Localized Unfolding at the Junction of Three Ferritin Subunits

A MECHANISM FOR IRON RELEASE?*

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How and where iron exits from ferritin for cellular use is unknown. Twenty-four protein subunits create a cavity in ferritin where iron is concentrated >1011-fold as a mineral. Proline substitution for conserved leucine 134 (L134P) allowed normal assembly but increased iron exit rates. X-ray crystallography of H-L134P ferritin revealed localized unfolding at the 3-fold axis, also iron entry sites, consistent with shared use sites for iron exit and entry. The junction of three ferritin subunits appears to be a dynamic aperture with a “shutter” that cytoplasmic factors might open or close to regulate iron release in vivo.

Ferritins are vesicle-like assemblies of 24 polypeptide (4-helix bundle) subunits that concentrate iron in cells by directing the formation of a ferric mineral in the hollow protein interior (8 nm diameter) (1–3). Effective cellular iron concentrations >1011 times the solubility of the ferric ion are achieved by ferritins, which are found in microorganisms, plants, and animals. The complexity and the sophistication of the genetic regulation of the ferritins, involving both DNA and mRNA (4–7), emphasize the central role of iron and ferritin in life. Rates of Fe(II) oxidation, translocation of Fe(II) and Fe(III) (1.0–2.0 nm), and mineralization are all controlled by the protein (1, 2). Fe(II) release from ferritin following reduction of the mineral is slow and poorly understood (8, 9) but is important for the biosynthesis of iron-proteins, such as those required in respiration, photosynthesis, nitrogen fixation, and cell division, (1, 2) and as dietary iron (10). How and where the iron exits from ferritin in vivo is not known.

We now show that localized unfolding in the assembled protein, at sites of cooperative subunit interactions, can increase the rate of exit of iron from ferritin. When conserved leucine 134 was replaced by proline (L134P), the protein assembled, oxidized Fe(II), and time-sequentialized Fe(III), but the time for complete dissolution of mineral (480 iron) in vitro was greatly decreased (5 min compared with 150 min for the parent protein). X-ray diffraction studies of crystals of H-L134P ferritin showed a flexible region localized near the termini of two subunit helices (C, D), which form the interfaces of subunit trimers and a channel. The results indicate that iron can exit from ferritin at the trimer subunit junction. A possible mechanism for regulated iron release in vivo could be localized disorder in the assembled protein, enhanced by cytoplasmic changes with effects analogous to the effect of H-L134P.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Ferritin Proteins—The coding sequence for H ferritin, H-L134P, K82Q, and H-L134P, R86Q were obtained by the mutagenesis of PJD5F12L134P sequence (12) with a Chameleon™ double-stranded, site-directed mutagenesis kit (Strategene). The oligonucleotides, 5'-CACCTGTTCTCC-CAGGTATCAGTCTCC-3', 5'GCTCTGGTTCCTGGA-CATCCTGAGA-CATCCTGAGA-3', and 5'GCTCTGGTTCCTGGA-CATCCTGAGA-3', were used as the mutagenic primers. The coding sequences for H-L134P, K82Q or H-L134P, R86Q were subcloned into a PET-3a vector (Novagen). The coding sequence for H ferritin was subcloned into a PET-9a vector (Novagen) to enhance expression, which was low in the PET-3a vector (Novagen). All of the recombinant ferritin proteins were expressed and purified as described previously (11–13).

Kinetic Studies of Iron Uptake and Release—The method for iron uptake was described previously (13). In iron release experiments, apoferritins (2.08 μm) were mineralized by the addition of a solution of ferrous sulfate at the iron/protein = 480:1 M/MOPS (pH 7.0) and 0.2 mM NaCl, followed by incubation for 2 h at room temperature and then incubation overnight at 4 °C (11–13). Iron release was initiated by the addition of 25 mM bipyridil, 25 mM FMN, and 2.5 mM NADH to reconstituted ferritin in 0.1 M MOPS (pH 7.0) and 0.2 mM NaCl (14, 15). The amount of iron released from ferritin was monitored at room temperature by the absorbance at 522 nm of the Fe(II)-bipyridil complex.

X-ray Diffraction—Crystals of H-L134P, K82Q or H-L134P, R86Q ferritin were obtained by the hanging drop method. The crystalization conditions were optimized, beginning with the sparse-matrix sampling method (16), with the best H-L134P, K82Q crystals being obtained by mixing 5 μl of a 10 mg/ml protein solution with an equal volume of 15% 2-methyl-2,4-pentanediol, 0.02 mM CaCl2, 0.01 mM sodium acetate buffer, pH 4.6. The best H-L134P, R86Q crystals were obtained in 25% isopropl alcohol, 0.1 mM sodium cacodylate, 0.2 mM MgCl2, 2° Bipyrimal-shaped crystals, ~0.5 × 0.2 mm, formed within 2 weeks. Diffraction data were collected both with a conventional rotating anode x-ray generator and a Siemens area detector and on beamline X-12C at National Synchrotron Light Source, Brookhaven National Laboratory. The data were processed by XGENEN and DENZO, and refinements were carried out with X-PLOR (17). Data statistics and final refinement statistics are listed in Table I. Program O (18) was used for model building and fitting to the electron density map.

RESULTS

Ferritin subunits fold and assemble spontaneously as iron-free proteins (1, 2, 19–24) with buffer in the cavity (22, 23).

1 In nature, the iron minerals in ferritin range in average size from

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TABLE I
Data and refinement statistics for frog H-L134P, K82Q ferritin

| Parameters                  | Value            |
|-----------------------------|------------------|
| Space group                 | F432             |
| Cell dimensions (Å)         | a = b = c = 182.8Å |
| Resolution limits (Å)       | 40–2.4 Å        |
| Number of reflections (unique) | 142,013 (10,541) |
| Rmerge (%)                  | 8.7              |
| (I)/σ                       | 6.2              |
| Solvent content (%)         | 62               |
| Number of atoms in the final model | 1345         |
| Number of reflection involved in refinement | 8392 |
| R value (%)                 | 20.1             |
| Rfree value (%)             | 24.1             |
| r.m.s.d. ideal bond length\(a\) | 0.012        |
| r.m.s.d. ideal bond angle   | 2.61             |
| r.m.s.d. ideal dihedral angle | 21.18        |
| r.m.s.d. ideal improper angle | 0.90          |

\(a\) r.m.s.d., root mean square deviation.

800 to 2500 iron/molecule and from microcrystalline to amorphous, particularly when the phosphate content is high (1, 2). The heterodispersity of mineral size in natural ferritin varies from 10 to 4000 iron/molecule and is much greater than in minerals reconstituted to 480 iron/molecule from the iron-free protein (15). To study iron release, Fe(II) was added to the recombinant ferritin proteins to form iron minerals of constant average size (480 iron/molecule).

The role of ferritin in iron release was compared for proteins that differed over 1000-fold in iron uptake rates and in the mechanism of iron oxidation (fast-ferroxidase sites and nucleation sites present, slow-only nucleation sites present). The initial rates of iron oxidation by proteins with ferroxidase sites and nucleation sites present, slow-only nucleation sites present. were 0.99 ± 0.02 (\(A_{1000/s}\)) for H ferritin, 0.081 ± 0.038 (\(A_{1000/s}\)) for H-L134P ferritin (13), and 1.55 ± 0.22 (\(A_{1000/s}\)) for M ferritin (13). The slower rate of iron uptake/ferroxidation for the H-L134P protein is coupled to a shift in the absorbance maximum of the initial Fe(III) complex from 650 to 550 nm (Fig. 1) and a slower decay (12, 13), but the formation rate is still within the range for fast ferritins and 100-fold faster than L ferritin (13). L ferritin has no specific ferroxidation site, and the initial rate of oxidation is 0.0037 ± 0.0017 (\(A_{1000/s}\)) (11).

Iron exit from the mineralized recombinant ferritins was triggered by reduction of Fe(III) with FMNH\(_2\)/NADH and trapping the Fe(II) as the Fe(II)-bipyridyl complex. No Fe(II)-bipyridyl complex was detected until the reductant was added, and essentially all (96%) of the Fe(II)-bipyridyl complex that formed could be separated from the protein by ultrafiltration (data not shown). Previous studies have shown that the reductive release of iron is independent of reductant/chelator size (8, 9). Rates of iron release from recombinant ferritins were biphasic (Fig. 2A). There was little difference in iron release rates among the recombinant ferritins with natural sequences.

In contrast to the wild type proteins (H, M, L), substitution of leucine 134, conserved in all ferritins (1, 2), with proline had a faster initial rate of iron release (Fig. 2) that was essentially monophasic. Complete dissolution of a ferritin mineral of 480 iron and release as Fe(II)-bipyridyl only required 5 min in the H-L134P protein compared with 150 min for the parent protein with L134.

Crystals formed by frog H ferritin with L134P showed localized changes in the subunit packing at the junction of three
subunits (Figs. 3 and 4). Because the crystals of H-L134P had a very large and complex unit cell, also observed for frog M ferritin (25), glutamine was substituted for lysine at position 82 (K82Q) or arginine at position 86 (R86Q) in the BC loop to allow formation of cubic crystals as previously observed for human H-K82Q ferritin (21). This crystal form of frog H-L134P, K82Q was isomorphous to human H-K82Q (21) and frog L ferritin crystals, making the structure solution straightforward. The electron density maps for both frog H-L134P, K82Q and H-L134P, R86Q ferritins are of high quality except at the ends of helices C and D and CD loop, residues 114–133. Despite thorough efforts to model this region using different conformers from the Protein Data Bank, the weak density remained throughout refinement, in contrast to all other high resolution ferritin structures determined to date (21–24). 1.8-Å resolution synchrotron data collected at Brookhaven National Laboratory showed the same weak density region, and as expected for a truly disordered region, the better the data quality, the weaker the density in this region. The data from this region were excluded from the refinement.

**DISCUSSION**

The sites in the ferritin structure for reductive iron release are different from the rapid oxidation sites: iron release rates differed little in fast (H, M) and slow (L) ferritins with natural sequences (Fig. 2), but oxidation rates varied over a wide range (11–13). Rapid oxidation occurs in the center of the 4-helix bundle that forms the ferritin subunit and involves residues from helices A, B, and C (1, 2, 21, 24). In contrast residue 134, which when changed to proline altered rates of iron exit, is near the N terminus of helix D (21–24). The backbone nitrogen of Leu-134 is hydrogen-bonded to the carbonyl oxygen of Leu-129, whereas the side chain of Leu-134 has a hydrophobic interaction with Leu-110 in helix C. A kink in the backbone of the helix D adjacent to residue 134, produced by deviations from the standard dihedral angles at positions 132 and 133 (24), determines precise positioning of the interhelical and intersubunit interactions of the D helix near the subunit trimer interface. The L134P protein will have changed intrasubunit hydrophobic interaction, hydrogen bonds, and intersubunit interactions.

H-L134P ferritin assembled from 24 subunits will have eight regions of local flexibility distributed symmetrically around the surface of the molecule caused by the changes at each junction of three subunits (Fig. 3). In other proteins, introduction of proline into a peptide can be without functional effect (26), the proteins adjusting conformation to accommodate the change (26–28). However, in ferritin, the effect of the proline substitution is amplified by disrupting both cooperative interhelical and intersubunit interactions (Fig. 3). The unfolded structure at the 3-fold axes can alter the behavior of assembled ferritin H-L134P in solution. For example, the flexibility of the structure will propagate to the channels for iron entry; the mutation led to a shift in the absorbance maximum of the initial Fe(III) complex (Fig. 1), a decreased rate of oxidation (13), and a decreased decay rate of the initial Fe(III) complex that fortuitously permitted its identification as Fe(III)-tyrosine (12). Whether Fe(III)-tyrosine is specific to H-L134P ferritin protein

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4 Two numbering systems have been used for ferritin sequences. The initial numbering scheme, based on the horse spleen ferritin sequence (34), can be used for all sequences (1, 2), but separate numbering for human ferritin sequences has also been used (21, 24). The uniform numbering system is used here; thus 4 must be added from residue numbering for the human H ferritin sequence to relate to the H/L equivalent numbering system.
Disorder at the 3-fold axis of the assembled H-L134P protein appeared to increase the accessibility of the mineral core to solutes such as reductants and chelators (Fig. 2). The structure of ferritin H-L134P contrasts with the high degree of order in the same region of crystals of recombinant H or L ferritins from frog, horse, and human wild type (Fig. 4) (21–24) or with amino acid substitutions in the A or B helices (22, 23). Conserved residues with carboxylate side chains contributed from three subunits line the channel at the 3-fold axis (1, 3, 21–24, 34), which is the site for both iron entry (1, 2, 29–33, 35) and iron exit (Figs. 2–4). In addition to metal ions, the carboxylate side chains at the junction of three subunits could facilitate exit and entry of protons involved in mineralization (hydrolysis) and mineral dissolution (−2.5 H+Fe).

Subunit interactions in ferritin occur between dimers and tetramers, as well as trimers (21–25, 34). The assembly of H-L134P ferritin subunits into the typical supramolecular ferritin structure, except at the subunit trimer junctions, emphasizes the contributions of the subunit dimer and tetramer interactions to the structure (1, 2, 36); subunit trimer interactions contribute more to entry and exit of iron. Localized flexibility of ferritin at the subunit trimer junctions, caused by substitution of proline for conserved leucine 134, acts like a camera shutter increasing the aperture. Regulated iron release in vivo could result from cytoplasmic molecules causing similar conformational changes in ferritin.

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