5-1998

The Role of Ferritin in Iron Regulation

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THE ROLE OF FERRITIN IN IRON REGULATION

by

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Thesis submitted in partial fulfillment
of the requirements for the degree

of

UNIVERSITY HONORS
WITH DEPARTMENT HONORS

in

Liberal Arts and Sciences

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UTAH STATE UNIVERSITY
Logan, UT

1998

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Introduction

Iron plays a critical role in biological systems. Its variable redox properties make it a tremendously versatile element. It is involved in numerous cellular processes including; oxygen transport, nucleotide synthesis, nitrogen fixation, electron transport and a host of metabolic reactions. Without it, nearly all life would cease to function. However, under certain circumstances iron can be extremely detrimental to biological systems and has been linked to numerous pathologies, including cancer, diabetes, Parkinson's disease, Alzheimer's and atherosclerosis. In the presence oxygen, Fe(II) is rapidly oxidized to Fe(III), and oxygen is reduced to potentially deleterious oxygen radicals (1). These radicals, especially the hydroxyl radical, are powerful oxidants, capable of oxidizing DNA, lipids and proteins (2). As a result, biological systems have developed intricate methods for maximizing the benefits of iron utilization while at the same time minimizing its destructiveness.

A key factor in the regulation of iron in mammalian systems is the iron-storage protein, ferritin. This 450 kD protein is responsible for the storage of excess intracellular iron. It is a heteropolymer consisting of 24 H and L chain subunits arranged in a hollow, spherical structure. The ratio between H and L ferritin subunits varies within cells and tissues. Twenty-three hundred Fe atoms, arranged as a ferric oxyhydroxide phosphate complex, can be stored in the ferritin core. Iron incorporated into ferritin will not catalyze the production of reactive oxygen species under physiological conditions and therefore provides the cell with a safe, bio-accessible form of iron.

Ferritin has numerous functions in mammals. In high iron demanding cells, such as

erythrocyte precursors, ferritin plays a critical role in the sequestration and stabilization of excess intracellular iron. In the spleen and liver, ferritin is involved in the uptake and distribution of iron as iron containing proteins are recycled. In the intestinal mucosa, iron is believed to play a role in the regulation of iron absorption from the gut. As levels of ingested iron increase, incoming iron is stored in mucosa ferritin, where the iron can be later mobilized for uptake or sloughed off(3). Ferritin is also believed to have a regulatory role in the growth of macrophages and granulocytes(4), although the mechanism by which this occurs is still unclear. The role of ferritin in mammalian systems is both diverse and widespread.

The Loading of Iron into Ferritin

The loading of iron into ferritin requires the oxidation of Fe(II) to Fe(III) followed by the subsequent incorporation of Fe(II) into the ferritin core. Since this reaction requires the transfer of electrons in an oxidative environment, the partial or complete reduction of molecular oxygen to either oxygen radicals or water is a distinct possibility(5). When iron is loaded in an environment lacking Fe(II) chelators, a mixture of reactive intermediates like $O_2^{\cdot -}$, H_2O_2, $OH^{\cdot}$, can be generated, most likely through a series of reactions known as the Haber-Weiss reactions:

$$Fe(II) + O_2 \text{ ----> } Fe(III) + O_2^{\cdot -}$$

$$O_2^{\cdot -} + HO_2^{\cdot} \text{ ----> } H_2O_2 + O_2$$

$$Fe(II) + H_2O_2 \text{ ----> } HO^{\cdot} + OH^{-} + Fe(III)$$

It is interesting to note however, that ferritin isolated from rat liver, which typically contains between 500-2300 atoms of iron per ferritin molecule, does not exhibit oxidative damage(6). This finding, coupled with the idea that large scale free radical generation *in vivo* would quickly lead to the death of the cell has lead to the hypothesis that a mechanism must exist inside the cell in which iron can be loaded into ferritin safely.

Some investigators have proposed that ferritin exhibits its own ferroxidase activity and is capable of loading itself (7-10). However, this mechanism seems unlikely since the putative ferroxidase activity is only observed *in vitro* when ferritin is loaded with iron in the presence of oxygen and with a Good's-type buffer (ie Hepes), which has been shown to stimulate iron oxidation. No such ferroxidase activity was observed when ferritin is loaded in buffers other than HEPES (11). Furthermore, the ferritin proteins loaded in HEPES buffer exhibit oxidative damage, as supported by the increase in carbonyl content (12). This oxidative damage is likely caused by free radicals produced in the loading process. Specific lysine and histidine amino acid residues at certain locations are especially susceptible this type of oxidation (11). These findings suggest that a better model for iron loading must exist.

Another proposed mechanism for the loading of iron into ferritin involves ceruloplasmin, a glycoprotein containing 5-7 cupric copper, which is synthesized and secreted by the liver. Ceruloplasmin is capable of catalyzing the four-electron reduction of an oxygen molecule to form two molecules of water, and is believed to be involved in the loading of iron into transferrin (12). Additionally, ceruloplasmin has been shown to be a catalyst for the oxidation and incorporation of iron into ferritin (13,14). Ferritin loaded in the presence of ceruloplasmin exhibits similar properties to ferritin isolated from tissues with respect to the stability of its iron core and the

maximal amount of iron that can be loaded into it (15). Furthermore, ferritin loaded by ceruloplasmin does not exhibit the oxidative damage characteristic of the ferritin loaded in a HEPES (Good's-type) buffering system. These observations suggest that ceruloplasmin is involved in the loading of iron into ferritin. A proposed mechanism for the reaction is shown in figure 1.

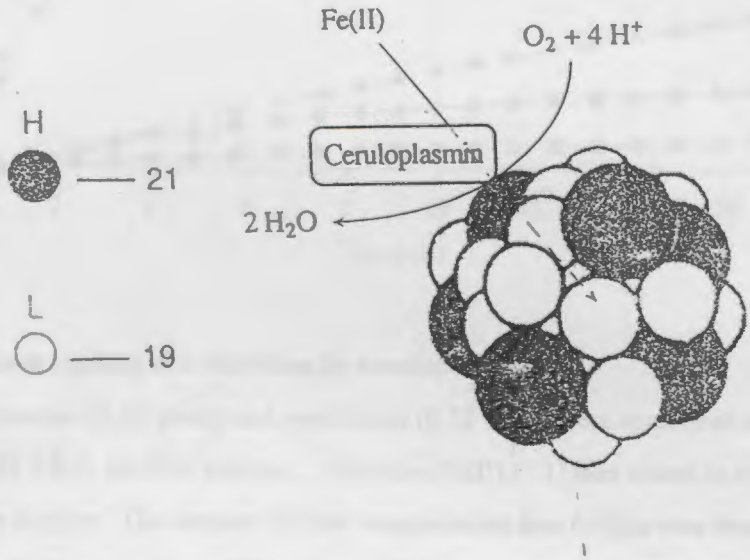

FIG. 1. The loading of iron into ferritin with Ceruloplasmin. In the presence of ceruloplasmin, iron can be loaded into ferritin. H and L represent ferritin H and L chains, rspectively (H-chain 21 kD and L-chain 19 kD).

Studies by Guo et al. (13), and Juan et al. (14), provide additional evidence in support of the ceruloplasmin loading hypothesis. Guo produced recombinant rat liver ferritin H and L chain homopolymers in a eukaryotic expression system (Baculovirus) and attempted to load them in the presence of ceruloplasmin. His data showed that only the H-chain ferritin homopolymers were capable of being loaded with iron. The results of his experiments are shown in figure 2.

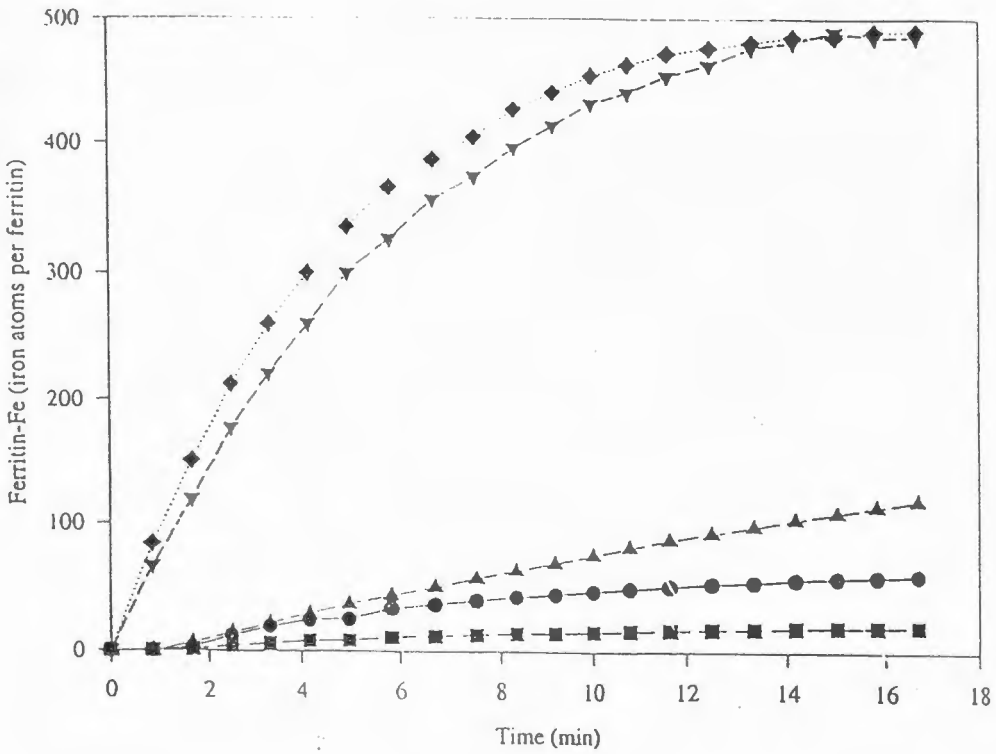

Fig 2. Iron loading into ferritins by ceruloplasmin.

Ceruloplasmin (0.22 nmol) and apoferritin (0.22 nmol) were incubated at 37 ^{0}C in 50 mM NaCl, pH 7.0 (1 ml final volume). Histidine:Fe(II) (5:1) was added to a ratio of 500 atoms of iron per ferritin. The amount of iron incorporation into ferritin was monitored spectrophotometrically at 380 nm and calculated using a molar extinction coefficient (E_{380}=1.03 $nM^{-1}cm^{-1}$). Results are expressed as atoms of incorporated iron per ferritin molecule. Data are representative measurements from at least three individual experiments. RH-Ft loaded by CP( ), rat liver ferritin loaded by CP ( ), rL-Ft loaded by CP ( ), rH-Ft loaded without CP ( ), rat liver ferritin loaded without CP ( ).

These results suggest that an association may have been occurring between the H-chain of ferritin and ceruloplasmin and that only the H-chain was involved directly in iron loading. Studies conducted later by Juan and Guo provide additional support for this hypothesis. Juan was able to determine the optimal loading ratios for ceruloplasmin and H-chain ferritin by producing

recombinant ferritin heteropolymers with 1-2 H chains per ferritin. These recombinant molecules were then loaded with iron using with varying amounts of ceruloplasmin. These studies suggested that the optimal ratio of ceruloplasmin to ferritin during iron loading was 1 mole of H chain ferritin to 1 mole of ceruloplasmin, providing further evidence that a specific interaction between ceruloplasmin and the H chain subunit of ferritin was occuring(14). The results of Juan's experiments are summarized in table 1.

TABLE 1

The Effect of Molar Ratios of Ceruloplasmin to Ferritins on Iron Loading

	rH_1L_{23}-Ft		rH_2L_{22} Ft		Horse Spleen Ferritin		Rat Liver		rH-Ft	
Molar ratio of ceruloplasmin to Ft	Final iron (atoms/ ferritin)	Nonoxidized Fe(II) (nmol)	Final iron (atoms/ ferritin)	Nonoxidized Fe(II) (nmol)	Final iron (atoms/ ferritin)	Nonoxidized Fe(II) (nmol)	Final iron (atoms/ ferritin)	Nonoxidized Fe(II) (nmol)	Final iron (atoms/ ferritin)	Nonoxidized Fe(II) (nmol)
0.5:1	330 ± 10	17.9 ± 0.4			345 ± 10	17.0 ± 1.0	—	—	—	—
1:1	450 ± 20	5.2 ± 0.5	465 ± 5	3.9 ± 0.4	460 ± 10	4.4 ± 0.5	450 ± 10	4.9 ± 0.6	450 ± 20	4.7 ± 1.0
2:1	415 ± 6	0	490 ± 6	0	420 ± 10	0	480 ± 10	0	485 ± 10	0
3:1	392 ± 4	0	435 ± 8	0	394 ± 6	0	494 ± 8	0	498 ± 10	0
4:1			410 ± 10	0			500 ± 10	0	500 ± 6	0
6:1							498 ± 8	0	500 ± 10	0
8:1							499 ± 7	0	500 ± 6	0

Note. Reaction mixtures (1 ml final volume) contained ferritin variants (0.11 μM) and various molar ratios (0.5, 1, 2, 3, 4, 6, or 8) of ceruloplasmin to ferritin variants in NaCl (50 mM, pH 7.0) at 37°C. 500 atoms of iron per ferritin as 5:1 histidine:Fe(II) (55 μM iron) were added. The reactions were monitored for 10 min at 380 nm. Iron content per ferritin (atoms per molecule of ferritin) and nonoxidized Fe(II) (nmol) were measured as described under Materials and Methods. Data are expressed as the mean ± SD of triplicate measurements from three individual experiments.

In both Guo's and Juan's studies, a maximum of 2300 atoms of Fe was loaded into the recombinant ferritin molecules, a number that corresponds well to the total iron incorporated into native ferritin.

Previous research has indicated that a narrow 1.0 A channel is present in the ferritin H-chain subunits that is absent in the L-chain subunits due to the presence of a salt bridge. The difference between the two subunits led to the postulation that this channel may be involved in the loading of iron into ferritin. In order to test this hypothesis, Guo et al. produced recombinant

L chain ferritin homopolymers that lacked this salt bridge as well as recombinant H-chain ferritin homoplolymers that contained the salt bridge, and then loaded them with iron in the presence of ceruloplasmin. The results of the experiments are shown in table 2.

Table 2

Incorporation of Iron into Ferritin by Ceruloplasmin

Ferritins	Initial iron atoms/ferritin	Incorporated iron atoms/ferritin	Non-oxidized Fe(II) nmol
Rat liver	45 ± 4	492 ± 8	ND[a]
rH-Ft	ND[a]	490 ± 5	ND[a]
rH-Ft mutant	ND[a]	5 ± 7	ND[a]
rL-Ft	ND[a]	3 ± 4	46 ± 3
rL-Ft mutant	ND[a]	151 ± 15	43 ± 4

Note. Conditions were as follows: apoferritin (0.22 µM) in 1 ml NaCl (50 mM, pH 7.0) with ceruloplasmin (0.22 µM) and histidine:Fe(II) (550:110 µM) at 37 °C. The reaction was for 17 min. Iron content per ferritin (atoms per ferritin molecule) and iron not oxidized (nmol) were measured under Materials and Methods. Data are given as the mean ± SE of triplicate measurements from three individual experiments.

[a] ND indicates not detectable by the method of iron assay as described in Materials and Methods.

The mutant L chain homopolymer was capable of being loaded with iron in the presence of cerulolasmin although the initial iron loading rates in the mutant L chain ferritin was significantly slower than for native ferritin. The recombinant H chain homopolymers were capable of being loaded with iron in the presence of ceruloplasmin. Additionally, the ferroxidase activity of ceruloplasmin was only found to be stimulated by the H chain homopolymers. These results provide further evidence in support of the ceruloplasmin/iron loading model.

Although there appears to be ample evidence in support of ceruloplasmin as an effective model for iron loading, questions regarding the possibility that such a mechanism occurs *in vivo*

have been raised. These questions seem to have been partially answered by studies involving Alzheimers, a known iron disorder in the brain, in which low levels of ceruloplasmin had a high correlation with the disease (16). It has also been shown that ceruloplasmin mRNA exists and is inducible in various organs including the lungs, synovia tissue, the testis, the choroid plexus, and the uterus.

The Production of Recombinant Human Ferritin Homopolymers

Although rat ceruloplasmin does appear to play a key role in the loading of iron into rat ferritin, it is unknown if a similar mechanism occurs in humans. One way to determine this is by using recombinant human ferritin and seeing if it can be loaded with iron by ceruloplasmin. By using recombinant ferritin, it is possible to avoid the high costs associated with handling human tissue. It is also possible to produce ferritin H and L chain homopolymers (24 H or L chain subunits only), which do not occur *in vivo.* The production of H and L chain homopolymers makes it possible to study their individual roles.

Cloning and expression of the human H and L chain ferritin genes. A human liver cDNA library was obtained from Clontech. Primers designed to amplify the human H and L chain ferritin genes via Polymerase Chain Reaction (PCR) were obtained from DNA Express Macromolecular Resources. Following PCR, the DNA was purified using a Qiagen PCR purification kit and electrophoresed using a 3% agarose gel. The results are shown in figure 3. Samples of DNA with the appropriate # of base pairs (lanes 2-6) were sent to the Utah State Biotechnology center for sequencing. Sequencing results confirmed that the purified PCR products were in fact the human ferritin H and L chain genes (lanes 2-4, H chain & lanes 5-6, L chain).

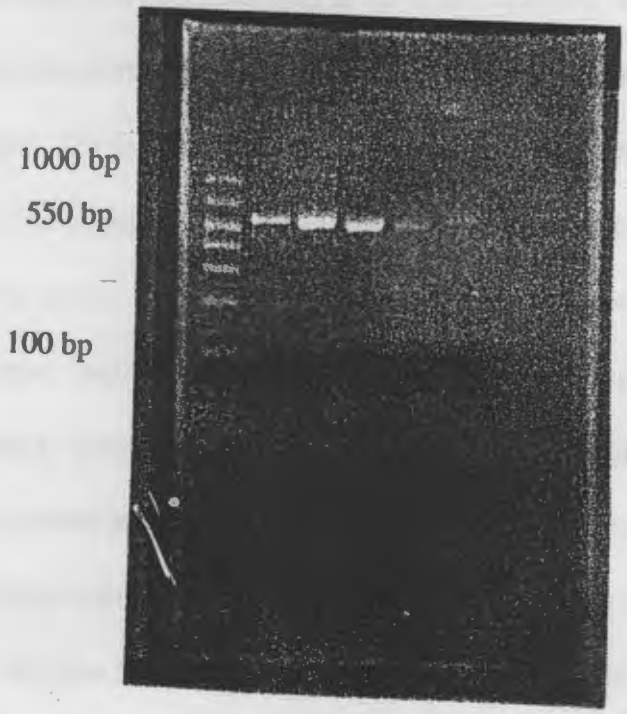

Figure 3. Lane 1,standard DNA 1-1000 bp marker. Lanes 2-4, human ferritin H chain gene + primer tail (552 bp + 9bp). Lanes 5-6, human ferritin L chain gene + primer tail (527 + 9bp)

The Baculovirus transfer vector PAcUW51 was obtained from Pharmingen. This vector contains a site for gene insertion under the control of the P10 promoter. This insert site has a Bgl II restriction enzyme recognition sequence and is therefore made accessible to genes by digestion with Bgl II. foreign genes by digestion with Bgl II. Corresponding Bgl II restriction enzyme sites were incorporated into the primer tags for the H and L chain genes prior to initial PCR. Restriction enzyme digest reactions were performed using the Bgl II restriction enzyme for both

the H chain and L chain genes and the PAcUW51 vector. Digested products were purified and added to a 50 ul ligation reaction mixture containing a total of 5ug insert DNA and 1 ug vector DNA. The ligation reaction was incubated at 16° C for 16 hours. Potential insert/vector plasmids were then transformed into DE3 *E.coli* cells, plated on ampicillin/agarose plates and incubated overnight. The fact that the PAcUW51 vector conveys ampicillin resistance to the host cell was a means of selecting only those cell colonies which had been effectively transformed with the PAcUW51 vector. Colonies which appeared on the ampicillin/agarose plates were tested for vector/insert incorporation using PCR and specially designed insert/vector identification primers. Positive clones were selected and used to inoculate 100 ml of LB media, and, after being incubated at 37° C overnight, the vector contained in the cells was harvested and purified using a Qiagen Midi-prep purification kit. To verify that the DNA inserted into the PAcUW51 was in fact the H or the L chain ferritin genes, PCR which amplified the 5' and 3' regions of the PAcUW51 on either side of the inserted gene along with the inserted gene were sequenced at the Utah State Biotechnology Center. The results confirmed the proper insertion of both the H and the L chain genes. Now that the H and L chain ferritin genes have been inserted into the Baculovirus transfer vector, it is possible to produce recombinant virus and begin the final steps necessary for the production of recombinant H and L chain ferritin.

Summary

Iron is critical for the survival of biological systems. However, Fe must be tightly regulated if it is to be utilized without causing any detrimental effects. In mammalian systems, the iron storage protein ferritin has evolved to store iron in a safe, bio-accessible form, eliminating the potential production of reactive oxygen species that could occur when iron is in

an aqueous environment.

Although some have proposed that the loading of iron into ferritin is accomplished by the ferroxidase activity of ferritin, this hypothesis seems unlikely for several reasons. First, the ferroxidase activity of ferritin can only be observed in a HEPES-type buffer system, which is known to promote ferrous iron oxdation. Secondly, ferritin loaded in a HEPES type system has been shown to be oxidatively damaged.

A more appropriate model for the loading of iron into ferritin would be the copper-containing ferroxidase, ceruloplasmin. This protein has been shown to load iron into ferritin without the production of reactive oxygen species. Research has also shown that an interaction may also occur between the H chain subunit of ferritin and ceruloplasmin, providing further evidence in support of this model. Recently, evidence has also been provided by Reilly et al. (17) suggesting that a ceruloplasmin-like protein may be in the membranes of several rat tissues, including heart. In the future, it will be interesting to see if either ceruloplasmin or a ceruloplasmin "like" protein will be found within cells. At this point, it seems likely. Future studies involving recombinant human ferritin homopolyers and heteropolymers should provide additional insight into the loading process and the specific interaction that occurs between the H chain of ferritin and ceruloplasmin.

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