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Ferritin Protein Nanocages Use Ion Channels, Catalytic Sites, and Nucleation Channels To Manage Iron/Oxygen Chemistry:

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Biochemistry

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Abstract

The ferritin superfamily is composed of ancient, nanocage proteins with an internal cavity, 60% of total volume, that reversibly synthesize solid minerals of hydrated ferric oxide; the minerals are iron concentrates for cell nutrition as well as antioxidants due to ferrous and oxygen consumption during mineralization. The cages have multiple iron entry/exit channels, oxidoreductase enzyme sites, and, in eukaryotes, Fe(III)O nucleation channels with clustered exits that extend protein activity to include facilitated mineral growth. Ferritin protein cage differences include size, amino acid sequence, and location of the active sites, oxidant substrate and crystallinity of the iron mineral. Genetic regulation depends on iron and oxygen signals, which in animals includes direct ferrous signaling to RNA to release and to ubiquitin-ligases to degrade the protein repressors. Ferritin biosynthesis forms, with DNA, mRNA and the protein product, a feedback loop where the genetic signals are also protein substrates. The ferritin protein nanocages, which are required for normal iron homeostasis and are finding current use in delivery of nanodrugs, novel nanomaterials, and nanocatalysts, are likely contributors to survival and success during the transition from anaerobic to aerobic life.

Keywords

Ferritin; protein nanocage; ion channel biomineral; oxidoreductase; di-iron enzyme

Introduction

Ferritins make life with iron and oxygen possible. The protein nanocages (Figure 1A) synthesize caged iron oxide biominerals that are iron concentrates for cell metabolism, and recovery sites for iron from oxidant-damaged proteins [1]. In bacteria and archaea, mini-ferritins are small ferritins (Dps proteins) [1,2] that protect DNA by combining H₂O₂ and Fe(II) to make protein-coated minerals; mini-ferritins use distinct reaction pathways [3-5]. Ferritin effects on viability and oxidant resistance and the consumption of Fe(II) plus H₂O₂

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or O₂ [6-9], suggest that ferritins contributed significantly to the transition from anaerobic to aerobic life.

The iron /oxygen nexus in ferritin function, illustrated by ferritin gene regulatory signals, is a keystone of iron homeostasis. While the role of iron in regulating ferritin expression is well known [10], recognition of antioxidant effects is more recent. In plants, oxidant regulation is preferential to iron and DNA only [11]. In animals, a regulatory feedback loop includes both ferritin DNA (ARE+ Bach 1 repressor) and mRNA (IRE + IRP repressor); the gene product, ferritin protein [12], consumes the iron and oxygen signals and shuts down the loop [13,14]. Direct Fe(II) -RNA interactions increase repressor (IRP) release [15] and an iron "F-box" (E3 ligase) increases free IRP degradation [16,17], linking IRE-mRNA/IRP binding and IRP degradation in the signaling cascade.

Ferritin Protein Cage structure

Hallmarks of the ferritin superfamily are the hollow, symmetrical, nanoparticles that self-assemble from 12 or 24 subunits and are 4- α -helix bundles. The hollow interior (Figure 1B), ~ 60% of the cage volume is filled with buffer and hydrated Fe (III)O mineral. In normal cells, the mineral occupies only part of the cavity. Variations in the mineral phosphate content coincide with variations in cytoplasmic phosphate concentrations indicating anion exchange between the cytoplasm and the liquid phase inside the ferritin protein cavity [18,19]. Other variants in the ferritin superfamily are the location of the active sites and oxidant substrates, amino acid sequence, cage size and crystallinity of the iron oxide mineral.

Only two sizes of ferritin cages are known, 12 subunits (mini-ferritins) and 24 subunits (maxi-ferritins) [1]. Cage diameters are 12 and 8 nm with interior cavities of ~ 8 and 5 nm, respectively. Ferritin cages also form minor, multimeric species, e.g. dimers (two cages) and trimers (three cages); long chains of ferritin cages have been observed in crystals [20] and bound to DNA in bacterial nucleoids [2,21].

Amino acid sequences vary up to 80%, masking the cage self-assembly code. Ruberythrins, sequence related to ancestral ferritins [22], cannot make cages. Some information on surface contacts in *E. coli*, BFR [23], is all that is available. Recently, a synthetic protein cage similar to a Dps with heme, assembled after modifying an unrelated, modified cytochrome c b₅₆₂ [24], indicating that the ferritin cage assembly code reflects secondary/tertiary structure rather than primary (sequence) structure. In bacteria and archaea all the subunits are the same in ferritin cages; each bacterial ferritin gene is expressed under different biological conditions [25]. Animal and plant ferritins by contrast, coassemble from mixtures of ferritin gene products to create tissue-specific ferritin cages with different mineralization rates and, in animals where one ferritin gene encodes a catalytically inactive subunit named L (Lacks catalysis) [26,27,28], to also create tissue-specific minerals. The need for such tissue specificity is not known but could relate to supramolecular interactions specific to each cytoplasm or mineral properties or both.

Wherever the oxidoreductase sites are in ferritin cages, initiation of mineralization at the sites removes Fe(II) and O₂ or H₂O₂ from the cell cytoplasm. In eukaryotic ferritins, with high sequence conservation, 60-85%, the catalytic sites are buried deep in the 4-helix bundles, with ligands from each helix. The catalytic product [di-Fe(III) O, the mineral precursor] is released to an interior channel for nucleation [29]. In BFR, by contrast the di-iron site is a cofactor site, near to the cavity surface and depends, for active site turnover, on a nearby, weak iron site with only two iron ligands that is on the inner surface of the cavity in the protein cage [3]. In mini-ferritins, active sites are usually, between two subunits with a small numbers of iron ligands, three for Fe1 and one for Fe2 [1,30] (Figure 2) contributed by

both subunits. The preformed di-Fe(II) sites in maxi-ferritins are absent and appear to require oxidant to bind the second Fe (II) substrate in mini-ferritins [4].

Ferritin mineral structure varies in size, composition and crystallinity. The mineral sizes are relatively independent of constraints imposed by the protein cage, contrasting with nanomaterials made in ferritins [31], and reflect iron bioavailability for mineralization, normally far below the maximum capacity. The average number of iron atoms/ferritin cage averages 1000-1500 normally, but average numbers can vary from zero to 2500; ferritin minerals formed in vitro are of more uniform sizes [32]. At toxic levels of cellular iron the average iron content/protein cage increases to averages as high as 3000-4000, usually associated with hemosiderin, insoluble material from damaged ferritin with exposed, chemically reactive minerals.

The phosphate content of ferritin minerals changes mineral crystallinity. The high phosphate content of bacterial cytoplasm or plant plastids leads to amorphous iron minerals in ferritin [18,33-35]. In the lower phosphate environment of animal cells, ferritin mineral disorder is more closely related to the presence of the animal-specific, catalytically inactive, L subunit [36]. The physiological significance of variations in ferritin mineral structure, less explored than the iron mineral size, may relate to mineral dissolution. In liver ferritin the less crystalline mineral is associated with large numbers of L subunits content, lacking nucleation channel residues and active sites, and with the release of iron to other tissues.

Iron entry into ferritin cages and protein catalysis

To reach the active sites in ferritin protein cages, the Fe (II) substrate traverses the protein cage from the cytoplasmic (outside) surface of the cage to the multiple active sites. Metal ions cocrystallized were aligned in the ion channels between the outer surface of the cage and pores on the inner cage surface (Fig 1A, 2 A). The ion channels are constructed by the juxtaposition of the same helix turn (helices 3-4) in each of three subunits around the three – fold symmetry axes of the protein cage; a constriction in the channel created by three conserved glutamates, is just large enough for a hydrated Fe(II) ion. The eight channels must distribute substrate to twenty-four active sites (Fig 1 C, 2 A). A distribution mechanism became clear from the cluster of three metal ions around the exits of the ion channels into the protein cage cavity; each of the three metal ions is oriented toward one of three catalytic sites by one of the three highly conserved aspartate residues contributed by each subunit that creates the channel (Fig 2A) [37]. Positioning of ferritin ion pores and channels at the junctions of three subunits is, thus, a functional feature required to deliver ferrous ion substrate to the multiple active sites in the cage, preserved in maxi- and mini-ferritins [37,38].

Two Fe(II) ions, after entering the cage and binding at the active sites react with O₂ and in eukaryotic ferritins, produces diferric oxo products; similar products are likely produced in BFR [25] and mini-ferritins [9,39] but no spectroscopic proof is available. Active site residues in eukaryotic ferritin are shown in Table 1. The first detectable intermediate, a blue, diferric peroxo intermediate (DFP), forms in milliseconds and decays in seconds to the di-Fe(III)O product, which is the mineral precursors [1,27]. The catalytic reaction can be written formally as:

Water coordinated to the Fe(II) substrate) plays an unknown role; many protons, ~ 2/ mineralized Fe, are released during iron mineralization. Neither the protonation state in the

oxo-bridged product nor the role of protons in product release are known. DFP reaction intermediates in eukaryotic ferritins also form in ribonucleotide reductases [40] and iron methane monooxygenases [41] [42], but intermediates in mini-ferritins, BFR and eukaryotic active site mutants of eukaryotic ferritins are incompletely characterized [9,25,26].

The di Fe(III) O reaction products are released into protein nucleation channels (Fig 2B), likely accompanied by proton transfers, which are poorly understood. Site turnovers are much slower (hours) than DFP decay and may reflect conformational changes. Evidence for localized conformational changes in ferritin included changing Fe-Fe distances during catalytic turnover [43], multiple active site conformations in Co (II)-ferritin co-crystals [37] and localized unfolding of the three fold-pores (Figure 3A) associated with rapid mineral dissolution. Given the tightly packed, very stable (>80°C), α -helical structure of ferritin protein cages, with many hydrophobic interactions, conformational changes will be very slow contributing to the slow active site turnover after rapid decay of DFP intermediates.

Mineral nucleation and ferritin protein subunit cooperativity

Di-Fe(III)O mineral precursors move 20Å through channels in the protein cage between the active sites and the mineralization cavity [29] (Fig 2B). During transit Fe(III)O tetramers and octamers form in the protein channels, based magnetic susceptibility, likely by hydrolysis of coordinated water due to the space constraints imposed by the channels themselves. The ferritin protein cage exerts control over mineral growth through the clustering of nucleation channel exits around the four-fold symmetry axes of the cage (Fig 3B) [1] at one end of the subunit; iron enters at the three-fold ion channels at the opposite end of each subunit. If all the subunits are catalytically active (H subunits), the mineral nuclei emerging from four proximal channels will coalesce into ordered mineral. Clustering of nucleation channels exits explains the highly ordered mineral in heart ferritin, which has a large number of H subunits, and the low mineral crystallinity of liver ferritin [36] which has a small number of H subunits and a large number of L subunits. The ferritin gene duplication in animals that encodes L subunits may relate to the biological advantage of forming more disordered minerals in liver ferritin, which supplies iron to other tissues, and the absence of enough phosphate to lower mineral order as it does in bacterial cells and plant plastids.

The three different ways that the ferritin protein cage contributes to ferritin mineral structure and organization are: 1. Coupling 2 Fe(II) with O₂ at oxidoreductase (ferroxidase) sites to make di-Fe(III)O mineral precursors; 2. Facilitating nucleation and nucleation geometry during transport through nucleation channels; 3. Enhancing ordered mineral buildup by clustering mineral nucleation channel exits. What makes ferritin reactions unusual is the combination in a single protein of multiple, very rapid (catalytic coupling of substrates), slow (nucleation and transport), and very slow (mineral accretion) reactions. When ¹³C-C solution NOESY was used to identify the nucleation channels, during multiple catalytic turnovers, several hours were needed to achieve spectral homogeneity, after rapid decay of the DFP [29], which matches the long turnover time of the active sites [44]; moving multimeric Fe(III)O species through 4- α helix protein bundles, is, predictably, slow compared to reactions of Fe(II) and O₂. Several observations suggest that the subunits are communicating with each other, possibly to enhance interactions of the mineral nuclei emerging from each nucleation channel around the four-fold axes. For example, crosslinks, between ferritin subunits, which minimize subunit flexibility, alter rates of iron oxidation/mineralization and mineral dissolution [45]. In addition, Fe(II) binding to the active sites has a Hill coefficient of 3 that can only be attributed to protein: protein cooperativity [46]. These observations, and the difficulty in observing single, functional ferritin subunits with wild type sequences, suggest that the minimal functional unit of ferritin is the protein cage itself.

Ferritin protein cages are currently used as templates for materials, iron-containing imaging agents and bioremediation of precious metals, rare earths, etc [31,47-49]. Ferritin protein cages have also been used as surfaces to concentrate catalytic centers [50,51]. However, most of the past nanodesigns used ferritin protein cages as stable, relatively inert surfaces onto which metal sites could be designed. Integrating the natural features of ferritin ion channels and mineral nucleation control [29,37] into the use of ferritins as templates is an unexplored area of nanodesign.

Ferritin iron exit (Mineral dissolution)

Recovery of iron from ferritin mineral requires addition of electrons, protons and water released during mineralization; Fe-O-Fe bridges are accompanied, generally by release of water. Ordered water occurs in high resolution, ferritin protein crystals, in and around the ion channels [37]. One water is coordinated to each Fe(II) at the di-iron oxidoreductase sites [46]. Where and how the protons and water enter and exit the protein cage and participate in product release, mineral nucleation and dissolution are unknown. The paths taken by Fe(II) ions exiting the protein cages of animal ferritins is partly known from effects of amino acid replacements [52]. Less is known in plant or bacterial ferritins, but it is known, in plants that ferritin accumulation is regulated during leaf and nodule development [53]. Iron in legume seed ferritin is readily absorbed by humans and could minimize nutritional iron deficiency [54]. In BFR, heme mineral dissolution appears to enhance electron transfer from external reductants to the mineral (S Yasmin S, SC Andrews, GR Moore, and NE Le Brun, to be published.) Rates of mineral dissolution are detected as formation of a colored iron-chelate such as Fe(II) -bipyridyl. Modified rates from amino acid substitution are similar in solution or in vivo [55].

The ion channels at the three-fold symmetry axes of ferritin cages manage Fe (II) entry and exit [37,38, and 52]. Localized unfolding around the pores (Fig 3A) is associated with very rapid mineral dissolution after addition of reductant. The pores are stabilized by conserved hydrophobic (Leu134-Leu110) ionic (Arg 72-Asp 122) [52] and hydrogen bond (N-terminus-Arg 72) interactions (T Tosha and EC Theil, to be published). Changes in ferritin pore folding can be detected as helix loss in CD analyses, induced either by amino acid substitution or, in wild type proteins, by heat or low 1-10 mM urea [52]. In vivo regulators could be specific small metabolites or proteins, or changes in molecular crowding.

Fe (II) ions generated on ferritin mineral surfaces may result from direct contact with exterior reductants through pore openings, or by electron transfer through the protein; either model fits current knowledge. The only available information about how Fe (II) travels from the mineral surface travels to the ion channels involves leucine 154 (Fig 3 B,D) [29], a residue the helix 4-5 loop, which connects the 4-helix bundle to a short, fifth helix (Fig. 1C); mutations in helix 5 are the only ones in eukaryotic ferritin that are compatible with human survival [57].

Conclusions

The ferritin superfamily, united by the large, hollow cage structure that self-assemble from multiple sets (subunits) of four helix bundle proteins, uses protein catalytic sites that bind diferrous substrates to synthesize oxo-bridged, di-Fe ferric complexes and initiate mineralization in the large cavity of the proteins cages; the cavities are ~ 60% of the cage volume and accommodate minerals with of thousands of iron and oxygen atoms. The second ferritin substrate, dioxygen in eukaryotes, can be dioxygen or hydrogen peroxide in archaea and bacterial facultative anaerobes. Ferritin gene expression is regulated by iron and/or oxidants; in animals regulation extends from DNA genes to include mRNA. In animals, the

ferritin regulatory structures are shared, and coordinately regulated with genes that make oxidant stress response proteins (ARE-DNA) or iron metabolism protein (IRE-RNA). Ferritins, found in all contemporary eukaryotes, bacteria and archaea, both anaerobic and aerobic, which are required to concentrate iron for cell nutrition and to sequester iron and oxidants during stress, likely contributed to the successful transition of life from anaerobic to aerobic atmospheres.

Recent studies identified ferritin cage structures for: 1. ion channels that distribute metal ions to each of three active sites with external pores that regulate mineral dissolution; 2. nucleation channels that also direct ordered mineral after Fe(II)/O₂ react at the active sites. Current, unanswered questions about ferritin include: Where and how do protons and water exit during mineralization and enter during mineral dissolution? How do electrons reach the mineral during mineral dissolution? How do Fe(III)O mineral nuclei move through the protein helices? What is the biological significance of differences in the crystallinity produced by different ferritin proteins? When normal iron homeostatic mechanisms fail in iron overload is there a change in ferritin pore dynamics (opening and closing)? Do viral protein cages/coats use the same self assembly codes as ferritins? If the iron minerals in ferritin did not increase above normal, i.e. if more ferritin protein were synthesized, would the iron toxicity due to hemosiderin (degraded ferritin with exposed mineral surfaces) decrease? When ferritins cages are used as nanomaterial templates, what nanomaterials can be designed using the ferritin channels for controlled nucleation to complement exiting models that modify cage surfaces? Can tissue specific ferritin cages be used to target drugs to specific tissue and subcellular sites for imaging or for cancer therapy? Ferritin studies have already provided enormous benefit in terms of fundamental science about iron oxygen reactions, protein nanocage and ion channel structures, in the clinic for assessing iron status, in nutrition for treating iron deficiency and in chemistry for heterogeneous catalysis. New studies continue to increase our awareness of the sophistication and complexity of ferritin protein cages and practical uses of the information. The ancient age of ferritin has given Nature billions of evolutionary years to tweak the complex protein cage structure for improved function. It is not surprising, then, that after only ~seventy years of human study large numbers of unsolved problems and questions remain about the ferritin protein cage family with answers that will expand biochemical knowledge and enhance innovation in nanochemistry.

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Figure 1. Ferritin protein cages

Iron passes into the cages through ion channels (16 Å) around the three-fold cage axes, connecting exterior and internal cage pores to distribute Fe(II) substrate to each of three oxidoreductase sites. In eukaryotic ferritins, di-Fe(III)O catalytic products enter nucleation channels (20 Å), exiting near other channels clustered around the four-fold axes, thus using the entire length of each subunit bundle. **A.** An outside view into an ion channel around the 3-fold axis (S. Haldar, PDB file 1MFR); gold-helix bundles, red-unfolded sections of three subunits around the eight pores. **B.** Cross section of a ferritin protein cage; gold-assembled helix bundles, grey-cartoon of a large mineral, found in iron overload; red/white: ion channel region (X. Liu, PDB file 1 MFR). **C.** A cartoon of iron moving through ferritin external pores to Fe (II) ion channels and to oxidoreductase sites, then through the Fe(III)O nucleation channels into the mineralization cavity, T. Tosha from [37] with permission. Spheres: Red-Fe(II); pink-Co(II), orange-Fe(III), green-Mg(II); Helices from subunits contributing residues to the ion channels: green-helix 1, pink-helix 2, and gray-helix 3; Arrows are paths of iron through the cage: blue-Fe(II) pink-Fe(III)O.

Figure 2. Iron entry into ferritin protein nanocages: Fe (II) ion channels, Fe(III)O nucleation channels and active site ligands

A. An ion entry channel and nearby active site (T. Tosha, pdb file 3KA3). Green-Mg(II); stick figures-aspartate 127 from gold-helix 1, blue-helix 2, pink-helix 3; gold ribbon-helix 1. **B.** A nucleation channel in the ferritin protein cage that receives the di Fe(III)O reaction products and produces multiferric oxo mineral nuclei: gold-ribbon depiction of a four helix bundle subunit; •- metal ions at a catalytic site; • •- diferric oxo-bridged catalytic products. (R.Behera, pdb file 1MFR).

Figure 3. Mineral dissolution in and ferrous iron exit from and ferritin protein cages

Ferritin biomineral dissolution is initiated by reductant, NADH/FMN, and monitored as Fe(II) bipyridyl (pink). Conserved amino acids maintain structures that protect the mineral from dissolution; amino acid substitution increases mineral dissolution rates. In vivo regulation might be physiological changes in urea [52], large protein regulators or protein crowding. **A, C.** (**A**) The ferritin protein cage viewed from outside; red - unfolded three-fold axes of H-L134P centered (S. Haldar, pdb file 1BG7); **C.** Kinetics of mineral dissolution in WT and H-L134P; **B, D.** Structure (**B**) of a ferritin protein cage viewed from the inside surface; red-folded four-fold axes (**D**) kinetics of ferritin iron mineral dissolution in wild type and L154A (Mineral formation rates is unaffected by L154A.)

Table 1

Iron Ligands at Di-iron Oxidoreductase (Ferroxidase) Sites In/On Ferritin Cages

	Fe1	Fe2	Detection Method	References
Maxi-ferritin-Eukaryotes	E,ExxH	E,QxxD/A/S	CD/MCD, Mutation	[1,28,46]
Maxiferritin- E. coli BFR	E,ExxH	E,ExxH	XRD (crystal soak)	[3]
MIniferritin (DpS)	H,D,D	(H)	CD/MCD, XRD	[4,5]