Published in final edited form as:

Prog Mol Subcell Biol. 2011 ; 52: 29–47. doi:10.1007/978-3-642-21230-7_2.

Maxi- and Mini-Ferritins: Minerals and Protein Nanocages

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Abstract

Ferritins synthesize ferric oxide biominerals and are central to all life for concentrating iron and protection against oxidative stress from the ferrous and oxidant chemistry. The ferritin protein nanocages and biomineral synthesis are discussed in terms of wide biological distribution of the maxi-ferritins (24 subunit $\pm$ heme) and mini-ferritins (Dps) (12 subunit), conservations of the iron/oxygen catalytic sites in the protein cages, mineral formation (step i. Fe(II) entry and binding, step ii. O₂ or H₂O₂ binding and formation of transition intermediates, step iii. release of diferric oxo mineral precursors from active sites, step iv. nucleation and mineralization) properties of the minerals, and protein control of mineral dissolution and release of Fe(II). Pores in ferritin protein cages control iron entry for mineralization and iron exit after mineral dissolution. The relationship between phosphate or the presence of catalytically inactive subunits (animal L subunits) and ferritin iron mineral disorder is developed based on new information about contributions of ferritin protein cage structure to nucleation in protein cage subunit channels that exit close enough to those of other subunits and exiting mineral nuclei to facilitate bulk mineral formation. How and where protons move in and out of the protein during mineral synthesis and dissolution, how ferritin cage assembly with 12 or 24 subunits is encoded in the widely divergent ferritin amino acid sequences, and what is the role of the protein in synthesis of the bulk mineral are all described as problems requiring new approaches in future investigations of ferritin biominerals.

2.1 Introduction

Ferritins are protein cages that synthesize ferric oxides (hydrated minerals), which are located in a central cavity of the protein, 5–8 nm in diameter (e.g., [Lewin et al. 2005; Liu and Theil 2005]) (Fig. 2.1). Ferrous ions and either dioxygen or hydrogen peroxide are the substrates for protein subunit- based catalytic sites, in the cage, that initiate biomineral synthesis. Iron minerals in ferritin have two major functions. First, ferritin minerals are nutritive iron concentrates that slowly release the iron for use in the synthesis of new iron centers for protein catalysts as in heme, iron–sulfur clusters, or iron bound entirely by protein in amino acid side chains (“non-heme iron”). Iron-containing proteins are key to electron transfer chains in respiration (heme proteins) and photosynthesis (iron–sulfur proteins) and play important roles in hydroxylation reactions (e.g., Stiles et al. 2009; Cojocaru et al. 2007) oxygen sensing (Semenza 2009), and reduction, exemplified by the synthesis of deoxyribose nucleotides from ribonucleotides, central to DNA synthesis and replication. Second, ferritins are antioxidants that consume iron and oxygen during mineralization. The two metabolic roles of ferritins are emphasized by genetic regulation with oxidants and iron (Pham et al. 2004; Hintze and Theil 2005; Hintze et al. 2007). In animals, both DNA and mRNA are regulated, and are selectively targeted by oxygen or iron

(Theil and Goss 2009). Iron and oxygen consumption to make ferritin minerals is part of a feedback loop where excess iron and oxygen activate ferritin DNA and mRNA to increase ferritin synthesis. Subsequent protein accumulation, which consumes iron and oxygen, decreases the signals and shuts down ferritin synthesis (Theil and Goss 2009).

The protein nanocages of the ferritin superfamily are currently known to occur in two sizes. First, 24 subunit ferritins were observed, in the middle of the twentieth century, in many plant and animal tissues by using electron microscopy with the density of the minerals as a reporter; later, other biophysical and molecular biological methods were used to identify ferritin in bacteria (reviewed in [Theil 1987]). Ferritins occur in all tissues with a wide range of concentrations that reflect environmental and developmental signals. In plants, ferritin is targeted exclusively to cell organelles, whereas in animals, ferritins are in the cytoplasm, and in the lysosomal and mitochondrial organelles. In bacteria, ferritin accumulations are particularly sensitive to the culture phase, environmental iron, and oxidant concentrations; in logarithmic cultures of *Escherichia coli*, e.g., ferritin concentrations are low unless extracellular stressors are added (Nandal et al. 2009). Smaller ferritins or mini-ferritins, discovered as large proteins induced by stress, protect bacterial DNA from damage by consuming ferrous ions and hydrogen peroxide; accordingly they were first named *DNA Protection During Stress* (DPS) proteins (Chiancone 2010). Only later, when protein crystal structures were obtained, was the ferritin family protein cage structure discovered (Grant et al. 1998). However, since the cages are built from only 12, 4 α -bundle subunits, with a smaller cavity that accommodates a smaller mineral (<500 iron atoms), they were called mini-ferritins to distinguish them from the larger, 24 subunit maxi-ferritins. Mini-ferritins, to date, are restricted to bacteria and archaea, contrasting with maxi-ferritins found in bacteria, archaea, and eukaryotes.

2.2 Ferritin Distribution in Organisms of Land and Sea

Ferritins can be identified based on primary sequence in the genomes of archaea, bacteria, and eukaryotes (Fig. 2.2a). Ferritins from organisms of each of the kingdoms have been purified or expressed, heterologously, with variable levels of characterization. Kinetic models and molecular pathways for the uptake of iron, the oxidation from ferrous to ferric, and subsequent reduction and release have been developed over time, especially for animal ferritins and bacterial ferritins (Theil et al. 2008; Chiancone and Ceci 2010; Le Brun et al. 2010). The multiple steps in ferritin biomineralization will be discussed in Sect. 3. In this section, the focus is on the distribution of the different types of ferritin and the conservation of the catalytic sites throughout the tree of life. The conservation of the secondary and quaternary structures, and the primary sequence of the catalytic sites among different ferritins is remarkably constant (Table 2.1), given the wide range of sequence differences.

2.2.1 Ferritin in Prokaryotes

Primary sequences for both maxi- and mini-ferritins are widespread in the genomes of prokaryotes, and even within the genome of one single organism. *E. coli* is an example of an organism that has gene copies for multiple ferritins. *E. coli* mini-ferritin (Dps), maxi-ferritin (FTNA), and bacterioferritin (BFR) were used as queries in BLAST searches in order to study the distribution of recognizable ferritins among prokaryotes (P -value $< 5 \times 10^{-5}$), and to generate alignments that provide insights into the conservation of active site residues. The term “recognizable” stresses the limitation of the approach to find homologous proteins based on sequence homology on its own. In the case of ferritin in particular, the three-dimensional protein structure is highly conserved and essential for its function. However, there are no means yet to screen multiple genomes for the presence of specific, quaternary, protein structures. The results of the sequence homology studies cannot be the “final picture” of the ferritin family, because of sequencing gaps. For example, sequenced archaeal

genomes, to date, are 12-fold fewer than sequence bacterial genomes (42 vs. 524). We only present a snapshot to illustrate the broad distribution patterns of mini-ferritins (Dps), maxi-ferritins, and bacterioferritins (BFRs), and the conservation of the catalytic sites.

The sequence similarity among microbial maxi-ferritins ranges from 24.2% to 100%. In a total of 566 genomes (524 bacteria and 42 archaea), 321 BFR homologues (sequence identity ranging from 24.2% to 100% and similarity from 42.6% to 100%, P-value $< 3.8 \times 10^{-5}$), 355 mini-ferritin (DPS) homologues (20.9–100%, 41.3–100%, P-value $< 3.4 \times 10^{-5}$), and 210 maxi-ferritin homologues (20.8–100%, 40.9–100%, P-value $< 4.3 \times 10^{-6}$) could be identified.

A group of 27 maxi-ferritin homologues (including *E. coli* FTNB) were discarded in the group when none of the active site residues aligned with the remaining 210 homologues. The sequence conservation in ferritin among all three groups, maxi-ferritins in bacteria + archaea, mini-ferritins in bacteria + archaea, and maxi-ferritins in eukaryotes is enormous and emphasizes that the requirements for forming the assembly of 4 α -helix into a hollow protein nanocage is coded in secondary/tertiary codes still to be defined.

Over 43% (230 out of 524) of the currently available bacterial genomes have more than one type of recognizable ferritin encoded in the DNA, indicating the importance of these proteins. This percentage is lower in archaea; only 2% of the sequenced genomes encode more than one type of ferritin, which was exclusively a combination of bacterioferritin and maxi-ferritin (Fig. 2.2b). While multiple gene copies of the same type of ferritin occur rarely in archaea (Table 2.2 and Fig. 2.2), many bacteria have multiple genes encoding functional ferritin proteins.

2.2.2 Ferritin in Eukaryotes

The distribution of ferritin genes in eukaryotes is fairly different; mini-ferritins and heme-ferritins (BFR) have not been found to date. Studying the distribution of ferritin genes in eukaryotic genomes using BLAST alignments is less straightforward than in prokaryotic genomes for a number of reasons: (1) the presence of introns; (2) large numbers of truncated or pseudogenes; (3) multiple gene annotations. Eukaryotic maxi-ferritins are mixtures of subunits encoded in different genes. In plants, all the genes encode H-type subunits that are catalytically active. The multiple names for the catalytic sites include oxidoreductase, ferroxidase, F_{ox}, FC.

Ferritin cages in animals are co-assembled from active H-type subunits or inactive subunits called L-subunits. Historically, H and L stands for heart, heavy, or higher; L stands for liver, light or lower, referring to the organ where the subunit is dominant or the mass, or migration in an SDS-PAGE gel. However, the only consistent parameter is catalytic activity since H and L combination are found in all tissues, and SDS gel mobility does not always relate to mass. Multiple genes encoding H subunits occur in fish, frogs, mice, humans, maize, soy, etc. where the H subunits are called H and M (frog, fish) (Dickey et al. 1987; Yamashita et al. 1996) or H and mitochondrial (human, mouse) (Corsi et al. 2002; Arosio et al. 2009) or H-1-4/ATF 1-4 (plant ferritins) (Dong et al. 2008; Briat et al. 2009). Some ferritins have been identified from intronless genes that possibly result from the high stability of the animal ferritin mRNA enabling copying by viral reverse transcriptases during infection. In prokaryotes, multiple ferritin genes are expressed at different times in the culture cycle or with different stimuli so that the bacterial ferritin nanocages assemble usually only one type of subunit.

To determine the distribution of eukaryotic ferritin sequences in Nature, human H-ferritin was used in a BLAST search against all eukaryotic curated protein sequences (the

UniProtKB/Swiss-Prot database, ExPasy) in order to generate alignments of animal and plant ferritins to get insight into the conservation of active site residues among the A and the B site. A total of 55 homologues sequences were identified (sequence identity ranging from 22% to 99.45% and similarity from 40% to 100%, $P\text{-value} < 67 \times 10^{-11}$) and the conserved active sites and variants are shown in Table 2.3. Inherent in the analysis is the constraint imposed by the absence of structural information, which will exclude therefore many ferritins. Such limits of the search tools can explain the apparent absence of ferritin in organisms such as *Saccharomyces cerevisiae*. The current list of ferritin homologues, nevertheless, demonstrates primary sequence features that are highly conserved, as first observed in the first comparisons of ferritins from bacteria and animals (Grossman et al. 1992).

2.2.3 Conservation of Active Sites in Ferritins of Prokaryotes and Eukaryotes

Catalytic events that initiate biomineral synthesis in ferritins occur in each ferritin subunit. Thus, ferritins have multiple catalytic sites, 24 or 12 in bacteria and archaea, 24 in plants, and up to 24 in animals, depending on the fraction of L subunits; the distribution of H and L subunits in a ferritin nanocage changes the degree of order/crystallinity of the ferritin mineral (St Pierre et al. 1991). At the oxidoreductase sites, ferrous ions transfer electrons to dioxygen or hydrogen peroxide. The presence of ferritins in contemporary organisms that are aerobes, facultative aerobes and anaerobes, as well as the ability of ferritins to use dioxygen and ferrous, or hydrogen peroxide and ferrous, suggest that the antioxidant properties of ferritin mineral synthesis may have contributed to the transition from anaerobic life as earth's atmosphere evolved.

A third metal site near the diiron catalytic centers has been observed in bacterial maxi-ferritins co-crystallized with various metal ions, and name site C or Fe 3 (Stillman et al. 2003; Crow et al. 2009); soaking in iron of co-crystallization with ferrous anaerobically indicates Fe 1, Fe 2, and Fe 3 (Crow et al. 2009). To date, no specific kinetic intermediates have been characterized in prokaryotic maxi-ferritins, contrasting with the well-described differic peroxo intermediate in animal ferritins (Pereira et al. 1998; Moënné-Loccoz et al. 1999; Bou-Abdallah et al. 2002); although changes in heme absorption have been observed during ferrous oxidation of heme-containing BFR (Le Brun et al. 1993). Most likely, Fe 3 represents an alternate iron-binding site related to ferrous ions traveling through the protein cage to the catalytic sites, especially since mutation of the ligands slows down oxidation (Treffry et al. 1998). Alternated conformations of ferritin protein cage side chains have been observed in high-resolution structures of ferritin, dependent on metals present during crystallization (e.g., Trikha et al. 1995; Toussaint et al. 2007) indicate the flexibility of the ligands at and near the active sites needed to move iron through the cage.

The ferritin catalytic sites are diiron sites related to the diiron cofactors sites in dioxygenases by simple, DNA codon differences in two amino acids of the Fe 2 site (Liu and Theil 2005). However, in eukaryotic ferritins, ferrous iron is substrates rather than cofactors, as in the oxygenases. Iron leaves the active sites, after catalytic coupling to dioxygen, as differic oxo products that are mineral precursors (Liu and Theil 2005). In prokaryotes, where primary structure varies as much as 80%, the ferritin protein cages are studded with catalytic sequences within each subunit or at the cavity surface between subunits that bind two iron atoms as cofactors, or are mono or diiron substrates sites (Chiancone and Ceci 2010; Le Brun et al. 2010). Since many of the amino acids at the ferritin catalytic sites are highly conserved, in both eukaryotes and prokaryotes (Table 2.3), the variable amino acids at and around the active sites provide significant tissue and species selectivity (e.g., (Tosha et al. 2008).

At the ferritin catalytic sites, the ligating residues for Fe 1(A) are highly conserved in prokaryotes and eukaryotes (91.3–100%). By contrast, Fe 2 (B) ligands vary considerably (46.2–93.3%) (Table 2.4). In general, iron site 2 has two, nonvariant residues and several variable residues that have specific kinetic effects and are genetically regulated so the abundance differ (Tosha et al. 2008). There appears to be some type of cooperativity among the catalytic sites of multiple ferritin subunits. Since each ferrous ion binds independently at the diiron sites in each ferritin subunit, and the other substrate, dioxygen, is absent, the cooperativity is likely protein: protein (Schwartz et al. 2008).

The diiron sites in maxi-ferritins of both prokaryotes and eukaryotes, Fe 1(A) and Fe 2 (B), are highly conserved (all above 90.9%). Conservation of site Fe 2 of mini-ferritins (46.2%) is low, unless the second most abundant site Fe2 motif is included a consensus sequence; thus mutation of aspartate to the glutamate, in the *E. coli* template, increases the conservation to 92.7%. A similar approach for bacterioferritin increases for Fe 2 ligands from 76.3% to 88.5%. However, in eukaryotic ferritins of organisms with multiple H subunit (catalytically active) genes, Fe 2 site differences as small as changing alanine to serine occur, and in recombinant ferritins identical except for the alanine/serine shift, have significant effects on K_{cat} (Tosha et al. 2008). Thus, while the sequence motifs for Fe 2 in both mini-ferritin and bacterioferritin consist of a few common motifs found in the majority of the ferritins, the variations could have functional significance.

2.3 Ferritin Iron Biomineral Formation

Iron biomineralization in ferritins is a multistage process with discretely defined steps such as ferrous substrate binding, oxidation, product (mineral precursor) release and, when the active sites are in the middle of the protein cage, mineral nucleation, as recently observed by ^{13}C – ^{13}C and magnetic susceptibility NMR (Turano et al. 2010). The multiphase process is illustrated in progress curves where data are collected at a general wavelength for Fe(III)O species (310–420 nm) as in Fig. 2.3. By contrast, when data are collected at the λ_{max} of the diferric peroxo complex (650 nm), a single phase of the reaction is observed (Fig. 2.3).

A goal for the future is deconvolution, both spectroscopic and kinetic, of the multiple intermediates in ferritin iron biomineralization. The different steps in ferritin biomineral synthesis are:

1. Fe (II) enters the protein cage and binds at the active sites
2. Oxygen or hydrogen peroxide binds with oxidoreduction and formation of transition intermediates
3. Release of ferric oxo mineral precursor
4. Nucleation and mineralization

2.3.1 Step i. Fe(II) Entry and Binding

Binding of Fe(II) to the active site has been studied directly by MCD/CD in a eukaryotic maxi-ferritin (Schwartz et al. 2008), and indirectly by soaking Fe(II) into an iron -free protein crystal (Crow et al. 2009), or by tryptophan quenching (Lawson et al. 2009; Bellapadrona et al. 2009). In maxi-ferritin, the two Fe(II) atoms appear to bind independently to Fe sites 1 and 2, with five ligands that include water and a “space” for dioxygen Fe binding. The Fe(II) binding sites in eukaryotic and prokaryotic ferritins are weaker than in BFR (Table 2.3), supporting the cofactor behavior of iron at BFR active sites (Le Brun et al. 2010). In mini-ferritins, the 12 active sites are saturated with 12 Fe(II) atoms (Su et al. 2005) except in proteins that can use dioxygen as the substrate, where 24 Fe(II) bind/cage (Liu et al. 2006). The differential affinities of Fe 1 and Fe 2 sites in mini-ferritins

are illustrated by protein crystal structures where the site occupancy of metals varies between one and two depending on the protein (Chiancone and Ceci 2010). In maxi-ferritins, the 24 active sites are saturated with 48 Fe (II), when the formation of the differic peroxo complex is the reporter (reviewed Liu 2005).

2.3.2 Step ii. O₂ or H₂O₂ Binding and Formation of Transition Intermediates

Ferritin catalysis is rapid (msec) and requires stopped flow measurements to monitor the early stages. For maxi-ferritins, formation of the differic peroxo intermediate, well characterized by UV-vis, Mössbauer, resonance Raman, and EXAFS spectroscopies (Pereira et al. 1998; Moëne-Loccoz et al. 1999; Bou-Abdallah et al. 2002), is a convenient spectroscopic probe. However, except for the heme changes during oxidation in BFR, oxidation is measured as Fe(III)O which does not allow separation of catalysis from subsequent mineralization steps, which hampers kinetic analyses. For mini-ferritins that preferentially use hydrogen peroxide as the oxidant (Chiancone and Ceci 2010; Su et al. 2005), the reactions are anaerobic and can involve uncharacterized intermediates with UV absorption properties similar to differic peroxo of maxi-ferritins (Liu et al. 2006).

2.3.3 Step iii. Release of Differic Oxo Mineral Precursors from Active Sites

Release of the Fe(III)O product of ferritin catalysis varies among ferritins. In BFR and most Dps, the ferric oxo product is thought to be released directly into the protein cavity, because of the proximity of the active sites to the cavity surface. In BFR, and possibly *Pyrococcus furiosus* ferritins, where two iron atoms are cofactors, the active sites are proposed to dominate dioxygen reduction while ferrous oxidation occurs in the cavity on protein or mineral surfaces with the released electron used to reduce the cofactor ferric iron and recycle the catalytic center (Le Brun et al. 2010). However, the properties of various ferric species remain uncharacterized. In eukaryotic ferritins, where the differic peroxo intermediate results from dioxygen and ferrous, the reactions produced is a differic oxo complex and multimeric ferric oxo complexes (Jameson et al. 2002). Even though the active sites in eukaryotic ferritin protein cages, like those in prokaryotic ferritins, have direct access to the internal cavity (Tosha et al. 2008), recent NMR evidence reveals that the differic oxo products of ferritin protein catalysts remain inside the protein cage and move along an interior trajectory of 20 Å through the protein cage to the cavity surface. During passage through the protein, reactions occur among the ferric oxo products of multiple catalytic cycles (Turano et al. 2010) (Fig. 2.4), leading to the formation of multinuclear ferric oxide complexes inside the protein cage itself. Whether this feature is an evolutionary advance in eukaryotic ferritins or a property yet to be detected in prokaryotic and archaeal ferritins, remains unknown.

2.3.4 Step iv. Nucleation and Mineralization

Nucleation and mineralization in prokaryotic ferritins, BFR, and mini-ferritins is considered to be essentially inorganic hydrolytic chemistry of ferric oxo complexes where the effective concentration of iron is increased by the volume of the cavity. Ferrous ions are propelled into the cavity from the exterior of the protein cages by high-affinity binding sites (active sites) that harvest ferrous ions moving into the cage through pores lined with negatively charged amino acids (Theil et al. 2008; Bellapadrona et al. 2009); in recombinant ferritins with only animal L subunits and no catalytic sites, oxidation and mineralization is ~1,000 times slower. Pores with negative charges are formed around the threefold axes in both mini-ferritins and maxi-ferritins; highly conserved residues control pore folding/unfolding which influences iron mineral dissolution more than mineral synthesis.

Eukaryotic ferritin protein cages appear to be much more active in mineralization than those in prokaryotes, which could mean either a different evolutionary path to achieve the

quaternary protein cage structure, or a lag in functional studies for prokaryotes. (See also comments in ref. Le Brun et al. 2010). In a recent surface, where the position of iron oxo mineral precursors was monitored as the ferric-induced broadening of resonance in ^{13}C – ^{13}C solution NMR spectra, the first two ferrous ions oxidized (48 Fe/cage) appeared as a dimer (magnetic susceptibility) distal to the active sites and away from the cavity surfaces (Turano et al. 2010). The addition of saturating Fe(II) for second, third, and fourth oxidoreductase cycles revealed the ferric oxo species moving through about 20 Å channels along the long axes of each four-helix bundle subunit to the inner edge of the protein cages. The ferric oxo mineral precursor that is produced by the multiple catalytic sites emerged into the cavity as multimers (mineral nuclei) of 4–8 Fe atoms. Since each subunit channel exit is near that of three others (near the cage fourfold axes), the ferritin protein cage of eukaryotes facilitates biomineral growth.

2.4 Ferritin Iron Biominerals

Iron biominerals in the center of ferritin protein cages of animals, plants, bacteria, and archaea are hydrated ferric oxides. The microcrystalline materials have been observed by electron microscopy, X-ray powder diffraction, Mössbauer spectroscopy, and more recently by scanning electron microscopy (SEM) (TEM) (Rodriguez et al. 2005; Dobson 2001). Mineralized iron in ferritins varies in the maximum number of mineralized iron atoms/cage varying from 500 in mini-ferritins to >4,000 in maxi-ferritins. However, in natural tissues, the average number of mineralized iron atoms in maxi-ferritins is much less, from 800 to 1,500, with a distribution from 1,000 to 3 or 4,000 Fe atoms/cage; ferritins reconstituted in the laboratory have much more homogeneous mineral sizes see review [Theil 1987]. When the mineral content of a ferritin protein cage is very high, damage occurs to the cage, likely from side reactions of repeated oxidoreductase catalysis, and an insoluble mass of damaged protein forms, called hemosiderin. In living systems, multiple mechanisms regulate both the cellular influx of iron and the synthesis of ferritin protein cages, so that hemosiderin only forms with abnormal regulation (disease). In the laboratory, the iron content of ferritin protein cages is controlled by the amount of ferrous iron added, which is usually up to 240 for mini-ferritins and up to 480–1,000 for maxi-ferritins (Theil 1987; Arosio et al. 2009).

All ferritin minerals contain some phosphate, but the amount varies. For example, in animals the phosphorous content is about 12% of the iron, whereas in plants and bacteria the phosphorous content approached 100% that of the iron (Lewin et al. 2005). The higher phosphate content in ferritin minerals minimizes order and leads to a more amorphous ferritin mineral. When iron minerals were reconstituted in empty ferritin protein cages with high phosphate (P: Fe = 4:1) and low phosphate (P: Fe = 0:1) in the solution, the phosphate-containing minerals were more disordered when analyzed by EXAFS, TEM, electron diffraction, and Mössbauer spectroscopy, and were independent of the proteins cages, which were bacterial (*Azotobacter vinelandii* or *Pseudomonas aeruginosa*) or animal (horse spleen) in origin (Mann et al. 1987; Rohrer et al. 1990).

The phosphate content of prokaryote cytoplasm is higher and more variable than the cytoplasm of eukaryotic cells, which explains the high phosphate content and greater disorder of ferritin biominerals in bacteria (Mann et al. 1987; Rohrer et al. 1990; Wade et al. 1993; Waldo et al. 1995). In plants, the plastid is evolutionarily related to prokaryotes and thus, even though ferritin protein is encoded in a eukaryotic nuclear gene, it is targeted to the plastid. The high phosphate content of plant ferritin minerals, then, is explained if the plant ferritin mineral is synthesized by the nanocage inside the plastid (Waldo et al. 1995), rather than in the cytoplasm where the nanocage is synthesized before transport to plastids. Whether the phosphate content of ferritin minerals is simply a reflection of the ambient

concentration where the mineral is formed or includes phosphate storage function, is unknown.

Order in ferritin minerals varies from microcrystalline to amorphous. The most dramatic effect is the effect of phosphate that distinguishes animal ferritin minerals from those of bacteria and plants, where the ferritin minerals appear to form in the prokaryotic-derived plastids. The properties of the mineral in mitochondrial ferritins of animal, only recently discovered and studied (Corsi et al. 2002) only at the level of the protein, is unknown. In animal tissues, the crystallinity of the ferritin mineral varies in different tissues and disease states (St Pierre et al. 1991). One contributing factor is the presence of catalytically inactive subunits, the animal-specific L subunit. Ferritins with a high L subunit content have more disordered minerals than those with few L subunits (St Pierre et al. 1991), which may relate to the recently detected role of the catalytically active subunits in directing nucleation and mineralization of ferritin minerals (Turano et al. 2010) (Fig. 2.4). The underlying mechanism of ferritin biomineral synthesis is ferric hydrolysis. Apparently, ferritin protein cages contribute to a much larger fraction of the mineralization process than previously thought, but the fraction of ferritin biominerals laid down without passage nucleation in the protein cage remains unknown.

2.5 Ferritin Iron Biomineral Dissolution

Recovering iron from the ferritin mineral core is crucial for the biosynthesis of heme and iron-sulfur cluster-containing proteins that are important for all forms of life. However, the exit of iron from the ferritin cage, which requires the delivery of electrons and protons to dissolve the mineral, is much less studied than entry/oxidation/mineralization. The reduction and subsequent release of iron from the ferritin cavity can be measured in an assay as first described by Jones et al. (1978), and has been used to study maxi-ferritins and mini-ferritins from plants, animals, bacteria, and cyanobacteria (Bellapadrona et al. 2009; Takagi et al. 1998; Richards et al. 1996; Castruita et al. 2007). Usually, NADH/FMN, or sodium dithionite is the source of electrons, added to mineralized ferritin protein in neutral buffer (Bellapadrona et al. 2009), with a chelator such as bipyridyl, as the reporter for iron exit the pink Fe(II)-chelate (λ 522 nm); other chelators such as desferrioxamine B (DFO) are also used. Curves for the formation of the Fe-chelator complexes are multiphasic and complex due to multiple sequential and simultaneous reactions; iron reduction, hydration of ferric oxo bridges in the mineral, transport of iron and reductants through the protein cage in the case of Fe(III) chelators, the competition between binding to the chelator or to the catalytic sites of the protein (Liu et al. 2007).

In both mini- and maxi-ferritins, iron leaves the mineral cage via pore structures in the protein that connect the inner cavity with the outside of the protein (Liu and Theil 2005; Bellapadrona et al. 2009). These funnel-shaped, hydrophilic pores are localized at the threefold symmetry axes by junctions of three subunits (Takagi et al. 1998). There are eight of these hydrophilic pores in maxi-ferritin and four in mini-ferritin. Experiments with the H-frog maxi-ferritin, using mutagenesis, protein crystallography, helix content (CD spectroscopy) have shown that the helix content around ferritin pores is sensitive to slight changes in temperature, ionic strength, or by the presence of low concentrations (1–10 mM) of chaotropes like urea or guanidine (Liu et al. 2003). Partial unfolding of these pore structures was observed simultaneously with an increase in rate of iron reduction and iron release from the proteins inner core illustrating the regulatory role of ferritin cage pores in controlling mineral/reductant access. Similar effects of threefold pore mutations were observed *Listeria innocua* mini-ferritin where replacement of conserved, negatively charged neutral amino acids changed the initial rate of iron release from the mineral core. Even though the amino acid sequences of mini-ferritin and maxi-ferritin subunits vary

considerably (up to 80%), quaternary structures have pores, at the junctions of matched helix turns in three subunits, which control reductant/chelator access/Fe (II) exit during demineralization of both maxi-ferritins and mini-ferritins. The turnover rate of ferritin minerals in vivo is not known but studies with natural maxi-ferritin minerals suggest it could be significant (Castruita et al. 2007).

Dissolution of ferritin minerals has a fast, pH-dependent initial phase and a slower, pH-independent phase in ~100–200 irons per cage are removed. These iron atoms may be anchored in the protein and represent iron still in transit through the proteins (Turano et al. 2010). The phosphate content of ferritin minerals also influences dissolution of mineral dissolution rates. Rates in the early phase of mineralization decreases when phosphate is high, whereas the later, slow phase is independent of phosphate (Richards et al. 1996). Such observations support the notion that phosphate replaces coordinated water and thereby blocks hydrolysis and formation ferric oxo bridges, which would have a larger effect on mineral nuclei than on bulk mineral.

What keeps ferritin minerals dissolving at a rapid rate? The cytoplasm of most cells is thought to be “reducing,” i.e., full of reductants. Increased rates of iron mineral dissolution occurred when ferritin threefold pores were unfolded, as observed in a crystal structure of a ferritin folded normally (Takagi et al. 1998). Thus, in normal ferritin cages, the folded pores keep reductant away from the ferric mineral. A search for other amino acid residues that stabilize pore folding in ferritin protein nanocages was performed using frog H maxi-ferritin, the protein in which pore unfolding was observed first (Fig. 2.5). (Takagi et al. 1998; Jin et al. 2001). Three highly conserved residues near the pores in three dimensions, but with no assigned function, were substituted with a variety of amino acids, each of which increased rates of mineral reduction and release of iron compared to the wild-type ferritin, identifying a set of amino acids that control pore opening. Even in wild-type protein, pore unfolding, measured as increased rates of reducing/dissolving ferritin minerals, is increased by low heat and low concentrations of chaotropes (both in the range of normal physiological conditions). Protein pore function was further probed with five peptides identified from a combinatorial phage display library of 10^9 peptides that bound to wild-type ferritin (Liu et al. 2007). One of the five binding peptides increased iron release as if it were unfolding the pores. Such results lead to the hypothesis that cellular proteins control folding/unfolding of ferritin pores and, thereby, control ferritin mineral/reductant/chelator interactions in vivo (Liu et al. 2003).

2.5.1 Perspectives

Ferritin protein nanocages, which synthesize hydrated iron oxide biominerals and protect them from uncontrolled cell reductants, are ubiquitous. Recognizable sequences are found in archaea, bacteria, plants, and animals, including humans. Ferritins concentrate iron in biominerals containing thousands of iron and oxygen atoms, to match cell need and to act as antioxidants that prevent corrosive ROS (reactive oxygen species) release from iron and dioxygen or hydrogen peroxide chemistry. Ferritin plays a role in normal development and in disease, as pathogens and host battle with iron and inflammatory oxidants. Hallmarks of ferritin family members are a quaternary protein cage structure self-assembled from polypeptide subunits and studded with: (1) catalytic sites that initiate mineralization, (2) pores that control iron entry and mineral reduction/dissolution, and (3) internal channels that facilitate during mineral nucleation. Ferritin subunits fold into 4 α -helix bundles; 12 subunit structures are mini-ferritins (Dps proteins), and 24 subunit structures are maxi-ferritins. The variable phosphate content of ferritin biominerals appears to relate to differences in environmental phosphate during biomineral synthesis. High-phosphate-induced minerals have biological relevance in plants and bacteria while biomineral disorder in low phosphate ion biominerals of animals depends on the relative abundance in the protein cage of subunits that are catalytically inactive (L subunits). The wide divergence among ferritin amino acid

sequences has two implications for future research: First, solving the self-assembly code for the cage, pores, and active sites will require new dimensions in informatics. Second, ferritins that escape identification by scanning methods emphasizing primary structure will be trapped by future scanning methods that incorporate features of secondary and tertiary structures to reveal more ferritin genes. Finally, the paucity of current information about the full role of the ferritin cage in biomineral synthesis, beyond catalysis and mineral nucleation, which is hampered by the sequence divergence, has obscured the answer to the question of the role of the protein cage in formation of the bulk biomineral. Clearly, the rapid advances in understanding function, structure, and biological importance of ferritin protein cages and iron biominerals still leave many questions for the future.

Acknowledgments

The authors are grateful for the contributions of all the members of the Theil Group. The writing of the manuscript and work described herein were supported by the CHORI Foundation (ECT), the NIH (DK20251) (LB and ECT), and a Rubicon Fellowship from the Netherlands Organization for Scientific Research (NWO), (LB).

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Fig. 2.1.

Maxi-ferritin protein cages, which synthesize iron oxide biominerals. View toward the threefold axis: (a) *Escherichia coli* bacterioferritin (pdb file: 1BFR, an example of a low phosphate ferritin biomineral (b) *Rana catesbeiana* (bullfrog) M-ferritin (pdb file: 1MFR, an example of a low phosphate biomineral. Red-a set of three subunits around one of eight Fe(II) entry/exit pores

Fig. 2.2. Distribution of the maxi-ferritin (FTNA and BFR) and mini-ferritin (Dps) genes in prokaryotes. **(a)** Venn diagram showing the distribution of the three different ferritin genes in 566 sequenced bacterial and archaeal genomes; **(b)** circular diagram showing the distribution for the bacterial (524) and archaeal (Dobson 2001) branches separately. **(c)** The number of genes encoding maxi-ferritins (24 subunits-210), mini-ferritins (Dps)(12 subunits-355), and bacterio-ferritins (24 subunits; 12 hemes-321) in 441 genomes

Fig. 2.3.

Overall ferritin oxidoreductase activity (Maxi-ferritin – 24 sites). (a) Absorbance at 650 nm of the differic peroxo (DFP) catalytic intermediate. (b) Absorbance at 350 nm of all Fe(III) species: DFP, differic oxo mineral precursors, other intermediates and mineral itself. The figure is modified from reference 24

**Fe²⁺ enters through ion channels
near subunit trimers**

Fig. 2.4.

Movement iron premineral through a eukaryotic ferritin protein cage. The data are from 13C–13C solution NOESY NMR obtained after the addition of 2 Fe (II)/F_{ox} site, for each of four catalytic turnovers. Modified from reference 35

Fig. 2.5.

Ferritin threefold pore influences ferritin demineralization rates. The eukaryotic model shows the mid-point constriction in the pore. There are eight ferritin pores in maxi-ferritins (Fig. 2.1) and four functionally analogous pores in mini-ferritins (Tosha et al. 2008)

Table 2.1

Ferritins with similar protein nanocage structure exhibit a wide range of primary structure (sequence identity/sequence similarity)

	<i>E. coli</i> FTNA	<i>E. coli</i> DPS	<i>E. coli</i> BFR	Human H	Human L
<i>E. coli</i> FTNA	–				
<i>E. coli</i> DPS	8%/34%	–			
<i>E. coli</i> BFR	12%/41%	9%/37%	–		
Human H	21%/47%	14%/39%	20%/38%	–	
Human L	20%/44%	12%/38%	17%/37%	53%/75%	–

Table 2.2

The distribution of ferritins among bacteria and archaea. Absolute numbers and percentages of the occurrence of recognizable maxi-ferritins, min-ferritins and bacterioferritins are presented from sequences of 524 bacterial genomes and 42 archaeal genomes obtained from BLAST searches against *E. coli* FTNA (ftnA/b-1905), DPS (dps/b-0812), and BFR (bfr/b-3336)

	No. homologous genes	No. genomes	No. bacterial genomes (% of total)	No. archaeal genomes (% of total)
Maxi-ferritin	321	225	221 (42)	4 (10)
Mini-ferritin	355	302	299 (57)	3 (7)
Bacterioferritin	210	193	178 (34)	15 (36)

Using the CMR database (J. Craig Venter Institute)

Table 2.3

Sequence patterns of putative, catalytically relevant Fe²⁺ ligands in mini- and maxiferritins. Ferritin homologues were identified by the alignment of prokaryotic (J. Craig Venter Institute) and eukaryotic genomes (Uniprot knowledgebase, Swiss-Prot). Templates: *E. coli* proteins FTNA, DPS, and BFR – prokaryotes and human H-FTNA – eukaryotes

Distribution	Example	Homologues (total)	Fe 1 (Site A) ^a	Fe 2 (Site B) ^a
Maxi-ferritins				
Prokaryotic FTNA	<i>E. coli</i> FTNA	196 (210 ^b)	E, ExxH	E, E, QxxE
Animal H-ferritin	<i>Human</i> H	33 (37 ^c)	E, ExxH	E, QxxA/S
Plant H-ferritin	<i>Soybean</i> H	17 (18 ^d)	E, ExxH	E, QxxA/S
Prokaryotic BFR	<i>E. coli</i> BFR	239 (321 ^e)	E, ExxH	E, ExxH
Mini-ferritins				
Prokaryotic DPS	<i>E. coli</i> DPS	164 (355 ^f)	H, DxxxE	HxxxD

^aDiiron sites in oxygenases and reductases are Fe 1 and Fe 2 (34); ferritin diiron sites were Fe A and Fe B before the similarity to the oxygenases was recognized.

^b*Prokaryotic FTNA active site variants*: E, (E/K)xxH (Fe 1), E, E, Qxx(Q/K/D/A/A) (Fe 2) in 14/210 homologues.

^c*Animal FTNA active site variants*: E, (E/G/S)xx(H/RD) (Fe 1), (E/V/K), (Q/V)xx(A/S/D/G/W) (Fe 2) in 4/37 homologues.

^d*Plant FTN active site variants*: E, HxxH (site A), E, QxxA (Fe 2) in 1/18 homologues.

^e*Prokaryotic BFR active site variants*: (E/L/H/Q/S/Y/W),(D/E/A/V/K/Q/D) xx (H/A/Y/Q/W/N/E/H/T) (Fe 1), (E/V/M/N/V/Q/G), (T/E/N/K/A/C/S/Q/V/xx(H/A/Q/Y/I) (Fe2) in 82/ 321 homologues.

^f*Prokaryotic Dps active site variants*: Fe1 100% conserved, Hxxx(E/M/Q/G) (Fe 2) in 191/ 355 homologues.

Table 2.4

Conservation of iron-binding amino acids at the catalytic centers of ferritins. Percentages of conservation of putative Fe²⁺ ligands in site A and B in mini-, maxi-, and bacterioferritin homologues based on the alignment of prokaryotic (J. Craig Venter Institute) and eukaryotic genomes (Uniprot knowledgebase, Swiss-Prot) as presented in Table 2.3

Protein	Fe 1 (Site A) ^a	Fe2 (Site B) ^a
Maxi-ferritin (pro)	99.5	93.3
Maxi-ferritin (euk)	92.7	90.9
Mini-ferritin (Dps)	100.0	46.2
Bacterioferritin	91.3	76.3

^aDiiron sites at the catalytic centers of oxygenases and reductases, Fe 1 and Fe 2, are homologous to ferritin A and B, which were named before the similarity to the oxygenases and FTNA became clear