferritin subunits on Fe$^{3+}$O/mineral formation (42).

An active role of H ferritin subunits in ferritin mineral nucleation and ordered iron mineral growth (11) provides an explanation for the physiologically significant and different combinations of catalytically active (H) and inactive (L) ferritin subunits in animal ferritins and the coincident variations in iron mineral order (43, 44). Ordered/more crystalline ferritin iron minerals will dissolve more slowly (release iron more slowly) than disordered ferritin minerals, which explains why increased L ferritin subunits increased cell proliferation: iron was released faster for critical iron proteins such as ribonucleotide reductase, which is rate-limiting for DNA synthesis. Decreased L subunits deceased cell proliferation (43). Moreover, heart ferritin, which functions in highly oxygenated tissue, has more H subunits, more antioxidant activity, and more crystalline (slow iron release) mineral (44). However, liver ferritin, which must release iron for other tissues, has a large number of L subunits and less ordered mineral. The disordered growth of ferritin iron minerals with large numbers of L subunits such as that from liver, is attributable to protein-independent, inorganic (45).

Conserved amino acids, both hydrophilic and hydrophobic, along the entire length of each ferritin four α-helix subunit bundle contribute to function in ferritin protein nanocages, based on the new data in this report, and contrasting with earlier studies that have focused on the functions of localized clusters of conserved amino acids related, e.g. to catalysis or mineralization (5, 38). Previously, conserved amino acids in ferritin that were not in localized clusters such as the oxidoreductase sites were assigned roles in folding and stabilizing the unique ferritin cage structure, with the exception of those around the 3-fold channels shown to regulate Fe$^{2+}$ release from the mineral and the cage (5) or those theoretically shown to create local electrostatic gradients for Fe$^{3+}$ entry through the ion channels (32). The amino acids studied here, which all have functional effects on iron oxidation and mineralization, are distributed over a distance of ~50 Å. From the Fe$^{2+}$ entry channel, formed by three subunits at the 3-fold cage axes, to the nucleation channel exits, at the other end of the subunit 4-helix bundles around the 4-fold cage axes, where mineral nuclei emerge, conserved ferritin subunit amino acid residues influence the process of iron biomineralization. How these studies relate to the ferritins with different active sites and/or cage locations as in bacteria and archaea is not known (46, 47). However, the results here suggest that the unusually high cage symmetry in ferritins, especially in the more highly evolved eu-ukaryotic ferritins, contributes to function emphasizing the role of cage geometry itself in function.

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