Ferritin: At the Crossroads of Iron and Oxygen Metabolism[1,2]

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ABSTRACT Iron and oxygen are central to terrestrial life. Aqueous iron and oxygen chemistry will produce a ferric ion trillions of times less soluble than cell iron concentrations, along with radical forms of oxygen that are toxic. In the physiological environment, many proteins have evolved to transport iron or modulate the redox chemistry of iron that transforms oxygen in useful biochemical reactions. Only one protein, ferritin, evolved to concentrate iron to levels needed in aerobic metabolism. Reversible formation and dissolution of a solid nanomineral-hydrated, iron oxide is the main function of ferritin, which additionally detoxifies excess iron and possibly dioxygen and reactive oxygen. Ferritin is a large multifunctional, multisubunit protein with eight Fe transport pores, 12 mineral nucleation sites and up to 24 oxidase sites that produce mineral precursors from ferrous iron and oxygen. Regulation of ferritin synthesis in animals uses both DNA and mRNA controls and genes encoding two types of related subunits with: *1*) catalytically active (H) or *2*) inactive (L) oxidase sites. Ferritin with varying H/L ratios is related to cell-specific iron and oxygen homeostasis. H-ferritin oxidase activity accelerates rates of iron mineralization in ferritins and, in animals, ferritin produces H_2O_2 as a byproduct. Properties of ferritin mRNA and ferritin protein pore structure are new targets for manipulating iron homeostasis. Recent observations of the high bioavailability of iron in soybean ferritin and efficient utilization of soybean and ferritin iron by iron-deficient animals, and of soybean iron by humans with borderline deficiency, indicate a role for ferritin in managing global iron deficiency in humans. J. Nutr. 133: 1549S–1553S, 2003.

KEY WORDS: • *ferritin* • *iron nutrition* • *chelators* • *protein pores* • *combinatorial mRNA*

Iron has been central to life since the beginning. Environmental dioxygen, a newer component of terrestrial life, was likely to have been the worst environmental pollutant of all time, but evolution has made oxygen so crucial that both iron and oxygen are absolute requirements for contemporary organisms. The chemistry of iron and oxygen under physiological conditions is corrosive (**Fig. 1**), producing insoluble rust and soluble oxy radicals. Ferritin evolved as the only protein able to solve the problem of iron/oxygen chemistry and metabolism. Aquated, ferrous iron is oxidized with oxygen to concentrate as many as 4000 iron atoms as a solid oxo-mineral in the center of the ferritin protein. Iron inside ferritin comfortably matches iron and oxygen chemistry with cellular concentration requirements of $\sim 10^{-4}$ M compared to the 10^{-18} M solubility of the ion, a gradient of 100 trillion fold (1,2). The biosynthesis of key proteins for iron and oxygen, including ferritin, is coordinately regulated by a combinatorial array of iron regulatory proteins (IRP)[4] and mRNA [noncoding iron-responsive elements (IRE)] structures that interact with varying efficiencies (3,4).

Combinatorial regulation of genes for Fe and O_2 metabolism

Combinatorial RNA IRE/IRP structural interactions (3–6) explain the differential effect of iron in a cell on the level of protein synthesis for proteins encoded in IRE-mRNAs (**Table 1**). One illustration of this are the different levels of induced synthesis of ferritin (iron metabolism) and mitochondrial aconitase (oxygen metabolism) in the liver of rats exposed to high levels of iron (7,8). Environmental signals that control the IRE/IRP combinatorial system include Fe, O (H_2O_2, anoxia), nitric oxide (NO) and IRP kinases (Table 1). Many signals acting through IRP kinases, and those as yet unidentified, could alter iron and oxygen homeostasis through the IRE/IRP combinatorial system (3,9).

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[4] Abbreviations used: IRE, iron-responsive element; IRP, iron regulatory protein.

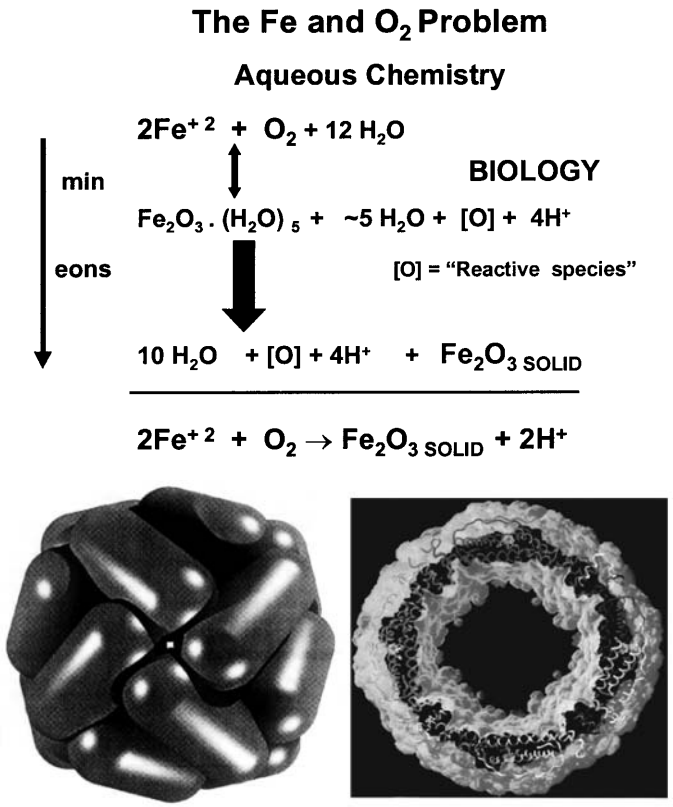

FIGURE 1 Reaction of iron and oxygen at physiological pH in water, and the ferritin structure. (*Top*) The reaction: Note the timescale for different reactions, reactive oxygen intermediates and the net release of protons. (*Bottom*) Ferritin Structure: (*left*) The outside of the molecule (12-nm diameter) showing the ellipsoidal subunits. (*right*) A cross section showing the internal cavity in black (8 nm in diameter) in which mineral forms with protein side chains shown in orange and cut away over the helical polypeptide subunit backbones, which are shown in multiple colors. Taken with permission from Trikha, J., Waldo, G. S., Lewandowski, F. A., Theil, E. C., Weber, P. C., and Allewell, N. M. (1994) Proteins 18: 107–118. (1994).

Ferritin genomics and expression

The impact of genomic research on knowledge of ferritin has been large (**Table 2**). Ferritin expression varies in response to signals acting on both mRNA (Table 2) and DNA (10). At normal levels of body iron, the expression of ferritin in the average cell is quite low (e.g., fibroblasts), which contrasts with cells that have specialized functions in iron and possible oxygen homeostasis (e.g., liver and heart). In many cells, both ferritin and iron uptake proteins are upregulated early in differentiation and downregulated as the amount of iron needed for cell-specific homeostasis is achieved, in analogy to whole-body iron uptake during growth and maturation. An example of the developmental changes in iron homeostasis at the cellular level is the erythroid cell where both ferritin and transferrin receptors increase during the normoblast stage and decrease in the erythrocyte (11). During normal growth and development, some specialized cells accumulate iron and ferritin for use by other cells later in development, exemplified by the erythrocytes of embryos and the liver of the fetus (12). In disease, other specialized cells and tissues accumulate iron and ferritin to detoxify excess iron and possibly oxygen. During stress and disease, ferritin accumulates in liver, spleen, kidney, heart and serum. During iron deficiency, the loss of ferritin and redistribution of iron from specialized cells and tissues "spares" average cells from iron deficiency, although the loss of tissue ferritin coincides with increased oxidative damage (13).

TABLE 1

IRE-mRNAs and signals for combinatorial mRNA regulation[1] of iron and oxygen metabolism

	IRE-mRNAs
Fe	Transferrin receptor, DMT1 (cell Fe entry)[1,2] Ferritin (cell Fe concentration)[1,3] Ferroportin (cell Fe exit)[1]
O_2	Mitochondrial aconitase[1,3] Aminolevlinate synthase[1,3]
Signals	Fe (chemical/biochemical form unknown) O (H_2O_2, anoxia) NO IRP kinases

[1] [2] RNA regulatory mechanism is mRNA dependent.
[3] mRNA turnover, degradation.
Ribosome binding, translation.

TABLE 2

Ferritin human genomics

1980s	~35 H and L hybridizable sequences
1986	Ferritin- H, L subunit genes cloned (~20 pseudogenes identified)
1995	Hyperferritinemia familial cataracts (mRNA)
2000	Ferritin gene deletion (mouse): embryonic lethal
2001	Adult onset basal ganglia disease (L-protein)
2001	Mitochondrial ferritin (mt-target sequence, intronless)
2003	Other genes found in "pseudogenes"?

Ferritin structure

Conservation of ferritin protein sequence, folding, tertiary and quaternary structure among plants and animals is very high, emphasized by the use of an animal sequence (frog) to clone the first plant (soybean) ferritin (14). In addition, structures of plant and animal ferritin are superimposable (15). Ferritin in contemporary bacteria diverges considerably in sequence, but not in secondary, tertiary and quaternary structure (1,2), suggesting evolutionary convergence with eukaryotic ferritins.

Ferritin is a large protein (12-nm diameter, 480,000 Da) with a large cavity (256 nm^3) for the mineral that is created by the spontaneous assembly of 24 ferritin polypeptides folded into four-helix bundles bound to each other by hydrogen and salt (ionic) bonds. The structure appears to have evolved as a patchwork of other proteins such as non-heme di-iron oxygenases that share with ferritin the binding of Fe and O_2. Iron in the dioxygenases is a cofactor, but iron in ferritin is a substrate. Ion channel/pore proteins share properties with ferritin Fe entry and exit sites and may be progenitors of ferritin pores. Protein mineralization surfaces are shared among ferritin and other proteins in matrices that form biominerals. The numbers of functional sites in ferritin are: one mineral cavity in the protein center, eight entry and exit pores on the outer surface, 12 mineral attachment sites on the protein cavity surface and a variable number of catalytic ferroxidase sites in the center of each subunit (3–24), depending on the number of H and L subunits per ferritin molecule.

H-ferritin subunits have an active ferroxidase site and occur in multiple forms in humans, animals, plants and bacteria. L-ferritin subunits have a degenerate ferroxidase site and the gene duplication to encode L-ferritin subunits is found only in vertebrate animals. H- and L-ferritin subunit expression is set by gene transcription during cell differentiation. Changes in ferritin H/L ratios are known to occur in animals or cells responding to very high levels of iron (16–19) or growth factors (20) and to hypertransfusion in humans with sickle cell disease and β-thalassemia (Hagar, Harmatz, Vichinsky, and Theil, 2003, unpublished results).

Ferritin function

Ferritin is required, not a luxury, as shown by the lethality of ferritin gene deletion during mouse embryonic life (21) and presence even in strictly anaerobic bacteria (22). In humans, the only diseases related to ferritin mutations were discovered very recently (Table 2) and are relatively benign or appear late in life. The human ferritin gene mutations that are compatible with survival modulate expression (4,23) or change the protein structure in a variable region (24), which suggests the lethality of mutations in major ferritin features.

Understanding the multiple functions of ferritin requires defining and studying each type of functional site (Fe entry, oxidation, translocation, mineralization and exit). How each functional site interacts with the others of the same type (eight Fe entry sites, three to 24 Fe oxidation sites, 24 Fe translocation sites, 12 Fe mineral attachment/mineralization sites and eight Fe exit sites) and of the different types adds a unique type of complexity to understanding ferritin function. However, many lessons can be learned from oxygenases and pore proteins.

When Fe^{2+} enters the ferritin protein pores, it rapidly (in milliseconds) readies the ferroxidase site buried within each H-ferritin subunit. The ferroxidase site activity that oxidizes Fe^{2+} and forms diferroxo-mineral precursors (Fe^{3+}-O-Fe^{3+}) is the function best characterized in ferritin. Mossbauer, resonance Raman and EXAFS spectroscopies (1,2,25) have been used to analyze the fast ferroxidase reaction intermediates (in milliseconds) by rapid mixing and freezing to trap enough of a blue (A650nm) diferric peroxo-transition state for the spectroscopic studies. Trapping the diferric complex in protein crystals has not been possible. The peroxo intermediate also forms in di-iron peroxo oxygenases. Iron is a substrate converted to the diferric peroxo intermediate in ferritin and is released into the interior of the protein as a diferric-oxo mineral precursor (**Fig. 2**). (Note that in di-iron oxygenases, the iron is retained at the active site as a cofactor, and O is activated for insertion into substrates such as methane, to make methanol). Ferritin releases H_2O_2 (26–28) and diferric-oxo mineral precursors (Fig. 2), leaving behind an active site that is altered for a fairly long time (26,29), presumably to allow peroxide to diffuse away before binding the next Fe(II) substrate atoms. In bacteria, the iron site in ferritin has a variety of Fe amino acid ligands. Heme is also a cofactor and mineralization does not produce peroxide (1). Other bacterial ferritin-like molecules do not bind oxygen, but rather bind peroxide and convert it to water (30).

H_2O_2 production, when iron and oxygen enter the protein to form the iron mineral, will reflect the H/L subunit ratio (**Table 3**). When the amount of extra iron in a cell is far above normal, as in transfusional iron overload or hereditary hemochromatosis, the combination of extra iron in the "labile iron pool" and extra peroxide from mineralizing iron may outstrip the activities of catalase, glutathione peroxidase and superoxide dismutase. In a number of cases during iron overload in animals or cultured cells, the H/L ferritin subunit ratio decreased, likely to minimize tissue damage from reaction of labile iron and peroxide, (16–19). Recently, a correlation between liver fibrosis and H/L ferritin subunit ratio was observed in humans with transfusional iron overload (Hagar and Theil, 2003, unpublished results).

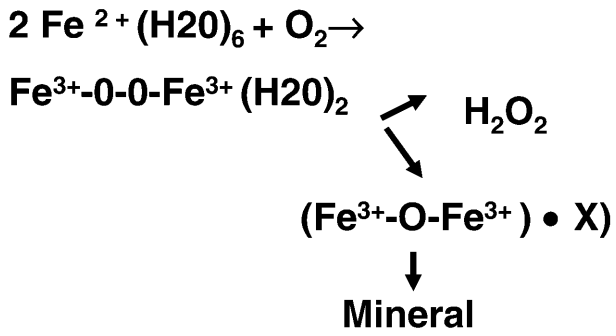

FIGURE 2 Ferroxidase site reaction in H-ferritin subunits.

Protein pores are the sites in ferritin where Fe^{2+} enters the protein from the cell or solution on the way to the ferroxidase site. In addition, Fe^{2+} leaves the protein as the mineral dissolves (reduction and rehydration) through eight pores formed at the junction of three ferritin subunits. Crystal structures of all L- and all H-ferritin subunits show the pores to be similar (1,2,31). Conserved amino acids important for iron entry, Asp 127 and Glu 131, were identified by amino acid substitution where decreases in oxidation rates were up to 100× (32,33). Fe^{2+} exit is controlled by a different set of conserved amino acids, Arg 72, Leu 110, Asp 122 and Leu 134, which have small to no effects on Fe oxidation but large effects (up to 30×) on iron exit rates triggered by NADH/FMN and detected by formation of colored chelator complexes outside the protein (31,34).

Identification of a "gate" in the ferritin pore occurred when the crystal structure of the –L134 mutated protein was examined (31). The entire helix-loop-helix of each subunit around the pore, amino acids 110–134, was completely disordered (unfolded), although the remainder of the subunit had folded and assembled normally, which was associated with enhancing the rate or reduction and chelation of iron in the ferritin mineral. When the ferritin polypeptide chain was extended by an insertion of five amino acids between 120 and 125 at the pore, the effect on Fe^{2+} exit was the same as the amino acid substitutions (34). The results suggest that gating the ferritin "pore" requires the set of interactions (hydrogen bonds: Leu 110/134, and ion pairs: Arg 72/Asp 122) with positions determined in part by the loop length. The selective advantage that conserved the exit pore amino acids in ferritin could be interactions with a cytoplasmic factor that "opens" the pore on demand. Recent studies with small chaotropic molecules show that the pore in the native protein is differentially accessible to

TABLE 3

Effect of changing H/L subunit ratio on maximum moles H_2O_2 produced (1000 Fe in ferritin mineral)

H/L subunit Ratio	All H	H/L = 0.5 (normal liver)	H/L = 0.18 (SCD)
H subunit/molecule	H:L = 24	H:L = 8:16	H:L = 3:21
H_2O_2	500	<170	<75

solute molecules (35) and support the idea of regulated opening and closing of the ferritin pore.

Translating ferritin structure/function and regulation for new strategies in medicine and nutrition

Both ferritin mRNA and ferritin protein contain structures that could be selectively targeted to synthesize more protein for efficient iron detoxification or to "open" the ferritin pores to remove more iron. Enhanced synthesis of ferritin or removal of ferritin iron could improve the current regimens for iron chelation therapy in transfusional iron overload associated with genetic anemias. The lesson learned from nature's use of ferritin as a regulated source of iron during the development of plants and animals suggests that ferritin might be an efficient source of dietary iron. Preliminary results are encouraging (36–38). Targeting ferritin in iron overload diseases to synthesize more ferritin protein is one approach to managing iron excess safely. Another approach is the faster removal of iron from existing ferritin molecules.

Manipulating the ferritin mRNA IRE can lead to increased synthesis of ferritin protein. During iron overload, a large increase occurs in the fraction of cellular iron that is bound to ribosomes to synthesize ferritin protein. However, a large fraction still remains inactive (39,40), apparently because some IRE/IRP interactions remain. Recent studies, which show that the ferritin IRE-specific structure folds in vivo and is accessible to bind small molecules the same way as in vitro (41), open the way for manipulating the ferritin IRE in vivo to increase the synthesis of ferritin protein and safe storage of iron. Overexpression of L-ferritin in humans due to IRE mutations has relatively few physiological effects (23), indicating the potential efficacy of controlled manipulation of the ferritin IRE to manage iron overload.

Opening ferritin protein pores to enhance iron chelation can lead to faster removal of iron from ferritin during iron overload. The ferritin pores are almost closed in native proteins when examined in crystals. Nevertheless, molecules larger than the pore size in the protein crystals reach the protein cavity in solution [reviewed in (1)], which suggests that the pores "flex" or "open". Recent studies have identified several features of "gating" the ferritin pores (31,34), measured in mutated proteins as changes in the rate of Fe^{2+} chelation, triggered by the addition of a reductant (FMN/NADH) or by blocking Fe^{2+} entry (42). Because small chaotropic molecules also open ferritin pores in the native structure without altering the global properties of the protein (35), it should be possible to develop new chelators targeted to ferritin pores that will remove iron more quickly than current chelators that are mainly targeted to iron outside cells or in the "labile iron pool".

Ferritin in nutrition

Iron deficiency is a human problem for which the treatment has been known since medieval times. Nevertheless, over 30% of the world's population is thought to suffer from iron deficiency anemia with associated economic and cognitive loss. Clearly the solution lies in improved access to bioavailable forms of iron. Many seeds such as legume seeds store iron in ferritin, which is a bioavailable iron source (36–38) (**Fig. 3**). When women with borderline iron deficiency consumed meals with intrinsically labeled soybean iron, high absorbance levels were also observed (43). The mechanisms of iron uptake into the gut in humans and animals are not known; nor is it known what impact the differences in the plant ferritin mineral (amorphous, high phosphate) and the animal ferritin mineral (crystalline, little phosphorus) have on digestion. Whether the differences in the subcellular location of ferritin in plants, inside a seed organelle, the amyloplast, or animal tissue, mostly in the cytoplasm, affect digestion and interaction with other food components such as phytate, is completely unknown. Experiments to analyze how ferritin absorption occurs from food have just begun, but the potential impact of ferritin, "nature's iron", on global iron deficiency could be immense.

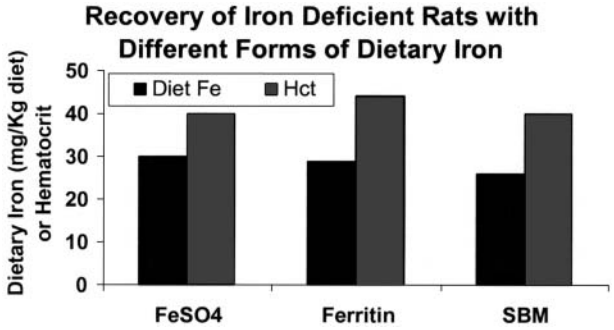

FIGURE 3 Bioavailability of iron in different dietary forms. Rats made anemic from restricted dietary intake of iron were divided randomly into three groups and fed equal amounts of dietary iron, in the form of horse spleen ferritin (ferritin) and soybean meal (SBM), where 70–98% of the iron is in ferritin and $FeSO_4$. Replenishment of iron for the erythron occurred at the same rate for all three forms of dietary iron. [Data from (36)]. Similar results were obtained with transgenic rice encoding the soybean ferritin gene (37).

The central role of ferritin iron in oxygen homeostasis is emerging. Crucial pieces of evidence are: *1*) the impact of deletion in animal models, *2*) the relative mildness of human mutations that survive, *3*) the conservation of unique protein structure and function, and *4*) DNA regulation that is integrated with combinatorial mRNA regulation. Complex regulation of ferritin and other proteins of iron and oxygen homeostasis create an integrated set of responses to iron and oxygen signals from the environment. Six Cs characterize ferritin structure/function and regulation.

- Concentrates Fe 10^{15} × solubility
- Catalyzes Fe^{2+} to Fe^{3+} mineral precursor
- Converts O_2 to H_2O_2, $Fe_2O_3 \cdot H_20$
- Combinatorial mRNA regulation
- Controls Fe availability, chelation
- Contributes dietary Fe.

Discovered early in the 20th century, ferritin began yielding its secrets at the century's end. The future understanding of ferritin and the uses for it in medicine and nutrition in the 21st century appear to be boundless.

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