Ferritin Contains Less Iron ($^{59}$Fe) in Cells When the Protein Pores Are Unfolded by Mutation

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Ferritins are cytoplasmic protein cages with an internal iron-oxide mineral. Gene deletion is embryonic lethal in mice (1); ferritins concentrate iron over the $10^{14}$-fold concentration gradient between cell need and iron solubility under physiological conditions (2, 3). During iron mineralization, ferritins use both Fe$^{2+}$ and O$_2$ and, in many pathogenic bacteria, Fe$^{2+}$ and hydroperoxide (4). Thus, ferritins also minimize oxidative stress by consuming both of the substrates that cause free radical damage in cells (Fenton reactions). Ferritin genes are now known to be part of the antioxidant response gene family (5, 6), in addition to being part of the iron responsive family (7, 8). The structure of ferritin protein cages includes the catalytic (“ferroxidase”) sites related to diiron protein cofactor sites (2, 3, 9) and eight pores for Fe$^{2+}$ entry (10, 11). The ferritin pores are gated to control localized folding/unfolding and protect the ferric mineral from reductants and Fe$^{2+}$ exit when they are closed/folded (12–15).

How cells regulate the recovery of iron from the ferritin mineral is only partly understood. A recent study in cells rendered iron-deficient by overexpression of the iron export protein ferroportin showed that iron was released from cytoplasmic ferritin followed by protein ubiquitinylation and proteasomal degradation (16). A number of other studies used cells with iron excess that increased the amount of ferritin protein mediated by increased DNA transcription (6), increased mRNA translation (7, 8), and decreased protein turnover (17, 18) and increased ferritin accumulations in lysosomes from autophagocytosis (19). Under such circumstances, lysosomal stability and lysosomal proteases contribute to recovering iron from ferritin, as well as from other iron proteins (16, 18, 19). In normal cells then, mechanisms exist for enhancing the removal of iron from the ferritin nanocages when cell need increases, as in growth or iron deficiency (16, 20–22).

Removing iron from ferritin in solution is very slow, even when reductants are added that mimic cytoplasmic reductants (12, 23–25), modeling well the slow rates of iron removal in vivo (26). However, mutation of any one of a set of four highly conserved amino acids at the junction of three subunits that form the pores greatly increases the rate of removing iron from ferritin when a reductant such as NADH/FMN is added (12, 13). Apparently, selective unfolding of the ferritin protein cage around the pores in wild type proteins is induced by mild heat or low concentrations of urea (CD spectroscopy) or in proteins encoded in mutated sequences (x-ray crystallography and CD spectroscopy), whereas integrity of the protein cage is maintained, to increase reduction of the insoluble ferric mineral to soluble Fe$^{2+}$ (12, 14).

We now report the effect of a pore unfolding mutation on the relative amount of $^{59}$Fe in the mutant protein compared with wild type, overexpressed to the same concentration in HeLa cells. Mutating leucine 138 to proline in human H ferritin, necessary because the well characterized frog ferritin H mutation at the homologous site, L134P, could not be expressed in cultured human cells, created a protein with similar solution properties to the frog model as follows: increased (17-fold) iron

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removal in solutions and decreased α-helix content, showing the conservation of ferritin pore structure/function. The amount of iron (59Fe) in the mutant human ferritin H was significantly (p < 0.01) lower than wild type ferritin when overexpressed to the same level in HeLa cells. Moreover, the 59Fe in cells expressing the mutant ferritin was significantly more accessible (p < 0.01) to chelators (Desferal®) compared with cells expressing wild type ferritin H. The combined results indicate a contribution of pore unfolding to the recovery of iron from ferritin minerals in vivo.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmids and Protein Purification**—Copy DNA for the coding region of human ferritin H (HuFtH),3 PCR-amplified from Marathon Ready HeLa cell cDNA (Clontech) and cloned in pUC19 plasmid (pUC19-FtH), was subcloned at the NdeI/BamHI site of pET3a (Novagen) for amplification in Escherichia coli, using the following two primers: forward, 5′-GACATATGACGACCGGTCACCAC-3′, and reverse, 5′-GTTGGATCTTAGCATTATACGTCGTCACAG-3′, respectively. A mammalian expression vector for HuFtH was constructed using pcDNA3.1 Directional TOPO expression kit (Invitrogen). Primers for HuFtH cDNA amplification from pUC19-FtH were as follows: forward, 5′-CACCATTCCACGAGTCCGC-3′, and reverse, 5′-TTAGCTGCTCCTCTTCCATGTCGCTG-3′, respectively. The amplified cDNA was ligated to the TOPO cloning site of pcDNA3.1/V5-His-TOPO vector according to manufacturer’s instructions. The codon for leucine 138 (leucine 134 in E. coli) expression and pET3a (E. coli protein expression) plasmids. Primers used were as follows: forward, 5′-CATGGAACACATATCCCGAACGAGTCCGC-3′, and reverse, 5′-GCTTTACCTGTCATTCCGGGTAAATGTGTCTCATATG-3′, respectively. All expression plasmids were analyzed (University of California, Berkeley, DNA Sequencing Facility) to confirm DNA sequence. The wild type and the pore mutant (HuFtH L138P) ferritins were expressed in E. coli BL21(DE3)pLysS (Stratagene) and were purified by the method described previously (27), using LB medium.

**Iron Uptake and Release**—Iron uptake, Fe2+ oxidation/mineralization, was monitored as the change in A250 nm. Ferritins were mineralized by mixing a freshly prepared ferrous sulfate solution (in 1 molar HCl) with recombinant apoferritin, to a final concentration of 4.16 μM protein cages, 2 mg iron (480 mineralized iron/protein cage), and 0.1 molar MOPS, pH 7.0, 0.1 molar NaCl, as described previously (12).

Iron release was initiated by adding reductant (NADH/FMN) (23) with bipyridyl as a chelator and reporter, as described previously (12, 14). Final concentrations were 0.1 mM MOPS, pH 7, 0.1 mM NaCl, 1.04 μM mineralized ferritin, 0.5 mM Fe3+, 5 mM NADH/FMN, and 2.5 mM bipyridyl. Fe2+ release was monitored as formation of the Fe2+-bipyridyl complex, outside the protein cages, as the changes in A250 nm. Initial rates (μmol liter−1 s−1) were calculated using the molar extinction coefficient for Fe2+-bipyridyl, 8430 M−1 cm−1 (23), from the slope of the linear plot (θ222 = 0.95–0.99) from 0 to 1 min, as we described before (14). The data are presented as the average of the results from three independent protein preparations for each type of ferritin with >2 replicates for each set of proteins, and the error is the standard deviation.

**CD Spectroscopy**—CD spectra were recorded on a P-180 spectrophotometer (Applied Photophysics, Surrey, UK) at 20 °C. The protein concentrations were 0.05 μM nanocages in 10 mM sodium phosphate buffer, pH 7.0. Spectra were measured from 200 to 300 nm with 0.1 nm resolution in a quartz cell with a 1-cm path length; data from 10 replicates were averaged. The α-helical content was estimated from the mean molar ellipticity at 222 nm (θ222) as follows: α-helical content (%) = −[(θ222 + 2340) × 100]/30300 (28).

**59Fe-Transferrin Labeling**—59FeCl3 in 0.5 molar HCl (PerkinElmer Life Sciences) was mixed with an aqueous solution of disodium nitrilotriacetic acid at a molar ratio of 1:5, as described in Ref. 29, to maintain iron as mononucleos complex. Lyophilized apotransferrin (human) (Sigma) was dissolved in HEPE buffer (250 mM HEPE, 10 mM sodium bicarbonate, pH 8), and 59Fe-nitrilotriacetic acid added, protein/iron = 1:19, to ensure all the 59Fe was bound to protein. After incubation at room temperature for 1 h and overnight at 4 °C, the buffer was exchanged for PBS using a PD-10 salt exchange column (GE Healthcare); the solution filter sterilized and protein concentration measured by the Bradford method (Bio-Rad). All the 59Fe was in transferrin, judged by native PAGE (4–12% Tris-glycine gel) (Invitrogen), autoradiography, and densitometry (ImageQuant, GE Healthcare).

**Cell Culture, Ferritin Overexpression, Labeling with 59Fe, and Effects of Deferoxamine Mesylate**—HeLa cells were maintained in modified Eagle’s medium (Invitrogen) supplemented with fetal bovine serum (10%) (Atlanta Biologicals), penicillin/streptomycin (100 units/ml) (Invitrogen), modified Eagle’s medium supplemented with nonessential amino acid (0.1 mM) (Invitrogen), and sodium pyruvate (1 mM) (Invitrogen) at 37 °C, 5% CO2. For transfection, cells were seeded at a density of 105 cells per well in antibiotic-free medium in 6-well plates. After 20 h of incubation, cells were transfected with pcDNA3.1/V5-HisA (Invitrogen) with or without ferritin sequences, using Lipofectamine 2000 transfection reagent (Invitrogen) at a DNA (μg) to reagent (μl) ratio of 4:20. Transient transfection of HeLa cells was used to avoid the possible toxicity of the ferritin pore mutant suggested by the failure to transfect the frog H L134P pore mutant ferritin and by the differential expression we observed of human wild type and L138P protein in HepG2 cells.

59Fe-Transferrin was added, after removing the transfection medium and washing twice with PBS and adding fresh medium, 20 h after transfection. The cells were labeled for 2 h, and the transfection concentration was 0.1 μM 59Fe-transferrin. After

3 The abbreviations used are: HuFtH, human ferritin H; DFO, deferoxamine B mesylate (Desferal®); HuFtH-WT, human wild type ferritin H; HuFtH L138P, human ferritin H with pores unfolded by substitution of a conserved amino acid in the pore/channel; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay.
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Removing the labeled medium and washing three times with PBS, $^{59}$Fe cells were collected in PBS by scraping, and the cells were sedimented by centrifugation. $^{59}$Fe was measured on the cell pellets in a solid state scintillation counter. In some experiments, medium with 10 μM Desferal® (Sigma) was added to washed $^{59}$Fe-labeled cells. The incubation was then continued for 20 h, followed by washing and collection of cell pellets and measuring $^{59}$Fe in cell pellets and culture medium. Cytoplasmic extracts were obtained from cell pellets resuspended in 40 μl of PBS, lysed by five freeze–thaw cycles, and clarified by centrifugation (13,000 rpm, 15 min, 4 °C). $^{59}$Fe was analyzed by scintillation counting, and protein concentrations were measured by the Bradford method (Bio-Rad).

$^{59}$Fe-proteins (20 μg of total protein) were resolved with native PAGE (4–12% Tris-glycine gel) (Invitrogen) and detected with autoradiography. Ferritin concentrations were measured with ELISA (Laguna Scientific) with a human liver ferritin standard. Values for endogenous ferritin concentration and $^{59}$Fe-ferritin in cell extracts were determined in parallel experiments. Visual examination of cells after iron or chelator treatment indicated no change in cell health. All experiments were repeated three times with independently plated cell cultures. The data presented are averages, and the error is presented as the standard deviation.

Western Blotting/Immunoprecipitation—Ferritin protein expression, wild type and mutant, was analyzed in the HeLa cell extracts described above, with 20 μg of protein. Protein separations used SDS-PAGE (4–12% Bis-Tris gels) and native-PAGE (4–12% Tris-glycine gels), blotting onto polyvinylidene difluoride membranes (X-Cell Blot Module, Invitrogen). After blocking in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 2.5 mM KCl, and 0.05% Tween 20) with 5% skim milk, ferritin was detected using rabbit anti-human ferritin IgG (Novus Biologicals) as the primary antibody (1:50,000 dilution) and goat anti-rabbit IgG HRP conjugate (Bio-Rad) as the secondary antibody (1:50,000 dilution). Antibody binding was detected with an Immun-Star™ HRP substrate kit (Bio-Rad). β-Actin was measured on the same blots by stripping ferritin antibodies with three changes of 0.2 M glycine, pH 2.8, over 30 min, and three washes with TBST buffer over 15 min, followed by the addition of HRP-monoclonal anti-β-actin antibody (Abcam) for 1 h at room temperature and development with Immun-Star™ HRP substrate (Bio-Rad). Human ferritin was applied to the gels over the concentration ranges of ferritin in the cell extracts as standards.

To immunoprecipitate ferritin from cell extracts, 0.5 mg of protein was used, extracted from pools of cells obtained from three culture wells, suspended in 0.6 ml of PBS, and lysed as described above. Rabbit anti-human ferritin IgG (Novus Biologicals) was used with a protein G immunoprecipitation kit (Sigma), according to manufacturer’s instructions. Ferritin was eluted in 100 mM glycine, pH 2.5, and both $^{59}$Fe and ferritin protein were quantitated (ELISA, Laguna Scientific, and human liver ferritin standard). Data from different experiments were normalized by subtracting endogenous ferritin values (transfection with empty plasmid) in each experiment.

RESULTS

Changing a Conserved Leucine to Proline Unfolds the Pores in Recombinant Human Ferritin H—Human ferritin H was selected to study the effects of ferritin pores unfolded by mutation because the more extensively characterized (12–14, 30) frog ferritin H pore mutant and frog H wild type could not be expressed in either HeLa, Caco-2, or HepG2 cells. Transfection of these cells with the mammalian expression vectors encoding frog ferritin H (wild type and H L134P, the pore mutant) severely affected cell survival, and the expression of these proteins was not detectable in the viable cells. We then turned to human ferritin H where the N-terminal extension of four residues, compared with horse or frog ferritins (31), shifts residue numbers by +4. Thus, we constructed human ferritin H L138P, which is homologous to L134P, a mutation in one of the conserved, pore gating residues. All four ferritin residues identified as affecting ferritin pore folding/unfolding are conserved not only in human and frog ferritins but all other known ferritins; the overall sequence identity of frog and human ferritin H is 65% (17).

Assembly of the recombinant human ferritin H L138P ferritin protein cage was normal (gel filtration) as in the homologous frog ferritin mutant (12). Moreover, the protein mineralization rate was essentially identical for wild type and H L138P mutant proteins, as observed previously (13). However, the iron release rate was increased (Fig. 1) similarly to the frog mutant protein (12), which confirms the role of the conserved leucine. The rate of iron release from human ferritin L138P was >4-fold larger than the wild type protein (Fig. 1). Moreover, the time required to reduce and release 50% of the mineral to chelators outside the protein cage (12) was 17-fold faster for human ferritin H L138P than for the wild type protein (Fig. 1, B and C).

A physical consequence of pore unfolding in ferritin is a loss of α-helix, detectable by CD spectroscopy (14); the overall α-helix content of ferritin is ~70%. A decrease of 8% in the helix content of the human H L138P ferritin (Fig. 1D) was observed (CD spectroscopy), close to that predicted if the eight pores were completely unfolded. The results are comparable with those in the frog H L134P. Thus, the solution properties of decreased helix content and rapid iron release, triggered by a natural reductant, are comparable for both the human H L138P and the homologous frog H L134P ferritins, emphasizing the ability of one of the conserved pore residues to alter iron release from ferritins with varied backbone sequences, by changing helix stability around the pores.

Pore Mutant Ferritin, Expressed and Assembled in HeLa Cells, Has Less $^{59}$Fe than Wild Type—We investigated the function of gated pores in vivo, by comparing $^{59}$Fe uptake and distribution in HeLa cells where HuFtH-WT or pore mutant, human ferritin H were overexpressed to comparable levels (Fig. 2); $^{59}$Fe was provided as $^{59}$Fe-transferrin. HeLa cells were selected as the test cell line because expression from transfected plasmids of mutant and wild type human ferritins was similar (Fig. 2), contrasting with HepG2 cells where expression of the mutant human ferritin was lower than wild type. The mobility of wild type and mutant human ferritins in transfected cells was identical to endogenous human ferritin in both denaturing and...
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To determine the impact of opening ferritin pores on $^{59}$Fe accumulation in vivo, we labeled HeLa cells with $^{59}$Fe-transferrin (0.1 mM) for 2 h. There was no significant effect of the mutant protein on total cell uptake of $^{59}$Fe (Table 1). When proteins in the cell extracts were resolved by electrophoresis in native gels, all of the $^{59}$Fe in the gel migrated with ferritin protein (Fig. 2B). $^{59}$Fe in heme (ethyl acetate/HCl extract) was undetectable (<2% of total $^{59}$Fe). The data emphasize the relatively large fraction of cell $^{59}$Fe that is in ferritin compared with heme proteins, FeS proteins, and the “labile”/chelatable iron pool; the amount of $^{59}$Fe in total soluble protein (cpm/ng protein) can be used to assess $^{59}$Fe-ferritin. When the counts/min $^{59}$Fe/ng of soluble protein were compared in cells expressing the wild type and pore mutant ferritins, the value was significantly higher ($p < 0.05$) in cells overexpressing wild type ferritin (Table 1). The data indicate that the pore mutant ferritin retained less $^{59}$Fe in vivo, because in solution at least iron uptake and mineralization were normal in the human ferritin H L138P mutant (Fig. 1). Direct determination of $^{59}$Fe in ferritin iron by immuno-precipitation (cpm/ng ferritin) confirmed the results of the $^{59}$Fe/ng of soluble protein. Ferritin with unfolded pores expressed in HeLa cells and analyzed after 2 h of labeling with $^{59}$Fe-transferrin had only ~28% $^{59}$Fe of the wild type ferritin (Fig. 3B), close to the ~5-fold faster Fe$^{2+}$ exit rates observed in vitro (Fig. 1B).

To see if longer incubation of the cells would further decrease the $^{59}$Fe/ng protein in cells overexpressing ferritin H with the pore mutation L138P, cells were incubated for 20 h after the removal of $^{59}$Fe-transferrin. At 20 h post-labeling
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TABLE 1

Unfolding ferritin pores by mutation decreases ferritin $^{59}$Fe in HeLa cells

| Distribution of $^{59}$Fe in HeLa cells (0.1 μM $^{59}$Fe-transferrin, 2 h)* | Transfection controlb | Wild type (HuFtH-WT)b | Open pore mutant (HuFtH L138P)b |
|---|---|---|---|
| Total cell $^{59}$Fe (cpm × 10$^{-3}$) | 4.77 ± 0.29 | 5.06 ± 0.33 | 4.62 ± 0.31 |
| Ferritin concentration (ng of ferritin/mg of total protein, ELISA) | 28.0 ± 6.14 | 115 ± 19 | 118 ± 20 |
| Cytoplasmic $^{59}$Fe (cpm/ng total protein) | 14.1 ± 1.4 | 20.7 ± 1.3*** | 16.0 ± 0.60 |
| $^{59}$Fe in the insoluble fraction after cell lysis (% of total) | 60.3 ± 1.3 | 47.3 ± 2.0*** | 54.6 ± 1.9 |

* The data are average from three independent experiments, and the errors are the standard deviation.

b Transfection control is plasmid with no insert; HuFtH-WT is plasmid encoding human wild type ferritin H; HuFtH L138P is plasmid encoding human ferritin H with pores unfolded by substitution of a conserved amino acid in the pore/channel.

d Data are significantly different from pore mutant (HuFtH L138P) or transfection control: ***, $p < 0.005$, and *, $p < 0.05$.

FIGURE 3. Mutation to unfold ferritin pores decreases the specific activity of ferritin in HeLa cells. Ferritin was immunoprecipitated from 0.5 mg of soluble protein (obtained from pooling 3 wells of cells, see “Experimental Procedures”) after transfecting and labeling with $^{59}$Fe (2 h incubation with $^{59}$Fe-transferrin); ferritin was eluted from the precipitates (100 mM glycine, pH 2.5) and analyzed for $^{59}$Fe and for ferritin protein by ELISA. Values for endogenous ferritin protein (1.6 ± 0.1 ng) and $^{59}$Fe-ferritin (690 ± 20 cpm) in eluates were determined in parallel experiments and used to normalize the data between experiments. A, total $^{59}$Fe eluted from the immunoprecipitate (IP). B, specific activity (cpm/ng ferritin) of overexpressed (plasmid encoded) ferritin. The data are the average of data obtained from three independent sets of plated cell cultures, and the errors are the standard deviation. Values are significantly different from wild type (HuFtH-WT): **, $p < 0.01$.

with $^{59}$Fe, cytosolic $^{59}$Fe was ~50% of the value at 2 h, 11.1 ± 1.1 and 7.9 ± 0.3 cpm/ng for cells with wild type or mutant ferritin compared with the 2-h values of 20.7 ± 1.3 and 16.0 ± 0.6 cpm/ng protein, respectively, for cells with wild type and mutant ferritin (Tables 1 and 2). In the presence of DFO for 20 h post-labeling with $^{59}$Fe-transferrin, the cytosolic $^{59}$Fe values were lower than 20 h without DFO, 8.1 ± 0.7 and 5.7 ± 0.4 cpm/ng protein, respectively (Tables 1 and 2). In all cases, the counts/min/ng soluble proteins was significantly lower ($p < 0.05$) for cells expressing open pore ferritin. In addition, the ratio of $^{59}$Fe/ng soluble protein for cells with mutant and wild type protein was constant, was 0.77 at 2 h of $^{59}$Fe labeling, and was 0.70–0.71 20 h after removing $^{59}$Fe-transferrin with or without DFO emphasizing the contributions of pore gating to the iron ($^{59}$Fe) in ferritin.

$^{59}$Fe Is More Accessible to Chelator Removal in HeLa Cell Ferritin H L138P (Pore Mutant)—Because ferritin with the mutated pore had less $^{59}$Fe than wild type ferritin (Table 1 and Figs. 2B and 3), we hypothesized that more iron would be accessible to iron chelators in the HeLa cells expressing ferritin with unfolded pores than in cells expressing wild type ferritin, as in solution (Fig. 1) (2, 30). Moreover, the ratio of $^{59}$Fe/ng soluble protein at 2 and 20 h were comparable in the absence of a chelator.

Deferoxamine mesylate, the therapeutic Fe$^{3+}$ chelator that predominantly targets iron in the labile pool of cells (26) was added to the HeLa cell incubation medium. DFO is widely used to create iron deficiency in cultured cells (16, 18). The DFO concentration used (10 μM) decreased immunodetectable ferritin protein (Table 2) proportionately in the transfection controls (endogenous ferritin) and in the cells overexpressing ferritin encoded in plasmids. However, DFO caused a disproportionately large decrease in the cytoplasmic $^{59}$Fe from cells with the open pore mutant ferritin protein compared with wild type (Table 2). In addition, a significantly larger percentage of $^{59}$Fe was in the culture medium for cells with the open pore ferritin after 20 h of incubation with the chelator, indicating that more of the $^{59}$Fe was accessible to DFO (Table 2). Interestingly the amount of $^{59}$Fe in the insoluble, cytoplasmic fraction of cells without DFO in the medium was higher in cells expressing the L138P mutant ferritin (Table 1) possibly reflecting $^{59}$Fe in transit. However even after 20 h of incubation in DFO, the amount of $^{59}$Fe remaining in the cytosol was significant (35–40%), although significantly lower for cells expressing open pore ferritin, indicating that ferritin pore gating is only one factor that influences the amount of iron ($^{59}$Fe) in ferritin.

DISCUSSION

Iron is recovered from the ferritin mineral inside the protein cage in vivo, by at least two mechanisms. During iron deficiency, iron is removed from ferritin followed by ubiquitinylating and proteasomal degradation of the protein cage (16), whereas in iron repletion/excess, autophagocytosed ferritin is degraded by lysosomal proteases, and the ferritin iron apparently is transported to the cytosol (18, 19). Iron in ferritin is protected from cytoplasmic reductants by the protein coat, and iron removal in solution is very slow (12, 24, 25, 32, 33), as it is in vivo (26). The proportional decrease in ferritin protein induced by DFO in endogenous ferritin, overexpressed wild type and overexpressed mutant ferritins (Table 2), suggests that cells accommodate to the overexpressed protein and sense the fractional decrease in ferritin concentration rather than the total amount, the regulatory mechanism (IRE regulation and/or protein degradation), or the ferritin pore structure. When the ferritin protein pores are unfolded or open, reductant access is increased and the speed of iron removal is accelerated in solution (12). The opening and closing of ferritin pores depends in part on a set of four amino acids that are conserved in all known ferritins; they appear to have no other effect on ferritin function except to control the rate of iron mineral reduction and chelation, based on studies of iron release rates, gel filtration, and protein crystallography of proteins with amino acid substitution (2, 13). Ferritin protein nanocage pores in wild type protein can be unfolded at temperatures and urea concentrations that
have no effect on the overall protein cage structure, based on CD spectroscopy and gel filtration (14, 34, 35). Ferritin pores are formed by six helices contributed by three subunits near the 3-fold symmetry axis of the protein cage, placing each pore roughly at one of the eight corners of a cube centered on the protein cage. Because each of the four conserved pore gating amino acids in a ferritin subunit are near the equivalent residues in the other two subunits that form the pore structure, the effect of a single mutation in any one gating residue is amplified by the proximity of the homologous residues in three-dimensional space (12–14). The high conservation of ferritin pore gating residues with no apparent function except controlling access between mineral and reductants (iron removal rates), as well as the recognition and unfolding of ferritin pores by a peptide selected from 10^9 in a combinatorial library (30), suggests that unfolding ferritin pores in vivo will also increase recovery of iron from the ferritin mineral.

The observations we now report support the hypothesis that selective unfolding of ferritin pores contributes to the rates of recovery of mineralized ferritin iron in vivo. We cannot exclude effects of the pore mutation on iron uptake in vivo, although they do not occur in vitro, and thus the possibility exists that our observations in vivo could include a novel change in iron entry rates into ferritin. The results show first that mutating one of the conserved residues, which stabilized the pore gates in the frog ferritin model in vitro, had the same effect in human ferritin in vitro; reduction and chelation of mineralized ferritin iron was faster in vitro, and the α-helix content was decreased (Fig. 1). Human and frog ferritin H differ in sequence by 35%. Second, the amount of 59Fe in the open pore mutant nanocaps (cpm/ng ferritin protein) was only 28% that of wild type ferritin in HeLa cells (Fig. 3), expressing equal amounts of one or the other plasmid-encoded protein. Finally, more 59Fe was available to DFO removal when cells expressed mutant, open pore ferritin (Table 2), showing that lower 59Fe in the ferritin mutant with open pores coincided with increased access to ferritin iron and/or a larger amount of 59Fe in the (chelatable) pool.

Ferritin gated pores share structural properties with channel proteins in membranes (2, 36). The recent identification, from a combinatorial library, of peptides that open or close ferritin pores, i.e. increase or decrease iron removal from ferritin, adds a functional analogy between ferritin pores and pores in peptide-regulated Ca2+, Na+, and K+ channel proteins (37). The peptide sequences occur in a number of proteins encoded in the human genome and may represent ferritin iron release chaperones that complement the recently identified ferritin iron delivery chaperone (38), but investigation of such an idea is outside the scope of this study. Three observations combined the following: (i) localized ferritin pore folding/unfolding, independent of cage structure and other functions such as catalytic ferrroxidation (2); (ii) the high conservation of ferritin pore gating amino acids with no detectable function besides regulating access between the ferritin mineral inside the protein cage and external (cytoplasmic) reductants in vitro and in vivo (Figs. 1 and 3 and Tables 1 and 2) (12, 14); and (iii) ferritin pore recognition by peptides that regulate pore folding in vitro (30) suggest that peptide or protein-binding partners of ferritin protein cages, under some physiological conditions in vivo, regulate ferritin iron pore function in analogy to other protein pores.

**REFERENCES**

1. Ferreira, C., Bucchini, D., Martin, M. E., Levi, S., Arosio, P., Grandchamp, B., and Beaumont, C. (2000) J. Biol. Chem. 275, 3021–3024
2. Liu, X., and Theil, E. C. (2005) Acc. Chem. Res. 38, 167–175
3. Lewin, A. C., Moore, G. R., and Le Brun, N. E. (2005) Dalton Trans. 21, 3597–3610
4. Chiancone, E., Ceci, P., Ilari, A., Ricabasci, A., and Stefani, S. (2004) Biochem. 17, 197–202
5. Torti, F. M., and Torti, S. V. (2002) Blood 99, 3505–3516
6. Hintze, K. J., and Theil, E. C. (2006) Cell. Mol. Life Sci. 63, 591–600
7. Hintze, M. W., Muckenthaler, M. U., and Andrews, N. C. (2004) Cell 117, 285–297
8. Theil, E. C., and Eisenstein, R. S. (2000) J. Biol. Chem. 275, 40659–40662
9. Schwartz, J. K., Liu, X. S., Tosh, B., Theil, E. C., and Solomon, E. L. (2008) J. Am. Chem. Soc. 130, 9441–9450
10. Chasteen, N. D., and Harrison, P. M. (1999) J. Struct. Biol. 126, 182–194
11. Arosio, P., and Levi, S. (2002) Free Radic. Biol. Med. 33, 457–463
12. Takagi, H., Shi, D., Ha, Y., Allewell, N. M., and Theil, E. C. (1998) J. Biol. Chem. 273, 18685–18688
13. Jin, W., Takagi, H., Pencorbo, B., and Theil, E. C. (2001) Biochemistry 40, 7525–7532
14. Liu, X., Jin, W., and Theil, E. C. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3653–3658
15. Theil, E. C., Liu, X. S., and Tosh, B. (2008) Inorg. Chim. Acta 361, 868–874
16. De Domenico, I., Vaughn, M. B., Li, L., Bagley, D., Musci, G., Ward, D. M., and Kaplan, J. (2006) EMBO J. 25, 5396–5404
17. Harrison, P. M., and Arosio, P. (1996) Biochim. Biophys. Acta 1275, 161–203
Ferritin Protein Pores and Fe Exit in Vivo

18. Kidane, T. Z., Sauble, E., and Linder, M. C. (2006) Am. J. Physiol. 291, C445–C455
19. Kurz, T., Terman, A., and Brunk, U. T. (2007) Arch. Biochem. Biophys. 462, 220–230
20. Lipschitz, D. A., Simon, M. O., Lynch, S. R., Dugard, J., Bothwell, T. H., and Charlton, R. W. (1971) Br. J. Haematol. 21, 289–303
21. Bothwell, T. H., Charlton, R. W., Cook, J. D., and Finch, C. A. (1979) in Iron in Biochemistry and Medicine (Jacobs, A., and Worwood, M., eds) pp. 564–589, Blackwell Scientific Publications, Oxford
22. Knutson, M. D., Oukka, M., Koss, L. M., Aydemir, F., and Wessling-Resnick, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 1324–1328
23. Jones, T., Spencer, R., and Walsh, C. (1978) Biochemistry 17, 4011–4017
24. Crichton, R. R., Roman, F., and Roland, F. (1980) J. Inorg. Biochem. 13, 305–316
25. Brady, M. C., Lilley, K. S., Treffry, A., Harrison, P. M., Hider, R. C., and Taylor, P. D. (1989) J. Inorg. Biochem. 35, 9–22
26. Porter, J. B. (2007) Am. J. Hematol. 82, 1136–1139
27. Waldo, G. S., and Theil, E. C. (1993) Biochemistry 32, 13262–13269
28. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) Biochemistry 11, 4120–4131
29. Schlabach, M. R., and Bates, G. W. (1975) J. Biol. Chem. 250, 2182–2188
30. Liu, X. S., Patterson, L. D., Miller, M. J., and Theil, E. C. (2007) J. Biol. Chem. 282, 31821–31825
31. Harrison, P. M., and Lilley, T. H. (1989) in Iron Carriers and Iron Proteins (Loehr, T. M., ed) pp. 123–238, VCH Publishers, Inc., New York
32. Ihara, K., Maeguchi, K., Young, C. T., and Theil, E. C. (1984) J. Biol. Chem. 259, 278–283
33. Mertz, J. R., and Theil, E. C. (1983) J. Biol. Chem. 258, 11719–11726
34. Theil, E. C. (2003) J. Nutr. 133, 1549–1553
35. Listowsky, I., Blauer, G., Enlard, S., and Betheil, J. J. (1972) Biochemistry 11, 2176–2182
36. Trikha, J., Theil, E. C., and Allewell, N. M. (1995) J. Mol. Biol. 248, 949–967
37. McDonough, S. I. (2007) Toxicon 49, 202–212
38. Shi, H., Bencze, K. Z., Stemmler, T. L., and Philpott, C. C. (2008) Science 320, 1207–1210