Expression and Iron Loading of Recombinant Ferritin Homopolymers

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EXPRESSION AND IRON LOADING OF RECOMBINANT FERRITIN HOMOPOLYMERS

by

Jia-Hsin Guo

A dissertation submitted in partial fulfillment of the requirements for the degree of

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in

Nutrition and Food Science

Approved:

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Logan, Utah

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ABSTRACT

Expression and Iron Loading of Recombinant Ferritin Homopolymers

by

Jia-Hsin Guo, Doctor of Philosophy

Utah State University, 1998

Major Professor: Dr. Steven D. Aust
Department: Nutrition and Food Sciences

Ferritin is an iron storage protein consisting of H and L chains to form a 24-subunit heteropolymer. Ceruloplasmin oxidizes Fe(II) and then loads the iron into ferritin. This research was conducted to determine which ferritin subunit is involved and whether a proposed iron-loading channel is required for iron loading by ceruloplasmin.

Recombinant rat liver H and L chain ferritin homopolymers, designated as rH-Ft and rL-Ft, respectively, were produced using insect cell-baculovirus and Escherichia coli expression systems. The expressed
rH-Ft strongly suppressed the growth of the host. The rH-Ft expressed in the E. coli contained approximately 150 iron atoms/ferritin and was observed to have protein damage, which was found to affect iron-loading by ceruloplasmin. The ferritin expressed in the E. coli system apparently was not proper for this iron loading study. Alternatively, the ferritins expressed in the insect cell-baculovirus system were utilized for this purpose. Ceruloplasmin was able to load iron into the rH-Ft, but not the rL-Ft. The initial rate of loading iron into the rH-Ft by ceruloplasmin was similar to that of native rat liver ferritin heteropolymer. Both the rH-Ft and the native rat liver ferritin could be maximally loaded with iron by ceruloplasmin up to 2,500 iron atoms/ferritin. When the rH-Ft or the native ferritin was present, the ferroxidase activity of ceruloplasmin was enhanced. No such enhancement was observed in the presence of the rL-Ft. This suggests that ceruloplasmin only associates with the ferritin H, but not L, chain during iron loading.
The role of an α-helix bundle channel in iron loading by ceruloplasmin was investigated by using site-directed mutagenesis. The channel in the rH-Ft was closed by mutation E62K and H65G to form a K62 to E107 salt bridge, which is thought to exist in the L chain. Conversely, the salt bridge in the channel of the L chain was removed by mutation K58E and G61H to form a channel similar to that in the four-α-helix bundle of the H chain. The initial rate of loading iron into the rL-FT mutant by ceruloplasmin was 50% of that for loading iron into the rH-Ft. When 500 atoms of iron per ferritin were used for loading, 98% loaded into the rH-Ft by ceruloplasmin in 5 minutes, but only 30% loaded into the rL-Ft mutant in the same time. The ferroxidase activity of ceruloplasmin was enhanced in the presence of the rH-Ft and its mutant, but not in the presence of the rL-Ft or its mutant. These results indicate that the association of ceruloplasmin and ferritin is required and the α-helix bundle channel is a channel for iron loading.
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CHAPTER I
INTRODUCTION

The goal of this research is to investigate the roles of the heavy (H) and light (L) chains of ferritin in iron loading by ceruloplasmin. In particular, this dissertation focuses on investigating which ferritin subunit is involved and whether a channel is required for the iron loading by ceruloplasmin.

Iron is required for nearly all cellular life. The variable redox properties of this transition metal permit its use as a cofactor in enzymes for electron transport and catalysis. However, Fe(II) is rather reactive and unstable when present in an oxygen-rich atmosphere. Unless being bound by proteins or other biological chelators, Fe(II) is rapidly oxidized to form insoluble iron oxide, i.e., iron rust. Moreover, reactive iron may provide a Fenton reagent to produce various deleterious oxygen radicals (1). Oxygen radicals, such as hydroxyl radical, are potent oxidants and can usually diffuse a few Å to oxidize surrounding biological molecules, such as proteins, lipids, and DNA (2).
Ferritin has evolved to avoid these problems. Insoluble iron oxide is enclosed inside ferritin to provide a soluble form of iron in the biological environment. Loading, storage, and release of iron are the fundamental processes by which ferritin serves as a reservoir to keep a supply of essential iron in a non-toxic and biologically available form inside the cell's cytoplasm. Loading iron into ferritin requires Fe(II), followed by an oxidation process to generate Fe(III), which is incorporated through the shell of ferritin into the protein core (3). However, the oxidation of iron can present a rather dangerous situation, the production of partially reduced species of oxygen. The donation of electrons to molecular oxygen can result in either partial reduction to form various oxygen radicals or complete reduction to form \( \text{H}_2\text{O} \) (4). Uncontrolled iron oxidation usually results in a mix of completely and partially reduced products. The products can be \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \( \cdot\text{OH} \), and \( \text{H}_2\text{O} \), which are generated by one-, two-, three-, and four-electron reduction of \( \text{O}_2 \), respectively. The generation of oxygen radicals by partial \( \text{O}_2 \) reduction
in vivo must always be controlled properly and confined in specific cellular compartments, such as mitochondria. Otherwise, the cell may be destroyed due to serious oxidative damages by the oxygen radicals.

Ferritin resides in the cell's cytoplasm and can store up to 2,500 atoms of iron (5). If iron oxidation in vivo is not well controlled during iron loading into ferritin, the oxidation will generate a massive amount of oxygen radicals, which may cause serious oxidative damage to the ferritin molecule and surrounding cellular compartments. However, this kind of damage was not observed on the native ferritin isolated from rat liver, which contained 500 to 2,300 atoms of iron per protein (6). Therefore, the partially reduced oxygen radicals are unlikely to be produced in vivo during the loading of iron into ferritin. Alternatively, di-oxygen may be reduced to form two molecules of H₂O during the loading of iron into ferritin by oxidizing four Fe(II) to Fe(III) and transferring four electrons to the oxygen molecule. This reaction is attractive because it does not generate any reactive species which may damage the cell, and may
be the most feasible reaction occurring in vivo for loading iron into ferritin. However, what is the biological enzyme or oxidant that can accomplish the Fe(II) oxidation and the complete reduction of O₂ during the iron incorporation into ferritin?

It was proposed that ferritin has its own ferroxidase activity such that incubation of ferritin with Fe(II) results in the iron incorporation into the iron core of ferritin (7-10). This hypothesis is controversial because the proposed ferroxidase activity of ferritin is dependent upon the use of a Good type buffer, such as HEPES, which itself can promote the autoxidation of iron. Without using this specific buffer, the ferroxidase activity of ferritin cannot be observed (11). In addition, a ferroxidase usually requires a transition metal, such as copper, residing in the enzyme's active site to act as an intermediator for the electron transfer of the iron oxidation reaction. Newly synthesized ferritin initially does not contain any transition metal. Although some studies proposed that ferritin can capture two atoms of iron to form a di-iron
center residing in the ferritin H chain (12,13), the function of this di-iron center for the ferroxidase activity of ferritin, however, is not conceivable because the recovery time of the di-iron center after oxidizing one iron atom may require several hours (14). Thus, the di-iron center is probably not relevant to the iron loading reaction. In addition, the most important argument against a ferroxidase activity on ferritin is that ferritin that has been loaded with iron in the Good type buffer suffers oxidative damage (11). When ferritin is loaded with iron in the Good type buffer, the total carbonyl content of ferritin is increased (6). This result indicates that oxygen radicals were produced during this type of iron loading and certain amino acid residues of ferritin were oxidized by the radicals. Histidine and lysine of ferritin were modified (11). The iron loaded into this ferritin was not as stable as that of native ferritin isolated from rat liver (6).

Ceruloplasmin is a copper-containing protein which can catalyze the Fe(II) oxidation and four-electron reduction of an oxygen molecule to form two molecules of
water (15). The ferroxidase activity of ceruloplasmin was proposed as a means of loading iron into transferrin (16). The ferroxidase activity of ceruloplasmin has also been observed for the catalysis of iron oxidation and deposition into ferritin (6,11,17-19). Ceruloplasmin may inhibit superoxide and ferritin-dependent lipid peroxidation by reloading iron into ferritin without the release of partially reduced oxygen species (20). The ferritin loaded by ceruloplasmin behaved similarly to ferritin isolated from tissues, with respect to the stability of the iron core and the maximal amount of iron contained in the protein (6,11). Most significantly, the ceruloplasmin-loaded ferritin did not have any oxidative damage (11). There is no doubt that ceruloplasmin has the ability to load iron properly into ferritin. The research presented herein focused on identification of the iron-loading channel of ferritin and how ceruloplasmin loads iron into ferritin.

Ceruloplasmin must associate with ferritin to place the oxidized iron directly into ferritin. Without association, oxidized iron may not be loaded within
ferritin but may precipitate out of solution. An example is tyrosinase, which has ferroxidase activity but is not capable of loading iron into ferritin (21). The association of ceruloplasmin and ferritin may be observed by the enhancement of ferroxidase activity of ceruloplasmin as the association may alter the conformation of ceruloplasmin, increasing its ferroxidase activity. An iron-loading channel may be required also for loading iron into ferritin. This channel in ferritin may be consolidated with the ceruloplasmin association site for iron loading.

Chapter III describes a study in which recombinant rat liver ferritin H and L chain homopolymers were produced by the insect cell-baculovirus expression system and the protein properties were characterized. Using ceruloplasmin, iron loading into the ferritin H and L chain homopolymers was determined. The iron complex employed in these experiments was histidine-chelated Fe(II) (at a molar ratio of 5:1), which has been shown as stable to autoxidation and a good substrate for ceruloplasmin (22). Many investigators have proposed the
ferritin H, but not L, chain as containing an α-helix bundle channel for iron loading using the HEPES buffer system. However, our laboratory used ceruloplasmin as an iron loading enzyme. It is noteworthy to know if the same iron loading channel of ferritin is involved for different iron-loading mechanisms. The results showed that ceruloplasmin only can load iron into the ferritin H, but not L, chain homopolymer. The iron-loading rates of the ferritin H chain homopolymer using ceruloplasmin were comparable with those obtained using native rat liver ferritin. A maximum of approximately 2,500 iron atoms was incorporated into both the ferritin H chain homopolymer and native rat liver ferritin. These results suggest that both ferritin H chain homopolymer and native rat liver ferritin have the same capacity for iron storage when ceruloplasmin is used as a ferroxidase for iron loading. The results also indicate the iron-loading channel may be present in the ferritin H, but not L, chain. In addition, an interesting feature of ferritin iron loading by ceruloplasmin is that native rat liver ferritin or the ferritin H chain homopolymer, but not the
ferritin L chain homopolymer, enhanced the ferroxidase activity of ceruloplasmin. The enhancement of the ferroxidase activity of ceruloplasmin may be due to the association of the ferritin H chain with ceruloplasmin. This association may alter the conformation of ceruloplasmin, increasing its ferroxidase activity. We believe this association is required for ceruloplasmin to safely load iron into ferritin, as mentioned earlier.

While producing the recombinant rat liver H and L chain ferritin homopolymers using *Escherichia coli* and insect cell-baculovirus expression systems, we noticed the ferritin H chain homopolymer had a suppressive effect on the growth of *E. coli* and *Spodoptera frugiperda* (Sf-21) cells. The ferritin H chain has been found to have a strong suppressive effect on several cell lines by other investigators (23-25). However, the suppressive effect of the ferritin H chain has not been tested in a recombinant expression system. It has been proposed that the suppressive effect of the ferritin H chain is due to its competition for cellular iron (23-25). If this hypothesis is true, then fully-loaded ferritin H chain
homopolymer should not suppress cell growth. This was tested by investigating the suppressive effect on *E. coli* and Sf-21 cell growth during the expression of the ferritin H chain homopolymer or adding various iron-containing ferritin H chain homopolymers into insect cell cultures (Chapter IV). Suppression was observed immediately when the ferritin H chain homopolymer was expressed in both *E. coli* and insect cell-baculovirus expression systems. Adding purified ferritin H chain homopolymers containing no iron, 1,970 iron atoms, or 2,520 iron atoms per ferritin also resulted in immediate suppression of Sf-21 cell growth. These results indicate that suppression may not be due only to the competition for cellular iron. The ferritin H chain has also been proposed as a regulator for cell growth (26).

Suppression of the expression system is probably due to interference of certain gene expression of the host by the ferritin H chain homopolymer. An investigation of the suppressive effect of ferritin H chain homopolymer, reported in Chapter IV, resulted in a strategy for the expression of rat liver ferritin H and L chain
homopolymers in both *E. coli* and insect cell-baculovirus expression systems.

A comparison of structural and functional differences of recombinant rat liver ferritin H chain homopolymers expressed in *E. coli* and insect cell-baculovirus expression systems is reported in Chapter V. Ferritin is an intracellular protein that does not contain any post-translational modification. The expression of the ferritin H chain homopolymer in *E. coli* and insect cell-baculovirus systems should result in proteins having the same structures and functions. However, we found that the ferritin H chain homopolymer expressed in the *E. coli* system had already contained approximately 150 iron atoms per protein, whereas the ferritin expressed in the insect cell-baculovirus system had none. In addition, the ferritin H chain homopolymer expressed in the *E. coli* system had more basic and multiple surface charges, compared to the theoretical values, whereas the ferritin H chain homopolymer expressed in the insect cell-baculovirus system had a single surface charge. The molecular weight of the
ferritin H chain homopolymer expressed in the *E. coli* system was smaller than that expressed in the insect cell-baculovirus system. Results indicate that certain amino acid residues of the ferritin H chain homopolymer expressed in the *E. coli* system had been modified, perhaps due to unregulated iron incorporation inside the *E. coli* cells. The ferritin H chain homopolymer expressed in the *E. coli* system had a less molecular weight compared to that expressed in the insect cell-baculovirus system. The ferritin homopolymer expressed in the *E. coli* system may be assembled with fewer than 24 subunits. The damaged ferritin was also found to have an unstable protein structure and different iron-loading curve compared to ferritins expressed in the insect cell-baculovirus system or isolated from rat liver. These results suggest that the expression of the ferritin H chain homopolymer should not be done using an *E. coli* system or more care should be used to avoid damage to the expressed protein.

Chapter VI describes a study to identify the iron channel for ceruloplasmin to load iron into ferritin. As
mentioned before, the channel may be present in the ferritin H, but not the L, chain because ceruloplasmin can only load iron into the ferritin H chain homopolymer. It has been reported that a narrow 1.0 Å channel is present in the four-α-helix bundle of the ferritin H chain, whereas this channel is blocked by the presence of a salt bridge (Lys 62 v.s. Glu 107) inside the ferritin L chain (27). If this channel is the iron-loading channel, then removal of the salt bridge to form a channel in the ferritin L chain or manipulation of the salt bridge to block the channel in the ferritin H chain should affect the loading of iron into ferritin by ceruloplasmin. It has been shown that removal of the salt bridge from the α-helix bundle channel of ferritin L chain homopolymer may cause the protein to become insoluble when the protein is expressed by the E. coli system (28). However, we have successfully expressed the opened iron-loading channel ferritin L chain homopolymer, as well as the closed iron-loading channel ferritin H chain homopolymer, using the insect cell-baculovirus expression system. Both proteins are soluble and form a multiple
subunit complex. The ferritin L chain homopolymer mutant could be loaded with iron by ceruloplasmin, but the ferritin H chain homopolymer mutant could not be loaded. These results suggest that the α-helix bundle channel is required for ceruloplasmin to load iron into ferritin. However, the initial rate of iron loading into the ferritin L chain homopolymer mutant using ceruloplasmin was 50% lower than the rates for loading of iron into wild type ferritin H chain homopolymer or native rat liver ferritin. The reason may be because the ferritin L chain mutant does not have the association site for ceruloplasmin to bind during the iron-loading reaction. Part of the iron oxidized by ceruloplasmin may not be properly incorporated into this ferritin. In addition, the ferroxidase activity of ceruloplasmin was only enhanced in the presence of the ferritin H chain and its mutant, but not in the presence of the ferritin L chain or its mutant. These results suggest that the ceruloplasmin association site is present in the ferritin H, but not the L, chain, and independent from the iron-loading channel.
REFERENCES


CHAPTER II

LITERATURE REVIEW

IRON NUTRITION AND BIOLOGICAL ROLES

Iron is the most abundant transition metal found in the human body. An estimation of the total body iron of a 70-kg adult male is about 4 grams, whereas for an adult female it is somewhat less, about 3 grams. Hemoglobin within the circulating red blood cells contains about 60-65% of the body iron. Approximately 10% of the body's iron is found in skeletal muscle, which is present in myoglobin, the cytochromes of the mitochondria, and iron-containing enzymes such as catalase. Other heme-containing proteins, iron-sulfur proteins, and iron-dependent enzymes contribute another 5% of the body iron. The body's remaining 20-25% iron is found in the storage protein ferritin, which is mainly distributed among the liver, spleen, and bone marrow cells (1,2). Only about 4 mg of iron circulates in the plasma bound to the iron transport protein transferrin, an $\alpha_2$ globulin with a molecular weight of approximately 77,000-80,000 (3).
However, the daily exchange of iron through transferrin is ten times this amount. Transferrin is a secreted protein and primarily synthesized in the liver (4,5). Normally transferrin acquires iron from cells of the iron storage compartments and incoming, absorbed iron from the gastrointestinal tract, and then delivers the iron to iron-demanding tissues, such as bone marrow (6), for production of iron-containing proteins. Excess iron is mainly transported to liver cells for iron storage in ferritin (7).

The total ferritin iron of North American males is about 1,000 mg, with females having about 400 mg (8). Stored iron is usually reflected in the amount of serum ferritin circulating in the plasma (9). In a healthy person, iron stores can be calculated from the serum ferritin as follows (10):

\[
\text{Iron stores (mg)} = \text{serum ferritin (\mu g/L)} \times 8 \quad [1]
\]

However, the rationale for this correlation and the physiological role of serum ferritin are still not well
elucidated. In addition, iron stores calculated as described above are not always reliable because serum ferritin is frequently increased in response to inflammation, infection, liver disease, or cancer (11). Therefore, caution is needed when this calculation is used.

In humans, the source of iron is mainly from the diet. The balance of body iron is highly controlled by iron absorption. Iron absorption is responsive to total body iron stores such that subjects with low iron storage, such as iron deficiency, absorb more iron (12). Increased erythropoietic activity also results in high iron absorption (13). Absorption inappropriate to the amount of storage iron does occur. For example, long-term consumption of beer brewed in iron vessels has been shown capable of increasing iron absorption and accumulating high amounts of iron in liver, spleen, and bone marrow (7). However, iron overload due to high absorption of dietary iron is uncommon. In addition, genetic hemochromatosis is one of the most common disorders of body iron imbalance. This disease is
associated with greatly increased, up to 50-fold, deposits of iron in the liver and other tissues due to abnormally high absorption (14). The biochemical origin for this abnormality is not well understood. Abnormally high iron stores are also found as a secondary effect due to ineffective erythropoiesis, such as thalassemia (15). This so-called "iron-overloaded anaemia" is associated with increased iron absorption and also treated by blood transfusion. The increased body iron cannot be eliminated because the amount and the route of the excreted iron are very limited.

The average daily Western diet contains approximately 10-20 mg of iron from which approximately 1-2 mg/day is absorbed by the mucosa of the proximal small intestine. This absorption balances the daily losses of iron from the body that occur mainly from exfoliated mucosa cells of the gastrointestinal tract, with smaller amounts lost from skin, urine, and bile (10). Approximately 80-85% of the absorbed iron is transported by transferrin to the bone marrow for incorporation into the hemoglobin of red blood cells. A
small portion of the absorbed iron is applied to the construction of iron-containing proteins, with the remainder delivered by transferrin to ferritin for storage (16).

Cells require a certain amount of iron for metabolism, replication, and expression of various proteins. These biological functions are conducted by a great variety of heme and non-heme iron-containing enzymes (17,18). The iron present in an enzyme can change its valence. The ability of iron to function across a wide range of redox potentials contributes greatly to its versatility. The most significant reactions catalyzed in the cell by iron are electron transfer (19), ATP generation (20), DNA synthesis (21), oxygen dismutation (22), and the oxidation/reduction of both organic (23) and inorganic (24) substances. The functional importance of iron makes it essential for growth and replication of living cells. Generally all life forms require iron except for certain species of bacteria such as lactobacillus, which need other transition metals such as Mn (25). Most of the iron
present in life forms is tightly restrained within proteins. There is no evidence that there is "free" iron present within the cell; however, a low molecular weight pool of chelated iron in vivo has been hypothesized (26, 27). It is generally believed that the "free" iron associated with a low molecular weight chelator in vivo, under normal conditions, is present in a stable ferrous form but has the potential for the mediation of deleterious reactions.

**BIOLOGICAL TOXICITY OF IRON**

Iron can be very damaging to biological substances. Minotti and Aust (28) reported that an Fe(II)-Fe(III) complex can serve as an initiator of lipid peroxidation in vitro. Most body iron is tightly bound within proteins to prevent this deleterious effect. However, a small amount of iron has been proposed to exist in cytosol associated with undefined physiological chelators, such as citrate and ATP (29), to form a so-called "low molecular weight pool of iron" (26, 27). The low molecular weight iron pool is thought of as being a
necessary vehicle in which the iron exchange between storage and transport iron pools occurs (30). The concentrations of these iron-chelator complexes are normally low, in which Fe(III) is less than 10^{-18} M and Fe(II) is about 10^{-8} M (31). This low molecular weight iron pool is thought to have the potential to generate deleterious oxygen radical species in abnormal physiological states, such as "oxidative stress" (32). Production of oxygen radical species can be achieved by iron, serving as a Fenton reagent, in an iron-catalyzed Haber-Weiss reaction (33-35) shown as follows:

\[
\begin{align*}
\text{Fe(II)} + O_2 & \rightarrow \text{Fe(III)} + O_2^- \quad [2] \\
2O_2^- + 2H^+ & \rightarrow O_2 + H_2O_2 \quad [3] \\
\text{Fe(II)} + H_2O_2 & \rightarrow \text{Fe(III)} + OH^- + \cdot OH \quad [4]
\end{align*}
\]

The reduction of O_2 by Fe(II) can produce O_2^- (Eq. [2]), which may dismutate to form O_2 and H_2O_2 (Eq. [3]). Further reduction of H_2O_2 by Fe(II) may produce the reactive hydroxyl radical (Eq.[4]). The hydroxyl radical is extremely reactive, causing lipid peroxidation (36),
DNA strand breaks (37), and degradation of other biomolecules (38), which may lead to the development of numerous toxicities and pathologies. Increased available body iron may increase the concentration of unregulated low molecular weight iron-chelator complexes (39), thus increasing the risk of certain iron-related diseases, such as heart disease, cancer, and Alzheimer’s.

High body iron has been found to contribute to the cause of heart disease (40). Unregulated body iron may serve as a Fenton reagent to produce the hydroxyl radical (32). The radical can lead to lipid peroxidation of cell membranes and subsequent cell damage. Evidence suggests that a high level of body iron may increase the chance of radical production, which may contribute to cardiac damage including ischemia-reperfusion studies (41,42) and hemochromatosis (43). Reduction of the body iron level may decrease the chance of heart disease. Studies examining the use of low doses of aspirin for prevention of coronary artery disease have been reported. Results indicate that plasma iron concentrations can be reduced by taking 3-4 g of aspirin per day (44). Myocardial
Infarction can be prevented by taking 0.3-1.5 g of aspirin per day (45).

Cancer is another major concern for iron overload. Results of several studies (46-48) indicate that abnormal iron metabolism and high body iron can be associated with carcinogenesis. However, the mechanism of iron-induced carcinogenesis is uncertain. Iron may generate oxygen radical species and modify DNA. In general, genetic alterations, such as mutations and deletions, most often result in a loss of function or life of the cell. The most likely mechanism by which iron could affect carcinogenesis may involve damage to mechanisms that restrain the multiplication of cells. For example, the mutations in p21 ras (49) and the α subunits of G proteins (50) show that the loss of a negative regulatory function within a protein results in constitutive activation and generation of signals to proliferate. Thus, iron overload may increase the chance of damaging the "tumor suppressor" gene, by which the tumor cell develops rapidly without control.
Alzheimer's disease is a neurological disease highly related to the role of iron and brain functioning in the aged. Iron is critical for normal neurological functions, but changes in iron metabolism in the brain are the major cause of brain dysfunction (51). Most of the iron in the brain is found in ferritin (51,52), although the concentration of brain ferritin is very low (53). The majority of the brain's iron, ferritin, and transferrin are located in the oligodendrocytes of both gray and white matter (51-53). Aging results in a relocation of ferritin and transferrin to the astrocytes in the basal ganglia, hippocampus, and amygdala (53). Low amounts of transferrin with high iron content have been found in the brain tissues of patients with Alzheimer's disease (51,52).

Iron is highly toxic to living cells. Consequently, iron uptake, transport, and storage in the body are extremely isolated and precisely regulated. Ferritin may decrease the risk of intracellular iron toxicity by maintaining iron in a stable storage state. In situations when an excess of iron enters the cell, more
iron will be deposited in ferritin. Excess iron can also
induce the synthesis of apoferritin to provide the cell
with a higher iron storage capacity. Such mechanisms are
developed to protect the cell from damage due to the
oxidative action of iron.

BIOSYNTHESIS OF FERRITIN

Since the reactivity of iron in an aerobic
environment may produce toxic oxygen radical species
(54,55), ferritin is present in nearly every kind of
tissue in order to prevent iron toxicity. The synthesis
of ferritin is mainly regulated by iron. Higher
eukaryotes have evolved a system that may quickly respond
to incoming iron primarily by post-transcriptional
mechanisms (56-61). However, transcriptional induction
also plays a role under certain conditions (62,63). The
biological significance of increasing ferritin synthesis
is to ensure that cells have more storage space for
incoming iron (64).

Originally, Munro and colleagues (65) proposed a
model for the rapid, transcription-independent induction
of ferritin synthesis in response to iron. In this
model, the ferritin mRNA is initially stored as a ribonucleoprotein (RNP)-associated and translationally resting form in the cytosol (65). Iron administration shifts the resting form of ferritin mRNA to a polysome-associated form that is translationally active (65). In rats, both H and L chain ferritin mRNAs have been shown to respond to iron to the same extent and in the same way, having a shift from a resting RNP fraction to the polysome fraction without an overall increase in total ferritin mRNA (65). Subsequent work has identified that the inactive form of ferritin mRNA was actually associated with a repressor protein with a molecular weight approximately 90 kDa (66).

Sequencing of rat liver H and L chain ferritin mRNA showed 210 and 168 nucleotide 5'-untranslated regions (UTRs), respectively (66). Within the structures of the first 75-nucleotide stem-loop, a sequence of 28 nucleotides has been found to be highly conserved within the putative stem-loops in the 5'-UTRs of H and L chain ferritin mRNA of humans (67-69), bullfrogs (70), and rabbits (71). The 28-nucleotide sequence is now known as
the iron responsive element (IRE). IRE structures are primarily involved in the regulation of the initiation of mRNA translation. With its 6-nucleotide loop sequence, CAGUGN (N is any nucleotide other than G), and especially its 5' C nucleotide, are highly critical for binding the repressor protein (72-78). Another important structure for the repressor protein binding is a conserved cytosine within a nucleotide bulge between the upper and lower stems.

Another factor essential for iron-dependent translational regulation is the presence of a cytosolic 98-kDa protein that is found in humans, mice, frogs, and fish (79). This protein is referred to as the iron regulatory protein (IRP) (80). IRP is also known as iron responsive element binding protein (IRE-BP) (81), iron regulatory factor (IRF) (82), ferritin repressor protein (FRP) (83,84), and p90 (85). Some investigators have proposed that binding IRP to the IRE prevents access of eIF-4F (the cap binding protein) to the 5' cap structure, resulting in a blockage of the initiation of translation (57,74,86). IRP bound to a 5' UTR IRE prevents
association of the 43S ribosomal preinitiation complex with ferritin mRNA (87). When iron levels are increased in the cell, IRP dissociates from IRE, which enables the initiation of translation of ferritin mRNA (56-58,61,88).

Another IRE has also been reported to be at the location of the 3' UTR of transferrin receptor mRNA (82,89,90). This IRE appears to be constructed with five successive stem-loops and bound by IRP in the absence of iron and dissociated in the presence of added iron. When bound to the IRE in the transferrin receptor 3' UTR, IRP provides protection for mRNA from degradation by nucleases (75,82,91,92). On the other hand, dissociation of IRP binding by iron uptake results in degradation of the transferrin receptor mRNA. The transferrin receptor is the protein required for cells to take up transferrin-bound iron. When intracellular iron is at a low level, the transferrin receptor mRNA will be effectively expressed, which results in a higher iron uptake. Meanwhile, the synthesis of ferritin will be suppressed. When the intracellular iron is at a high level, ferritin gene expression will increase to expand the space for
iron storage and transferrin receptor expression will
decrease to decrease incoming iron. Thus, the
coordinated regulation of iron storage and iron uptake is
established by ferritin and transferrin receptor
syntheses. The transferrin mRNA also contains an IRE­
like sequence in its 5' UTR, but the effect on
translation is minimal, and its response to iron is the
opposite of that of ferritin (93). At this time, the
actual function of this IRE-like structure of transferrin
is not understood.

The IRP has been purified from human (94) and rabbit
(95) livers and its genes have been localized on
chromosome 9 (96). A second protein, IRP-2, which also
binds to IREs with a high affinity, has been isolated
from humans (96) and rodents (66,97). The human IRP-2
has a gene sequence closely similar to IRP-1 and is
located on chromosome 15. The protein size of IRP-2 is
approximately 105 kDa and does not react with IRP-1
antibodies (97). The distribution of IRP-1 is greatest
in liver, intestines, and kidneys, whereas that of IRP-2
is highest in the intestines and the brain (97). The
significance of the two IRPs is not clear. The IRP-1 sequence aligned well (with 30% overall identity and 57% similarity) to the amino acid sequence of the Krebs cycle enzyme aconitase (78,97-103). Moreover, the gene for cytoplasmic aconitase is also located in chromosome 9. It was suggested and confirmed (99) that IRP-1 and cytosolic aconitase are the same protein, but have two different functions under different conditions. The biological function of cytosolic aconitase enzyme activity has not been well defined. The level of intracellular iron is the key factor that enables the reversibility of aconitase/IRE binding activity of IRP-1. However, IRP-2 does not show aconitase activity.

IRP-2 binds to the same IREs as does IRP-1, but its specificity varies slightly. Guo et al. (98) found that IRP-2 binds to the rat L chain ferritin mRNA IRE with an affinity equivalent to that of IRP-1. However, IRP-2 binds in vitro more strongly to the ferritin H chain mRNA IRE than to any other (77). The iron response of IRP-2 also differs strikingly from that of IRP-1 (97,98,104-106). The IRPs both respond to iron, but via different
pathways. IRP-1 is post-translationally converted between active and inactive IRE-binding forms. IPR-2, however, is induced following iron starvation through renewed synthesis of stable IRP-2 protein (101,107), and its inactivation by iron reflects degradation of IRP-2 protein (104,106) by a translational-dependent mechanism (107). Henderson et al. (106) proposed that IRP-2 might also bind to wild-type IREs, and exclusively to a unique set of IRE-like targets. These observations implied that each IRP may regulate its own type of IREs. However, other kinds of IREs have not been found in vivo.

Induction of ferritin synthesis by cell differentiation or by cytokines results in the selective expression of the H vs the L chain ferritin. This process is accomplished by both transcriptional (108-110) and translational (111-113) mechanisms. The effect of iron appears to be independent of the cytokine effects. The acute response of the polypeptide, interleukine-1α (IL-1α), has been shown to induce the synthesis of the ferritin H chain (110). Another acute response protein, tumor necrosis factor-α (TNF-α), has the same effect on
ferritin synthesis. TNF-α preferentially induces ferritin H chain synthesis in muscle cells (114) and myoblasts (110). Interferon-γ (IFN-γ) and TNF-α induce ferritin H chain mRNA levels, whereas interleukin-1β (IL-1β) has no effect on this induction (115). None of these cytokines had much effect on the levels of mRNA for L chain ferritin. IL-1β, IFN-γ, and TNF-α may decrease the overall rate of iron uptake. This effect may be due to the reduction of the transferrin receptor expression, but is not yet completely understood. In human hepatoma cells, IL-1β may increase the synthesis of both H and L chain ferritins at translational levels. The induction of L chain ferritin synthesis is accompanied by a shift of L chain ferritin mRNA from monosomes to polysomes (113). Finally, a 20-nucleotide sequence within the ferritin H chain 5' UTR, which is distinct from IRE, is responsible for the enhancement of translation by IL-1β (112).

Nitric oxide (NO) may also affect the expression of the ferritin gene (116,117). NO has a strong reducing power which may disrupt the iron-sulfur cluster in IRP,
by which the IRE-binding ability of IRP is induced. This may be the mechanism for connecting iron metabolism to the inflammation response that is mediated by NO.

**STRUCTURE OF FERRITIN**

Ferritin has a huge globular structure with a large hollow internal cavity and a molecular weight of approximately 450,000. The protein shell has a diameter of the order of 120-Å and the internal cavity is approximately 80-Å (118-120), which can provide enough storage space for up to approximately 2,300 atoms of iron as a ferric oxyhydroxide phosphate complex (121). Ferritin is a multi-subunit heteropolymer. The subunits have been identified as two different kinds (122-124): L chain, which predominantly resides in liver ferritin and usually contains a molecular weight of approximately 19,000; and H chain, which predominantly resides in heart ferritin and usually contains a larger molecular weight of approximately 21,000. Native ferritins are usually assembled in various proportions of H and L chains in a 24-subunit complex. In mammalian ferritin, the amino acid sequences of H and L chains show about 55% identity
and 75% similarity. About 95% of the ferritin H chain residues and about 85% of the ferritin L chains are identical between species (122-124). The three-dimensional structures of ferritin of various species are highly conserved. Both H and L chains of ferritin consist of a bundle of four long α-helices, usually designated as A, B, C, and D, and a fifth short α-helix, designated as E, lying at the C-terminal at an angle of about 60° to the bundle axis (Fig. 1). Helices A and B are anti-parallel, as are helices C and D. A long β-loop, designated as L, connects helices B and C. Helices A and C and the loop L are present on the external surface of the ferritin molecule, whereas helices B and D are present on the internal core (Fig. 2). The overall dimension of each ferritin subunit is about 25x25x50 Å (125-127).

Each ferritin molecule is assembled from 24 structurally equivalent subunits (Fig. 3). The ferritin 24-subunit complex is arranged as a shell of 4-fold, 3-fold, and 2-fold symmetry axes (Fig. 3). Two anti-parallel subunits, H-H, H-L, or L-L, initially pair with
FIG. 1. The three dimensional structure of single subunit of ferritin.
FIG. 2. Tetragonal crystal structure of native ferritin. The external surface of ferritin is away from the paper and the internal core of ferritin is into the paper.
FIG. 3. Schematic model of the ferritin 24-subunit complex. Each subunit is represented as a sausage with caps E (helix E) and N (N-terminus) indicated (129).
their long apolar interface, hydrophobic side chains of helix A and loop L (128), to form 2-fold symmetry axes (129-131). At the 3-fold axis, eight narrow hydrophilic channels, traversing the ferritin shell, are formed near the N terminal of each subunit. Due to the presence of conserved metal-binding residues, Asp 127 and Glu 130, in these channels, they have been illusively suggested as the channels for transport of iron in and out of ferritin (132-138). The interface of the 4-fold axis forms six large channels which are very hydrophobic. Twelve leucine residues line up in the channel. The function for the hydrophobic channel is still unclear. However, they have been proposed to be used for entrance and exit by iron chelators and iron-chelator complexes (139). There are also many hydrophobic contacts between the ends of the paired subunits (both N and C terminals) and the residues of helices C and D lying along the outer edge of another subunit pair. These interactions also serve for the assembly of the ferritin complex. Many hydrogen bonds and salt bridges, in addition to hydrophobic interaction, leading to a compact structure, may account
for the high stability of the ferritin molecule. Due to its highly rigid structure, ferritin can resist up to 8 M urea (pH 7.0) or a temperature of 70°C. Complete disassembly of ferritin requires extended incubation at pH 3.0 or at 100°C in the presence of 2% sodium dodecyl sulfate and β-mercaptoethanol or dithiothreitol (pH 7.0).

The structures of ferritin subunits are all closely similar. When crystals of ferritin with different ratios of H to L chains were compared, the X-ray diffraction patterns for were similar (140,141), indicating that a large amount of structural homology is conserved. For example, most peptide backbones of human H, rat L, horse L, and bullfrog L chains of ferritin can be superimposed to within about ± 1.0-Å (142). Computer modeling has also predicted that plant ferritin is similar to mammalian ferritin, although X-ray crystallography data are not yet available (143). The three-dimensional structures of native horse spleen L chain (129,144), recombinant rat liver L chain (125), recombinant human H chain (125), and recombinant bullfrog L chain (127,142), as determined by X-ray crystallography, have been well
documented, and can be obtained from the Protein Data Bank of Brookhaven National Laboratory (Upton, NY). The Protein Data Bank can also be accessed through the World Wide Web at the address of http://pdb.pdb.bnl.gov/. It is very important to know how to use this Protein Data Bank, because the information on the three-dimensional structures is not only for the satisfaction of our visual perception, but more significantly, provides the position and orientation of each amino acid residue which may be involved in the important functions of the proteins. The information has made the study of protein function through genetic engineering techniques more effective and reliable. By utilizing this information, computational chemistry can also investigate protein folding energy, dynamics of protein movement, protein-substrate and protein-protein interaction, and drug design, which are among the most valuable and promising studies occurring in modern protein chemistry and in the pharmaceutical industry.

Ferritin isolated from mammalian tissues consists of a mixture of various ratios of H and L chain
compositions. Those ferritins rich in the H chain are often acidic, whereas molecules rich in the L chain are basic. In general, L-rich ferritins have a relatively high average iron content, 1,500 Fe atoms per ferritin or more, whereas H-rich ferritins have a relatively lower average iron content, less than 1,000 Fe atoms per ferritin (124). The iron is stored in the cavity of ferritin. The protein cavity is in contact with the iron at several carboxyl residues (145). These carboxyl residues, especially glutamic acid, are defined as iron nucleation sites (146,147), because they are good candidates of ligands for Fe(III) binding. Even though the amino acid sequences and protein folding are very similar, interestingly, the number of nucleation sites is different between ferritin H and L chains. The ferritin L chain shows a greater number of glutamic acid residues on its cavity than does the H chain ferritin (148). Since L chain rich ferritin usually can stably retain more iron in vivo, researchers believe the function of the ferritin L chain is for iron storage. However, the role of the glutamic acid residue and the nature and
order of the molecular events leading to iron core formation are not well understood.

That ferritin can be reconstituted from apoferritin by the addition of Fe(II) in the presence of oxygen was reported in 1968 (149). The occurrence of iron incorporation directly indicates that iron must be able to pass through the protein shell of ferritin. The previous hypothesis for the iron loading channel, identified as "intersubunit channel model" (129,150), proposed that the small interstices between the subunits at the 3-fold symmetry axes form functional pores or channels for this purpose. X-ray diffraction has shown that this kind of intersubunit channel has a minimal diameter of approximately 4-Å (129). The size would allow iron associated with small biological chelators to pass through the channels. In addition, these intersubunit channels present two conserved carboxyl residues, Asp 127 and Glu 130, from each subunit, six carboxyl residues in total. They were considered as being the potential transition ligands for iron (132-138). However, this hypothesis was disproved when the
recombinant H and L chain ferritin homopolymers were produced. An iron-loading study sowed that only the H chain ferritin homopolymer was active in the iron-loading reaction, but not the L chain. Nevertheless, research studies have found that both the H and L chain ferritin homopolymers have a similar arrangement of amino acid residues in the intersubunit channels (139). Such results suggest that the intrasubunit channels are unlikely relevant in contributing to iron-loading functions.

Recently, improved X-ray diffraction structure analyses of ferritin, accompanied with genetic engineering studies, have led to the recognition of a narrow 1.0-Å channel, so called the “intrasubunit channel model,” in the H chain ferritin molecule. This channel is buried within the four-α-helix bundle. The length of the channel is about 17-20-Å, which is about the thickness of the ferritin shell. It is proposed that iron can pass through this channel of the ferritin H chain in the protein shell to reach the iron core. *Interestingly*, this channel is blocked in the ferritin L
chain by the presence of a salt bridge (K58 vs E103) between helices B and C (151). The corresponding residues, E62 and E107, of the ferritin H chain are both glutamic acids. Therefore, the electrical repulsion of these two glutamic acids keeps the intrasubunit channel of the ferritin H chain opened. This conformation has been considered as a major protein structure difference between the ferritin H and L chains, which is relevant to the iron-loading function of ferritin.

**BIOLOGICAL FUNCTIONS OF FERRITIN**

Ferritin can be defined as a repository in which unneeded iron can be stored within the cell in a non-toxic form, and from which iron can be mobilized when required either within that particular cell, or in other cells. In mammals, we can distinguish a number of distinctive features of the function of ferritin in different organs. In cells of erythrocyte precursors, a high iron-demanding cell, transferrin carrying Fe(III) binds to specific receptor sites on the cell’s membrane. Transferrin is taken up into the endocytic vesicles by
receptor-mediated endocytosis (152-154). Release of the transferrin iron within the endocytic vesicles is probably due to the decrease of the internal pH value and reduction of bound Fe(III) to Fe(II) (155,156). After release, iron will be used for heme and iron-containing protein syntheses. If the incoming iron is unneeded, it is then incorporated into ferritin. The iron incorporation process involves oxidation of the Fe(II) to Fe(III). However, no physiological chelator is known for carrying this iron before it is used or stored, although the proper chelator is apparently required for keeping intracellular iron stable and soluble. At the end of the red blood cell’s life span, cells are phagocytized by the spleen. The porphyrin is excreted after conversion to bilirubin in the bile and the iron is kept. Iron is first stored in ferritin, and can be subsequently mobilized from the tissue by transferrin (157). The mobilization of ferritin iron requires the reduction of Fe(III) to Fe(II).

In the liver, an iron housekeeping tissue, iron is taken up by the cells when iron saturation levels of
transferrin are high and deposited first into ferritin (158). The iron is most likely kept within these cells for storage. The ferritin can also incorporate iron released intracellularly from the breakdown of other iron-containing proteins during their turnover. Mobilization of such iron probably involves reduction of iron to Fe(II). The necessary physiological reductant and chelator for this iron are unknown. In the liver, the balance between the deposition of iron in and mobilization of iron from ferritin is likely decided by the demand for iron from other organs.

Ferritin also occurs in the cells of the intestinal mucosa, where it has been thought to have some roles in regulating the amount of dietary iron absorbed from the gut. It was suggested that there is a mucosa ferritin for iron which retains iron to prevent further entry of iron through the mucosa into the body (159,160). Dietary iron is absorbed by the mucosa cells and transferred to ferritin. The needs for iron by the body are reflected in a low iron saturation level of transferrin and the high expression of the transferrin receptor on the mucosa
cell membrane, under which condition the iron of mucosa ferritin can be delivered into the blood stream by transferrin (161). Once the body does not need more iron, iron will be accumulated in mucosa ferritin. When the mucosa ferritin is saturated, no further iron is taken up by the gut. The remaining iron in the mucosa cell at the end of 7-10 days of cell life is lost when the cell is sloughed (162).

Ferritin is an intracellular protein commonly sequestered in subcellular components, such as lysosomes and vesicles (163,164). However, small amounts of ferritin are normally present in the serum. Serum ferritin is glycosylated, although the structure of the carbohydrate is unknown. It has been indicated that serum ferritin is immunologically similar to liver and spleen ferritin and has a relatively low iron content (200 to 500 atoms of iron per protein) as compared to liver and spleen ferritin (1,500 to 2,000 atoms of iron per protein) (165). Some of the serum ferritin probably originates from tissue damage because of its tight correlation in response to various inflammatory
conditions. The presence of glycosylated coating indicates that serum ferritin is secreted from the cell. However, no transcript with appropriate signal sequences for serum ferritin has been identified. Serum ferritin contains a high proportion of the L chain (166). There is no direct evidence that serum ferritin in the body has functional roles. However, various ferritin receptors have been found on the liver cells (167-169), on lymphocytes and erythroblasts (170,171), and on many different cell lines (172-174). These receptors can bring serum ferritin into the cell by endocytosis. The ferritin receptors on the liver cell membrane have different specificities with respect to the H and L chains of ferritin (168), whereas those on the lymphocytes and other cell lines are specific for the H chain of ferritin (168,170-172). According to these observations, serum ferritin seems to have distinct roles for the transfer of iron among different cells due to the different specificities of the ferritin receptors.

Ferritin has also been proposed to have a regulatory role in the growth of granulocytes and macrophages (175).
This novel capability is specifically associated with the ferritin H chain, which was found to have a suppressive activity on various cell lines in vitro and in vivo (170,171,176,177). Initially, the hypothesis for this suppressive activity was the ferritin H chain can bind to the specific H chain receptors and, due to some unidentified reasons, interfere with cellular iron uptake. Some researchers speculate that the ferritin H chain can compete with available iron in cells (178). If this is the case for the suppressive activity of the ferritin H chain, the binding receptor will not be necessary to affect the growth of the cells. However, blocking the binding by using anti-ferritin antibodies has been shown to hinder the suppressive activity of the ferritin H chain (179). No reason has been given to explain why the binding of the ferritin receptor is necessary, nor has evidence been provided to show that antibody-bound ferritin H chain loses its ability to bind cellular iron. Our group has found that iron availability is not the only reason for the suppressive activity of the ferritin H chain. We demonstrated that
iron fully loaded recombinant H chain ferritin homopolymers can perform similar suppressive activities on insect cell growth as does ferritin loaded with less or little iron (180). In addition to the suppressive activity of ferritin H chain on cell growth, it has also been shown that both H and L chain rich ferritins can suppress antibody production (181). Furthermore, ferritin can inhibit the expression of mRNA in the Epstein-Barr-virus transformed human B-lymphoblastoid cell lines (181). We also observed that the recombinant H, but not L, chain ferritin homopolymer can inhibit in vitro globin mRNA translation (Abedi and Aust, unpublished data). From these observations, we believe that cell suppression by ferritin H chain is more likely due to a regulatory effect on gene expression, rather than competition for cellular iron. However, the mechanism of this regulation is far from clear.

LOADING IRON INTO FERRITIN
BY CERULOPLASMIN

The key function of ferritin is storing iron. However, the mechanism by which iron is placed into
ferritin is unknown. It has been shown that when Fe(II) is added to native apoferritin in Good buffers, e.g., HEPES and MOPS, in the presence of O₂, an oxidation process results in the deposition of iron within the ferritin shell (182). Some researchers have proposed the ferritin H, but not L, chain has the responsibility for the iron deposition mechanism because of a "ferroxidase center" located at its four-α-helix bundle. The surmised catalytic sites of this center have been identified as E27, Y34, E62, H65, E107, and Q141 by analyses of X-ray diffraction and genetic site-directed mutagenesis of human H chain homopolymers (125,150). The substitution of these residues at the center of ferritin H chain to the corresponding residues of ferritin L chain, i.e., E27A, Y34F, E62K, H65G, E107A, and Q141E, leads to the elimination of ferroxidase activity (183-185). In addition, the formation of an early oxidation intermediate, a μ-oxo-bridged Fe(III) dimer, specifically in the ferritin H, but not L, chain during the initial stage of the iron loading reaction has also been suggested (186). This intermediate, also called the di-
iron center (187), is thought to act in concert with the "ferroxidase center" for iron oxidation and incorporation into ferritin (183,185). However, our group does not believe there is any ferroxidase activity in ferritin (188). We found the so-called ferroxidase activity of ferritin is dependent upon the use of a buffer, which promotes the autoxidation of iron (189). It is known that Good buffers, which are often used by other researchers for the iron loading studies, may promote the autoxidation of Fe(II) to Fe(III) for depositing iron into ferritin (190). We demonstrated that the proposed ferroxidase activity of ferritin was not observed when Good buffer was replaced with saline. Saline did not promote the autoxidation of iron (189). In addition, a serious concern of using this type of buffer for iron loading is that this type of autocatalytic event generates oxygen radicals (190) which would be very damaging to nearby biological substances in vivo, such as ferritin. Our group has demonstrated that iron deposition into ferritin in the presence of Good type buffers is directly related to the rate of iron
autoxidation, during which time the basic amino acids, lysine and histidine, of ferritin are oxidized (189). Moreover, the formation of the iron core within the ferritin is in a form which is not as stable as the iron core of isolated native ferritin (189). We have also noted that the overall stoichiometry of Fe(II) oxidation to O$_2$ consumption was close to 2 (191). This observation indicates that O$_2^-$, H$_2$O$_2$, and ·OH, mainly H$_2$O$_2$, are formed. In addition, sequentially loading various amounts of iron into ferritin in the HEPES buffer system results in an increase in the amount of carbonyl residues in ferritin (191). Again, the evidence of increasing carbonyl residues suggests that ferritin incorporated iron in the Good buffer has been damaged, which is unlikely to occur in a biological iron loading system in vivo.

We previously found that ceruloplasmin can load iron into ferritin to prevent lipid peroxidation in vitro (192). Ceruloplasmin, a glycoprotein containing 5-7 cupric copper, is normally synthesized in the liver and secreted into serum (193). Ceruloplasmin is generally considered as a copper conveyer which carries 90-95% of
the plasma copper (194). Ceruloplasmin is a ferroxidase which may be involved in the loading of iron into transferrin (195). Rydén (196) reported that ceruloplasmin can catalyze the oxidation of four Fe(II) by direct four-electron reduction of oxygen to form water. The reaction is shown as follows:

\[ 4 \text{Fe(II)} + 4 \text{O}_2 + 4 \text{H}^+ \xrightarrow{\text{CP}} 4 \text{Fe(III)} + 2 \text{H}_2\text{O} \]  

We (189, 191, 197-199) and others (200, 201) have intensively studied the use of ceruloplasmin as a ferroxidase to load iron into ferritin. Ferritin iron loaded by ceruloplasmin behaves very similarly to that of ferritin isolated from tissues, with respect to the maximal amount of iron that can be loaded or found in native ferritin (189). The stability of ferritin's iron core loaded by ceruloplasmin is also comparable to that of native ferritin (189). Therefore, there is no doubt that ceruloplasmin has the ability to load iron into ferritin. However, the question is what the biological significance of this reaction may be in vivo. Does
ceruloplasmin exist in every tissue? It has been shown that abnormally low levels of brain ceruloplasmin have a high correlation of iron disorder diseases of the brain, e.g., Alzheimer’s disease (202). We (203) and others (204-209) have also demonstrated that, besides liver tissue, the mRNA for ceruloplasmin exists and is inducible in a variety of different organs (lung, synovia tissue, testis, choroid plexus, and uterus). Use of ceruloplasmin as the ferroxidase to load iron into ferritin is also attractive because iron oxidation by ceruloplasmin results in the reduction of molecular oxygen completely to water without releasing partial reduced oxygen species (196). We demonstrated that neither amino acid residue damage nor carbonyl residue formation occurred when ceruloplasmin was used as the ferroxidase to load iron into ferritin (189).

To further understand the ceruloplasmin loading system, the mechanism of loading iron into recombinant H and L chain ferritin homopolymers was investigated. It is known that the deposition of iron into ferritin requires that iron be presented in the ferrous form
followed by oxidation, because ferritin does not appear to incorporate iron present in the ferric form (197). According to this, when ceruloplasmin loads iron into ferritin, ceruloplasmin must associate with ferritin and directly convey the oxidized iron through a loading channel into the ferritin core. Otherwise, the oxidized iron obviously will precipitate out of solution before being loaded into ferritin (Fig. 4). In other words, ferritin needs to provide certain available sites for association with ceruloplasmin. Thus, an "optimal" ceruloplasmin-ferritin ratio must exist to maximize the iron loading reaction. A lower ratio of ceruloplasmin-ferritin association can result in a lower initial rate of iron loading. A higher ratio of ceruloplasmin-ferritin association may increase the initial rate until the association of ceruloplasmin and ferritin is "saturated." At this ratio, ferritin may be loaded with iron by ceruloplasmin at a maximal rate and the greatest amount because each ferritin is associated with a maximal amount of ceruloplasmin. Further addition of ceruloplasmin does not increase the "saturation" level
FIG. 4. The essentiality of ceruloplasmin and ferritin association for iron loading. With association, ceruloplasmin can load iron into ferritin (A). Without association, ceruloplasmin oxidized iron cannot be loaded into ferritin. H and L represent ferritin H and L chains, respectively.
because no more ceruloplasmin can associate with ferritin. Nonassociated ceruloplasmin not only has no "place" to load iron into ferritin, but it competes with ferritin-associated ceruloplasmin for oxidizing iron. The iron oxidized by nonassociated ceruloplasmin will not be incorporated into ferritin, but instead will precipitate in the solution. Therefore, the total incorporated iron will be less (Fig. 5). We believe the proper association not only guarantees the capability of ceruloplasmin to load iron into ferritin, but also is very important evidence to show the biological significance of the ceruloplasmin-loading system. We have demonstrated that horse spleen ferritin, which contains approximately one H and 23 L chains per molecule, can associate with only one molecule of ceruloplasmin maximally. Higher ratios of ceruloplasmin to ferritin cause a decrease of the total iron loading due to the competition for iron oxidation by non-associated ceruloplasmin. Lower ratios of ceruloplasmin to ferritin cause a decrease in the initial rate of iron loading (199). This observation has also been confirmed
FIG. 5. The effect of molar ratios of ceruloplasmin to ferritin H chain on iron loading. Molar ration of ceruloplasmin and ferritin: (A) less than 1, (B) 1, (C) greater than 1. H, L, and Cp represent ferritin H and L chains, and ceruloplasmin, respectively.
by using ceruloplasmin to load iron into recombinant rat liver ferritin heteropolymers, which contained either one H and 23 L or 2 H and 22 L chains per molecule. We found the optimal ratio of ceruloplasmin to "one H and 23 L chains" ferritin was one and to "2 H and 22 L chains" ferritin was 2. This indicates the association site of ceruloplasmin is on the ferritin H chain and the number is one (199). Interestingly, Treffry et al., in 1995, reported that ferritin did not accumulate iron oxidized by ceruloplasmin (210). However, their work unintentionally and indirectly confirms our observations. They found that higher concentrations of ceruloplasmin not only help little toward the iron loading rate, but worsen the iron loading amount. They concluded that ceruloplasmin is not the right enzyme to load iron into ferritin because the kinetics of iron loading by ceruloplasmin at high concentrations were confusing to them. The amount of ceruloplasmin they used was more than the number of association sites on the ferritin. Unfortunately, Treffry et al. did not investigate further to determine the correct concentration, which is much
lower than what they used, of ceruloplasmin for this study.

The association of ceruloplasmin and ferritin can also be indicated from the changing enzyme activity of ceruloplasmin because protein-protein association may cause a conformational change in proteins. We found that the ferroxidase activity of ceruloplasmin can be enhanced in the presence of the H, but not L, chain ferritin homopolymer (198). This enhancement of ferroxidase activity of ceruloplasmin by the H chain ferritin homopolymer, we believe, is highly relevant to their association. This association can be observed by applying ceruloplasmin through a column which has been initially cross-linked with ferritin. The ceruloplasmin can be retained by this ferritin-linked column after washing 10X the bed volume with phosphate buffer (Guo and Aust, unpublished data). Recently, Reilly and Aust (211) found that the reduced ceruloplasmin has higher affinity to ferritin than the oxidized ceruloplasmin. They studied, spectrophotometrically, the enhancement of the ferroxidase activity of ceruloplasmin by observing the
rate changes of oxidation-reduction of ceruloplasmin copper at 610 nm (211). The results reconfirm that the ferritin H, but not L, chain can cause this effect. By competition binding of ceruloplasmin and its 10-amino acid peptide to the putative binding region of the H chain ferritin homopolymer, Juan and Aust (unpublished data) determined the dissociation constant for this association. All of these results convince us that the association between ceruloplasmin and ferritin does occur and it may be the way that ceruloplasmin loads iron into ferritin in vivo. In addition, the results also indicate that a correct physiological concentration of ceruloplasmin may be required to properly load into ferritin in vivo.

Moreover, since we found that only the recombinant H chain ferritin homopolymer can be loaded with iron by ceruloplasmin, we believe there must be a channel on the ferritin H chain which can be used by ceruloplasmin to load iron. According to preliminary crystallography data, computer prediction suggests a narrow 1.0-Å channel exists through the four-α-helix bundle of each H chain.
ferritin, as discussed in the earlier part of the “intrasubunit channel model” of this chapter. This channel in the L chain of ferritin is blocked by the presence of a salt bridge connecting K58 and E103. To further study this iron loading channel, we used site-directed mutagenesis to alter the amino acid sequences in this channel. Site-directed mutagenesis, K58E and G61H, on the rat liver L chain ferritin was done to construct an iron loading channel similar to that in the four-α-helix bundle of the H chain of ferritin. On the ferritin H chain, E62K and H65G mutations were performed to construct a salt bridge similar to that believed to exist in the ferritin L chain. We found that both variants expressed in the insect cell-baculovirus expression system were able to form soluble multi-subunit homopolymers. The H chain mutant homopolymer could not be loaded, whereas the L chain mutant homopolymer was loaded with iron by ceruloplasmin. However, the initial rate of loading iron into the L chain mutant homopolymer by ceruloplasmin was 50% of that for loading iron into the ferritin H chain homopolymer (212). The ferritin L
chain mutant does not contain a ceruloplasmin association site and ceruloplasmin cannot correctly load iron into ferritin without a proper ceruloplasmin-ferritin association. Furthermore, the ferroxidase activity of ceruloplasmin was also enhanced in the presence of the ferritin H chain and its mutant, but not in the presence of the ferritin L chain or its mutant (212). Obviously, the ferritin L chain and its mutant cannot associate with ceruloplasmin. These results suggest the role of a four-α-helix bundle channel for loading iron into ferritin by ceruloplasmin. Secondly, the association of ceruloplasmin and ferritin is absolutely required for iron loading. The ceruloplasmin association site of ferritin is only on the H chain and only one for each subunit.

**EXPRESSION OF RAT LIVER FERRITIN HOMOPOLYMERS**

Our group has studied the iron-loading mechanism with various native ferritins from different species and organs, such as horse spleen and rat liver (189,191). We have been unable to find native homopolymers of either H
or L chain ferritins. Therefore, the role of the H and L chains in iron loading may not be clearly defined by only studying native ferritins. The production and study of the H or L chain ferritins homopolymers are thus necessary to characterize the structural and functional differences of these two ferritin subunits. Various recombinant H and L chain ferritin homopolymers of different species and organs, such as human liver (183,213), horse spleen (214), mouse liver (215), and bullfrog red blood cell (216), have been successfully expressed and studied extensively. The gene for the rat liver L chain ferritin has been sequenced and expressed by a recombinant expression system as well (126,217). Although the gene for the rat liver ferritin H chain was sequenced several years ago (218), no biosynthesis of the homopolymer has been reported. The reason why the expression of rat liver H chain ferritin homopolymer has not has not been reported in not known.

In the present study, we have cloned the genes of the H and L chains of rat liver ferritin into expression vectors to investigate the functions of the two chains
During the process, we noticed the H chain had a strong suppressive effect on growth of the host. The yield of expression was poor, due to the limitation of the host growth when the recombinant H chain ferritin was present.

On the other hand, none of these effects were observed when the recombinant L chain ferritin was expressed. As mentioned earlier, the ferritin H chain has a novel suppressive activity on various cell lines. It may be the expressed ferritin H chain can also repress the host expression system. We found the suppressive activity of the rat liver ferritin H chain was significantly stronger than the human H chain.

The reason for this difference between the rat and human liver ferritin H chain is unknown. Due to the strong suppressive activity, expression of the recombinant rat liver H chain ferritin homopolymer was very difficult. Two expression systems, i.e., *E. coli* and insect cell-baculovirus expression systems, were tried and developed to overcome this expression problem.
We found that in the presence of expressed rat liver H chain ferritin homopolymer, either intracellularly or extracellularly, may immediately limit the growth of the host.

Effectively deferring the expression of a cloned gene until complete growth of the host can be a means of avoiding the toxicity of an expressed protein. According to the literature (219,220), the insect cell-baculovirus expression system may be one of the best candidates for this purpose, because the system usually expresses the cloned genes at a very late stage during the baculovirus life cycle. In other words, the expression of the cloned gene only occurs when all required enzymes for the insect cell and the baculovirus are completely produced. Therefore, the expression of the rat liver ferritin H chain gene will not affect the performance of the expression system, with the exception of the growth of insect cells, which, however, can be easily remedied by using a greater number of cells for infection. We have successfully expressed both the recombinant rat liver H and L chain homopolymers and concluded a study of their
functions for iron loading in the ceruloplasmin loading system (197).

An *E. coli* expression system could be another good candidate for the expression of the rat liver H chain ferritin, because this system might be able to provide a time-saving, economic, and alternative way for production of recombinant protein. Unfortunately, *E. coli* cannot tolerate the repressive activity of expressed rat liver ferritin H chain homopolymer. When we first tried to express the cloned rat liver ferritin H chain gene in the *E. coli* system using an expression vector pDR540 (from Pharmacia) which contained a very strong fusion promoter, the *tac* promoter, we found that *E. coli* ceased growing when transformed with the recombinant pDR540 vector. The reason might be that the *tac* promoter of pDR540 was so strong such that the rat liver ferritin H chain gene was expressed before the inducer, isopropyl-β-D-thiogalactoside (IPTG), had been added. Obviously, regulation of this *E. coli* expression system has a leakage problem which commonly exists when using the lac operator and the repressor for expression regulation.
Therefore, a better expression regulation method was needed for expressing rat liver H chain ferritin for use in an *E. coli* expression system.

The strategy for designing this expression system was to construct an expression vector that would be able to stringently control the time of expression of the inserted gene. We found it better to regulate the expression of the RNA polymerase for the promoter, such as the T7 promoter, for the inserted gene rather than to directly control the expression of inserted gene. The former method could lengthen the processing time for expression if regulation was leaking.

In this research, the gene for rat liver ferritin was inserted in the modified pBR322 under the control of the T7 promoter. The T7 mRNA polymerase gene was present in the chromosome of the host JM109(DE3) and under the control of the *lac* promoter. According to our results, this *E. coli* expression system produces both recombinant rat liver ferritin H and L chain homopolymers, although the growth of the host with the rat liver ferritin H chain gene before adding IPTG was still relatively slower.
than the wildtype JM109 (DE3) or JM109 (DE3) containing rat liver ferritin L chain gene. This problem was corrected by using a similar *E. coli* expression vector pET-11b, which has been commercialized by Novagen Inc. (Madison, WI).

In addition to the similar construct of the *E. coli* expression system as mentioned above, this expression system contains an additional lac operator gene between the T7 promoter and the cloned gene, and an extra copy of lac repressor gene within the host genome. With this added regulation, the *E. coli* system can produce approximately 10 times more of recombinant rat liver ferritin H chain homopolymer than the previous system (180).

The structures and functions of the recombinant rat liver ferritin homopolymers expressed in the *E. coli* system have also been studied (Guo and Aust, unpublished data). We found that the ferritin H chain homopolymer expressed from *E. coli* system already contained iron in the protein core, whereas the ferritin H chain homopolymer expressed from the insect cell-baculovirus
The results of protein structure studies indicate that the protein shell of the ferritin H chain expressed in the *E. coli* system were modified, due to the nonphysiological oxidation of iron in the cell of *E. coli*. This protein modification resulted in the ferritin H chain homopolymer having a smaller molecular weight, greater and multiple surface charges, instability, and an unnatural iron loading pattern (Guo and Aust, unpublished data). The results suggest that the recombinant rat liver ferritin H chain homopolymer expressed in the *E. coli* system, which is most commonly used by other researchers, may be not appropriate for an iron-loading study. Further studies to prevent the oxidative damage of the expressed proteins are needed.

**SUMMARY**

The structures and functions of ferritin have been studied for decades. The techniques of X-ray crystallography, molecular biology, and computational chemistry contribute significantly to investigations in this field. However, the physiological system for
loading iron into ferritin has not been well defined. Previously, the ferritin H chain was proposed to have ferroxidase activity for iron loading. However, we believe that the ferroxidase activity of the ferritin H chain actually does not exist, and this type of iron oxidation may lead to an oxygen radical generation and ferritin shell damage. As an alternative, use of ceruloplasmin as a ferroxidase for this purpose may be the most acceptable loading method. Ceruloplasmin can oxidize and load iron into ferritin by directly reducing oxygen molecule to water without producing oxygen radical species. Without this enzyme, iron oxidation and loading into ferritin becomes a very dangerous task, which may even occur inside the cell of *E. coli*. The physiological role of ceruloplasmin for loading iron into ferritin still needs further investigation.

REFERENCES


CHAPTER III

EXPRESSION AND LOADING OF RECOMBINANT HEAVY AND LIGHT CHAIN HOMOPOLYMERS OF RAT LIVER FERRITIN

ABSTRACT

The full-length genes for the H (heavy) and L (light) chains of ferritin isolated from a rat liver cDNA library were amplified using polymerase chain reaction. Each was inserted at the unique BglII site downstream of the p10 promoter of the baculovirus transfer vector pAcUW21. The genes were transferred separately to infectious Autographa californica nuclear polyhedrosis virus (AcNPV) expression vectors after in vivo homologous recombination. Ferritin homopolymers of either H or L chain were expressed up to approximately 1.5 mg per 100 ml of infected cultures (2.0 X 10⁶ cells/ml) of Spodoptera frugiperda, Sf-21, 4 days postinfection. Both recombinant H chain ferritin (rH-Ft) and recombinant L chain ferritin (rL-Ft) assembled as multi-subunit

complexes with predicted electrophoretic mobility.
Neither rH-Ft nor rL-Ft homopolymers had ferroxidase activity in 50 mM NaCl, as we have reported previously for native ferritin [D. DeSilva, D.M. Miller, D.W. Reif, and S.D. Aust (1992) Arch. Biochem. Biophys. 293, 409-415]. When ceruloplasmin, a copper-containing protein, was used as a ferroxidase, rH-Ft loaded iron at rates comparable those obtained with native rat liver apoferritin, but rL-Ft failed to load any iron. The initial rate of Fe(II) oxidation catalyzed by ceruloplasmin was increased in the presence of rH-Ft or rat liver ferritin but not in the presence of rL-Ft. A maximum of about 2500 atoms of iron were incorporated into both rH-Ft and rat liver ferritin. These results demonstrate that both rat liver rH-Ft and rL-Ft homopolymer can be properly produced by baculovirus expression system and ceruloplasmin can only load iron into H chain ferritin. The physiological significance of these results is discussed.
INTRODUCTION

The primary function of ferritin is to serve as storage protein for iron in animals, plants, fungi, and bacteria (1). Transferrin can carry up to two atoms of ferric iron in the plasma and enters cells through receptor-mediated endocytosis. Iron is then released into cytoplasm. In the cell, iron can be utilized in iron-containing proteins, such as ribonucleotide reductase, aconitase, and cytochromes, or can be stored within ferritin (2). Ferritin synthesis is regulated by intracellular iron. In situations when the intracellular iron concentration is high, iron triggers the synthesis of apoferritin to provide the cell with greater iron storage capacity (3). The ferritin can protect the cell from damage due to iron-catalyzed production of reactive oxygen species (4). Ferritin may also be responsible for keeping iron in a metabolically accessible form (5).

Ferritin has a large globular conformation with an approximate molecular mass of 450 kDa. It has a large hollow cavity which can provide enough space for up to approximately 2,500 molecules of the iron as a
polynuclear ferric oxyhydroxide-phosphate complex (6).
The protein shell of ferritin is assembled as a 24-subunit heteropolymer of various ratios of two types of subunits, heavy (H) and light (L) chains, depending upon the tissue of origin (7). Even though H and L chains of ferritins share 55% homology, they are different in size, surface charge, immunological reactivity (8), and presumably iron-loading properties (9).

The mechanism of iron loading into ferritin has been extensively studied, but several questions still remain. It has been suggested that ferritin H chain has ferroxidase activity for iron loading into ferritin (10, 11). When native apoferritin was incubated with ferrous ammonium sulfate in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer, it accelerated the rate of Fe(II) oxidation and iron incorporation (10). It was later shown that H chain, but not L chain, had this ferroxidase activity for iron incorporation (11). However, we found that ferritin had no ferroxidase activity when the HEPES buffer was omitted (12). It is known that Good buffer may promote the autoxidation of
iron (13). Secondly, during the autooxidation of iron, oxygen radicals are produced (14) which may be harmful to amino acid residues of ferritin. Previously, our laboratory reported that iron deposition into ferritin in the presence of Good buffers was directly related to the rate of iron autooxidation and that during this process some basic amino acids of ferritin were oxidized (12). In saline solutions, histidine-chelated Fe(II) was oxidized by ceruloplasmin and the iron was deposited into apoferritin by ceruloplasmin without oxidative damage to amino acid residues of ferritins (12). Additionally, ceruloplasmin-loaded ferritin had the same iron-binding and releasing properties as did native ferritin (12). These results suggest that ceruloplasmin may play a physiological role as the ferroxidase that loads iron into apoferritin. Alternatively, it may serve as a model for an as yet unidentified ferroxidase for this purpose.

Copper has been recognized as an essential nutrient for animals because insufficient dietary copper may lead to a type of anemia which can only be cured by copper supplement but cannot be improved by treatment with iron
Copper, which is mainly transported by ceruloplasmin (CP) in plasma, has been reported highly related to iron homeostasis (16). Ceruloplasmin, a blue copper-containing α2-glycoprotein, is normally synthesized in the liver and secreted into plasma (17). The expression of ceruloplasmin was also found in heart (18), lung (19), synovial tissue (20), lymphocytes, monocytes, granulocytes (21), testes (22), and uterus (23). Even though the majority of ceruloplasmin is present in serum, ceruloplasmin can be transported across the membrane of cells via receptor-mediated endocytosis (24).

Native ferritins are various proportions of H and L chains and are not found to exist as homopolymers. Therefore, the role of H or L chain ferritin in iron loading may not be clearly defined by studying native ferritins. Such a study can, however, be more easily accomplished with homopolymers of H or L chain ferritin and the best sources of homopolymers are those expressed by recombinant DNA technology. Homopolymers, which can only be obtained by recombinant DNA techniques, will
therefore be available to study functions which reside in only one protein chain. Site-directed mutagenesis will also be used to investigate amino acid differences between the two protein chains required for or involved in specific functions. Here we report the cloning of rat liver H and L chain ferritin cDNA in a baculovirus system for expression in insect cell culture (25). We also compare the structural and functional characteristics of the recombinant rat liver H chain ferritin (rH-Ft) and L chain ferritin (rL-Ft) homopolymers compared to those of native rat liver ferritin. In this study, neither rH-Ft nor rL-Ft homopolymer had ferroxidase activity in saline solution. The rH-Ft and rat liver ferritin were loaded with iron by ceruloplasmin to the same extent. Additionally, iron loading by ceruloplasmin was observed in rH-Ft, but not in rL-Ft. This may be because that H chain is the only subunit in ferritin providing an iron-loading route or channel.

MATERIALS AND METHODS

All enzymes used for DNA manipulations were purchased from Boehringer-Mannheim Biochemicals
(Indianapolis, IN) or United States Biochemicals (Cleveland, OH) and used according to manufacturers' instructions. The oligonucleotides for polymerase chain reaction (PCR) or DNA sequencing were synthesized by an automated DNA synthesizer (Applied Biosystems, Foster City, CA) in the Utah State University, Biotechnology Center Service Laboratory. GeneAmp PCR System 9600 and PCR reagents were obtained from Perkin-Elmer Cetus (Foster City, CA). The λ-bacteriophage rat liver cDNA library was purchased from Clontech (Palo Alto, CA). Baculovirus transfer vector pAcUW21, linearized baculovirus BaculoGold, and insect cells Spodoptera frugiperda (Sf-21) were obtained from PharMingen (San Diego, CA). Serum-free insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS). Rat serum and livers were obtained from Pel-Freez Biologicals (Rogers, AR). Other chemicals were purchased from Sigma (St. Louis, MO) except L-histidine, which was obtained from Kodak (Rochester, NY).

Construction of recombinant baculovirus transfer vector with rat liver H or L chain ferritin gene. The
genes for the H and L chain of rat liver ferritin were isolated from the \( \lambda \)-bacteriophage rat liver cDNA library using Southern blots and then preserved in 50% glycerol at 4°C. The \( \lambda \)-bacteriophage, with either the H or L chain ferritin gene, was used as a template for amplification of the genes by PCR employing the GeneAmp kit (Perkin-Elmer Cetus). Two oligonucleotides, corresponding to the H chain ferritin gene nucleotides 1 to 21 of the coding strand (relative to the A [+1] of the initiator ATG), 5'-GCCG\^GATCCATGACCCCGCTCTCCCTCG-3', and to nucleotides 529 to 549 of the non-coding strand, 5'-GACC\^GATCCCTTAGCTCTCATCACCAGTCC-3', and another two oligonucleotides, corresponding to the L chain ferritin gene nucleotides 1 to 21 of the coding strand, 5'-GGAG\^GATCCATGACCTCTCATCAGTCGT-3', and to nucleotides 532 to 552 of the non-coding strand, 5'-CACC\^GATCC-CTAGTCGTCTTTCAGAGGTG-3', were synthesized with the addition of a nine-base fragment with a BamHI site (underlined; the "^" symbol indicates the position of cleavage site) to the 5' end of each oligonucleotide. The resulting amplified DNA fragments were cloned into
the unique BglII site of plasmid pAcUW21 downstream of the p10 promoter. Both constructs were sequenced using the Sequenase polymerase (United States Biochemicals) and \([\alpha-^{35}S]\)dATP (Du Pont, Boston, MA), to ensure that no change was introduced into the DNA sequences of the inserts.

The PCR amplifications were performed in 100-μl reaction mixtures including 75 pmol of the individual primers, 20 nmol of each dNTP, 0.8 μg of recombinant \(\lambda\)-bacteriophage of H or L chain ferritin, 10 μl of 10x PCR Buffer II (Perkin-Elmer), and 10 μl of 25 mM MgCl\(_2\). After preheating for 5 min at 94°C, AmpliTaq (0.5 units) was added and the PCR cycle was started. Twenty-five cycles were carried out with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. An additional 5 min at 72°C was used for the last cycle.

*Expression of recombinant H and L chain ferritin homopolymers.* Recombinant pAcUW21 (2.0 μg) containing either the H or L chain gene was cotransfected into Sf-21 cells together with 1.0 μg linearized viral DNA.
BaculoGold in serum-free medium EX-CELL 401 and lipofectin (GIBCO BRL, Gaithersburg, MD). The details of the cotransfection method were described by O'Reilly et al. (26). The recombinant virus for rat liver H or L chain ferritin expression was obtained after three rounds of plaque assay using visual screening for the occ+ phenotype. Further confirmation that the recombinant virus contained a full-length gene of H or L chain ferritin was achieved using specific primers corresponding to H or L chain ferritin gene for PCR restriction mapping. The general methods of PCR restriction mapping were as described by Webb et al. (27). Sf-21 cells were grown in EX-CELL 401 serum-free medium with 50 units/ml penicillin and 50 µg/ml streptomycin at 27°C. For the production of recombinant proteins, monolayers of cells were infected with the recombinant baculovirus at a 20 multiplicity of infection in 15 ml for 48 h. Infected cells were seeded in 100-ml suspension cell cultures at a density of 2 x 10^6 cells/ml and harvested 4 days postinfection.
Purification of recombinant H and L chain ferritin homopolymers. The infected cells were centrifuged at 3,000 g for 10 min and the resulting pellet was subjected to three freeze-and-thaw cycles. The lysed cells were centrifuged at 5,000 g to remove cell debris and unbroken cells. The supernatant was concentrated to 5 ml using an Amicon protein concentrator with a 300,000-Dal molecular weight cutoff membrane (Amicon, Beverly, MA). The concentrated preparation was heated at 65°C for 15 min and centrifuged at 5,000 g for 30 min to remove denatured proteins. The supernatant was subjected to Econo-Pac 10DG column chromatography (Bio-Rad Laboratories, Hercules, CA) and eluted using 50 mM Tris (pH 7.5) for desalting. The eluant was then applied to a DEAE Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) and washed with 50 mM Tris (pH 7.5). Ferritin was eluted using 200 mM NaCl in 50 mM Tris (pH 7.5). The fractions containing ferritin were pooled and concentrated to 3 ml with an Amicon protein concentrator (Centriprep-100, 100-kDa cutoff). FPLC was performed at 4°C with a Sepharose CL-6B column (50 x 1.6 cm, Pharmacia
Biotech) using Chelex-100 (Bio-Rad Laboratories)-treated 50 mM NaCl (pH 7.0) at a flow rate of 0.5 ml/min. Fractions which exhibited maximal absorption at 280 nm were collected.

Preparation of rat liver ferritin and apoferritin. Native ferritin was purified from the rat livers as described by Thomas et al. (28) except that the chromatography step over Sephadex-G-200 column was replaced with Bio-Gel (Bio-Rad Laboratories) column and followed an additional Sepharose CL-6B column (Pharmacia Biotech) on a Pharmacia Biotech FPLC system. The ferritin fractions were pooled and centrifuged at 105,000 g for 4 h. The ferritin pellet was resuspended in 50 mM phosphate buffer (pH 7.0). Apoferritin was prepared by using thioglycolic acid and the ferrous iron chelator, \( \alpha,\alpha' \)-dipyridyl, as described by Dognin and Crichton (29).

Gel electrophoresis. To determine the purity and apparent molecular weight of the recombinant H or L chain ferritin, sodium dodecyl sulfate (SDS) denaturing gel electrophoresis of protein samples was performed on a Bio-Rad Mini-PROTEIN II electrophoresis cell using 12%
single percentage Ready Gel (Bio-Rad Laboratories) and SDS electrophoresis buffer. Native rat liver ferritin as well as other protein standards were used to determine the molecular weight. A 7.5% Ready Gel (Bio-Rad Laboratories) and electrophoresis buffer without SDS were used for non-denaturing gel electrophoresis to assess subunit assembly. Native rat liver ferritin was used as a reference to estimate the protein surface charge and the integrity of the 24-mer of the recombinant ferritins. Protein was stained with Coomassie brilliant blue. Iron staining was performed by immersing the non-denaturing gel in 0.1% potassium ferrocyanide in 1 N HCl for 30 min, which was then rinsed with water before drying.

**Ferritin iron-loading reactions.** Ceruloplasmin and apoferritins used in the iron-loading studies were desalted into Chelex-100-treated 50 mM NaCl (pH 7.0) using an Econo-Pac 10 DG column. Rat ceruloplasmin was prepared as described by Ryan et al. (30). Ferritin iron loading by ceruloplasmin were performed as outlined by de Silva et al. (12). Iron loading into ferritin was monitored at 380 nm ($E_{380} = 1.03 \text{ mM}^{-1}\text{cm}^{-1}$). The iron-
loading reactions were terminated by adding 1 mM ferrozine and the ferrous iron remaining quantified at 564 nm with a Shimazu UV160U spectrophotometer ($E_{564} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$) after which 1 mM desferrioxamine was added. An Econo-Pac 10 DG desalting column was used to remove the nonincorporated iron, ferrozine and desferrioxamine. Protein assays were performed by using bicinchoninic acid assay kits (Pierce, Rockford, IL) and total iron assays were performed as described by Brumby and Massey (31).

RESULTS

Construction of recombinant baculovirus transfer vector with the rat liver H and L chain ferritin gene. BamHI DNA fragments of the coding regions for rat liver H or L chain ferritin gene were obtained using PCR techniques and subsequently cloned into the unique Bgl II site of the baculovirus transfer vector pAcUW21. The recombinant plasmids were examined, using restriction enzyme analysis, to obtain the transfer vectors in which H or L chain ferritin gene was oriented in the same 5' to 3' direction as the p10 promoter gene. The DNA sequences of both inserts were determined using the dideoxy
termination procedure and confirmed to be 100% identical to the published sequences of the coding regions for the rat liver H and L chain ferritin genes (32,33), respectively.

Expression of recombinant H and L chain ferritin homopolymers. The recombinant H or L chain ferritin baculovirus expression vectors were obtained after cotransfection of the recombinant transfer vectors and linearized baculovirus BaculoGold into insect Sf-21 cells for homologous recombination. The transfer vector pAcUW21 is polyhedrin-based, which contains a pair of polyhedrin promoter and polyhedrin gene upstream of the p10 promoter. When the homologous recombination occurred, both the inserted gene and the polyhedrin gene in the pAcUW21 were transferred into baculovirus BaculoGold. Because both the polyhedrin promoter and the p10 promoter initiate expression at a very late stage of postinfection, the downstream genes are expressed approximately at the same time (34). Since the polyhedrin protein forms a crystal-like structure around the virus bundles, the time of expression of recombinant
ferritins may be estimated by observing the formation of polyhedrin crystal within the nuclei of infected cells by light microscopy. More than 90% of insect cells contained a visible polyhedrin crystal inside the nuclei after 3 days of cotransfection. To isolate polyhedrin-positive recombinant baculovirus, the supernatant fluid was harvested and titrated onto a confluent monolayer of Sf-21 cells overlaid with 0.5% agarose. After incubation, plaques showing occlusion bodies were selected. After three plaque purifications, high-titer stock virus was obtained. The H or L chain ferritin genes in the recombinant virus were identified using PCR restriction mapping (results not shown).

Expression of recombinant ferritins in insect cells grown in 250-ml spinner flasks was examined by using SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 6A and B). Partial purified recombinant H or L chain ferritin and rat liver ferritin were used to identify the expressed H or L chain ferritin. The expressed polyhedrin (29.0 kDa) and recombinant ferritin H (21.6
FIG. 6. Time course of expression of rH-Ft and rL-Ft in Sf-21 cell infected with the recombinant baculovirus, which contained the coding sequence for rat liver H and L chain ferritin, respectively. Recombinant baculovirus-infected Sf-21 cells were harvested every 24 h and analyzed for the expression of rH-Ft (A) and rL-Ft (B) by SDS-PAGE. The infected cells (6 ml) were harvested and centrifuged at 3000 g for 10 min. The resulting pellet was resuspended in 0.5 ml 50 mM Tris (pH 7.0). Ten microliters of the resuspended cell was subjected to SDS-PAGE. After electrophoresis, the SDS gels were stained by Coomassie blue. In A and B, lanes 0, 1, 2, 3, 4, and 5 represent the days of cell harvest postinfection; lane Rat represents the native rat liver ferritin; lanes H and L represent the recombinant ferritin H and L chains, respectively.
kDa) and L (21.0 kDa) chains appeared on the second day of postinfection and reached the highest on the fourth day of postinfection. The infected cells started to lyse on the fifth day of postinfection and to release the expressed ferritin out of the cell. Both rH-Ft and rL-Ft homopolymers were produced by the respective recombinant baculovirus at approximately 1.5 mg per 100 ml of infected insect cell cultures.

*Purification and characterization of recombinant H and L chain ferritin homopolymers.* The rH-Ft and rL-Ft homopolymers were purified to homogeneity by heat treatment at 65°C for 15 min followed by anion exchange on a DEAE Sepharose Fast Flow column and then by gel filtration on a Sepharose CL-6B column. The purities of the proteins were analyzed by SDS denaturing gel (Fig. 7). The purified rH-Ft and rL-Ft consisted of a single band of protein with apparent molecular masses 21.0 and 20.6 kDa, the molecular mass predicted from the DNA sequence being 20,995 and 20,674 Dal, respectively. The electrophoretic mobilities of rH-Ft or rL-Ft on SDS-PAGE were identical to the corresponding subunit of native rat
FIG. 7. Subunit size and purity of expressed recombinant rat liver H and L chain ferritins. Purified ferritins were resolved under denaturing conditions on 12% polyacrylamide gel and stained for protein with Coomassie blue dye. Lanes A and D, native rat liver ferritin; lane B, recombinant rat liver H chain ferritin; lane C, recombinant rat liver L chain ferritin. Molecular weight markers are shown on the both outer lanes (lane M) of the gel and the molecular mass of markers are indicated on the left.
liver ferritin. The electrophoretic mobilities of the rH-Ft and rL-Ft homopolymers on a nondenaturing 7.5% polyacrylamide gel agreed with the relative molecular masses and charges of native rat liver ferritin (Fig. 8). These results indicated that both recombinant proteins formed multi-subunit complexes similar to native rat liver ferritin.

Analysis of iron loading of recombinant H and L chain ferritins. The initial and final iron contents of each ferritin and residual, nonoxidized Fe(II) for each iron-loading reaction are shown in Table I. Native rat liver ferritin treated with thioglycolic acid was found to contain about 49 ± 3 atoms of iron per ferritin molecule (Table I) using total iron assay as described under Materials and Methods. No iron could be detected in the rH-Ft or rL-Ft homopolymers using the total iron assay method (Table I).

When 0.22 nmole of the apoferritins was incubated with an equimolar amount of ceruloplasmin and a 500-fold excess of histidine-chelated Fe(II) (5:1) in 50 mM NaCl (pH 7.0), iron loading was observed with native rat liver
FIG. 8. Identification of homopolymer formation of recombinant rat liver H and L chain ferritins on nondenaturing polyacrylamide gel. Approximately 10 µg of the purified recombinant H chain ferritin (lane A) and L chain ferritin (lane C) were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. Rat liver ferritin (lane B) was used as a reference. The gel was stained for protein with Coomassie blue dye.
**TABLE I**

The Effect of Ceruloplasmin on Incorporation of Iron into Ferritin

<table>
<thead>
<tr>
<th>Ferritins</th>
<th>Initial iron atoms/ferritin</th>
<th>Final iron atoms/ferritin</th>
<th>Non-oxidized Fe(II) nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>w/o CP</td>
<td>w/CP</td>
</tr>
<tr>
<td>Rat liver</td>
<td>49 ± 3</td>
<td>58 ± 5</td>
<td>484 ± 20</td>
</tr>
<tr>
<td>rH-Ft</td>
<td>ND*</td>
<td>20 ± 3</td>
<td>490 ± 22</td>
</tr>
<tr>
<td>rL-Ft</td>
<td>ND*</td>
<td>-</td>
<td>20 ± 15</td>
</tr>
</tbody>
</table>

Note. Conditions (1 ml final volume) were 0.22 μM ferritin in NaCl (50 mM, pH 7.0) at 37°C in the absence or presence of 0.22 μM ceruloplasmin (CP). Iron was added as 5:1 histidine:Fe(II) (110 μM iron). The reaction was for 20 min. Iron content per ferritin (atoms per ferritin molecule) and iron not oxidized (nmol) were measured as described under Materials and Methods. Data are given as the mean ± SE of triplicate measurements from three individual experiments.

*ND indicates not detectable by the method of total iron assay as described in (31).
ferritin and rH-Ft homopolymer, but not with rL-Ft (Fig. 9). According to the total iron assay, 484 ± 20 iron/ferritin (97% of added iron) and 490 ± 22 iron/ferritin (98% of added iron) were loaded into native rat liver apoferritin and rH-Ft homopolymer, respectively, by ceruloplasmin. Little or no iron was loaded into any apoferritin in the absence of ceruloplasmin (Table I). The amount of Fe(II) remaining with the ferritins incubated without ceruloplasmin was measured to determine if they had any ferroxidase activity. The amount of Fe(II) remaining was 97 ± 3 nmol (88% of added iron) for rat liver apoferritin and 103 ± 4 nmole (94% of added iron) (Table I), indicating that neither rat liver apoferritin nor rH-Ft homopolymer had ferroxidase activity.

Iron staining of the ferritins upon electrophoresis after the iron loading revealed that the iron that ceruloplasmin oxidized in the presence of rL-Ft was not incorporated into this homopolymer (Fig. 10). Staining of iron in native rat liver ferritin and rH-Ft
Iron loading into ferritins by ceruloplasmin. Ceruloplasmin (0.22 nmol) and apoferritin (0.22 nmol) were incubated at 37°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 $E_{380} = 1.03$ mM$^{-1}$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 ($\bullet$), rat liver ferritin loaded by CP ($\blacktriangle$), $rL$-Ft loaded by CP ($\blacktriangledown$), $rH$-Ft loaded without CP ($\bigcirc$), rat liver ferritin loaded without CP ($\blacksquare$).
FIG. 10. Iron staining of various ferritins on nondenaturing polyacrylamide gel. Rat liver apoferritin, recombinant rat liver H and L chain ferritin homopolymers loaded with iron by ceruloplasmin were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. Iron-loading mixtures (1 ml) contained 0.22 nmol ceruloplasmin and various ferritins (0.22 nmol). Histidine-chelated ferrous iron (11 μmol) was added sequentially five times. Iron loading was terminated by adding 1 mM desferrioxamine. The mixtures, except the sample in lane H, were passed through a desalting column to remove the non-bound iron. Approximately 10 μg of purified native rat liver ferritin (lane A) was used as a reference. The rest of the samples (10 μg protein) were applied as follows: rat liver apoferritin (lane B), rat liver ferritin loaded with iron (lane C), rH-Ft (lane D), rH-Ft loaded with iron (lane E), rL-Ft (lane F), rL-Ft after iron loading (lane G), and iron oxidation by CP alone (lane H). The gel was stained for iron by incubating with 0.1% potassium ferrocyanide in 1 N HCl for 15 min. The positions of CP and various ferritins are indicated on the left. These were identified by staining an identical gel with Coomassie blue. The oxidized iron on the lane H of the gel appeared as a long smear.
homopolymer by the same method confirmed that iron was loaded into both of these proteins (Fig. 10).

The effect of native rat liver ferritin, rH-Ft, and rL-Ft on the ferroxidase activity of ceruloplasmin is shown in Fig. 11. The oxidation of histidine-chelated Fe(II) in NaCl solution without adding ceruloplasmin or ferritins was insignificant. In the presence of ceruloplasmin the initial rate of Fe(II) oxidation was 0.3 nmol/min. This ferroxidase activity of ceruloplasmin was not changed by adding rL-Ft (0.3 nmol/min). However, the ferroxidase activity of ceruloplasmin was enhanced by adding H chain-containing ferritins, such as rH-Ft (100% H chain) and native rat liver ferritin (~30% H chain). The initial rates of Fe(II) oxidation by ceruloplasmin in the presence of rH-Ft and rat liver ferritin were increased up to about 4-fold (1.2 and 1.1 nmol/min, respectively). This enhancement of ferroxidase activity of ceruloplasmin was only dependent on the presence of H chain in the ferritin but not dependent on the percentage of H chain in the ferritins.
FIG. 11. The effect of apoferritin on the ferroxidase activity of ceruloplasmin. Ceruloplasmin (0.22 nmol) was preincubated at 37°C in Chelex-treated 50 mM NaCl (pH 7.0) in the absence or presence of various apoferritins (0.22 nmol) for 1 min (1 ml final volume). The reactions were initiated by addition of histidine:Fe(II) (5:1) to a final iron concentration of 11 μM. Aliquots of the reaction were removed over time and placed in 1 mM ferrozine. The ferrous iron remaining was determined using $E_{562} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$. Data are given as the mean ± SE of at least three individual experiments, each performed in triplicate. ○, Fe(II) alone; ■, Fe(II) plus ceruloplasmin; ▲, Fe(II) plus ceruloplasmin and recombinant H chain apoferritin; ▼, Fe(II) plus ceruloplasmin and recombinant L chain apoferritin; ●, Fe(II) plus ceruloplasmin and rat liver apoferritin.
To explore the extent of iron loading for rH-Ft and rat liver ferritin, loading of iron per ferritin into either apoferritin by ceruloplasmin was done sequentially in increments of 500 atoms per ferritin. Iron loading into both rH-Ft and rat liver ferritin resulted in the same loading curve and reached approximately 2500 atoms of iron per ferritin. Further additions of iron or ceruloplasmin did not result in loading more iron into ferritin (Fig. 12).

DISCUSSION

Several studies by other laboratories have compared iron loading between human H chain ferritin homopolymer and rat liver L chain ferritin homopolymer (10,35). However, no reason was provided for why rat liver H chain ferritin was not used in those studies. In our laboratory, we found that the growth of host *Escherichia coli* JM109 was suppressed for an unknown reason when the recombinant expression vector of rat liver H chain ferritin was transformed into the host. No expression of rH-Ft was observed, though the rat liver H chain ferritin gene was detected inside the host using PCR. This
FIG. 12. Sequential loading of iron into apoferritins by ceruloplasmin. Reaction mixtures contained rat liver apoferritin or recombinant H chain ferritin (0.22 nmol) and CP (0.22 nmol) in 1 ml of 50 mM NaCl. Iron was added in increments of 500 atoms per ferritin as histidine:Fe(II) (5:1). The additions of iron and CP were indicated by arrows. Iron incorporation into ferritin was monitored spectrophotometrically at 380 nm and calculated using a molar extinction coefficient $E_{380} = 1.03 \text{ mM}^{-1}\text{cm}^{-1}$. Results are expressed as atoms of iron incorporated per ferritin molecule. Data are representative measurements from at least three individual experiments.
observation may be because the presence of rH-Ft can affect the growth and differentiation of certain cell types (36-38). We report here that the expression of the rH-Ft and rL-Ft homopolymers in Sf-21 cells can be done using the baculovirus expression system and provides a preliminary characterization of the expressed proteins.

Preliminary experiments have shown here that the baculovirus expression system can produce rH-Ft and rL-Ft up to 1.5 mg/100 ml of cell culture. These results indicate that the host insect cell can tolerate the impact of rH-Ft, though suppression of cell growth of the insect cell host can still be observed during the rH-Ft expression (to be published). The expression of either rH-Ft and rL-Ft started on the second day and reached the maximum on the fourth day postinfection. Expression of polyhedrin was observed during this time frame as well. The infected cells started to lyse on the fifth day postinfection, so the expressed protein was harvested prior to that time to simplify purification.

Both the expressed rH-Ft and rL-Ft were found in the full-assembled multi-subunit complexes. One unique form
of homopolymer was observed for each protein. The electrophoretic mobility of rH-Ft and rL-Ft homopolymers on a 7.5% nondenaturing gel indicated the different protein surface charges between rH-Ft and rL-Ft homopolymers. The computed pI values for H and L chain ferritin based on the peptide sequences are 5.85 and 5.96, respectively. The electrophoretic migration of rH-Ft homopolymer was much faster than that of rL-Ft homopolymer, whereas the migration of native rat liver ferritin was between that of the rH-Ft and rL-Ft homopolymers because native rat liver ferritin is a heteropolymer which contains various ratios of H and L chains. According to SDS denaturing gel analysis, the band of both rH-Ft and rL-Ft were shown to be the correct molecular mass compared to the corresponding subunit of native ferritin. No impurities were detected on the SDS denaturing gel. Besides, both recombinant proteins were soluble and stable in 50 mM Tris buffer (pH 7.0) or 50 mM NaCl (pH 7.0) at 4°C for months. These results indicate that the cloned gene were expressed and purified recombinant proteins were obtained.
Native rat liver ferritin and rH-Ft homopolymer loaded iron at almost identical rates in 50 mM NaCl using ceruloplasmin as a ferroxidase. Loading of native rat liver ferritin or rH-Ft homopolymer with iron in 50 mM NaCl without ceruloplasmin resulted in very little iron inside the ferritin. Even though a slow iron oxidation rate was observed with the rL-Ft homopolymer in the ceruloplasmin-loading system, iron staining of a non-denaturing gel reveals that the iron did not enter into the rL-Ft homopolymer. These observations indicate that the initial rates of iron loading in this loading system are related to the presence of ceruloplasmin, but not to the ratio of H to L chain.

An interesting feature of ferritin iron loading by ceruloplasmin is that native rat liver ferritin or rH-Ft homopolymer, but not rL-Ft homopolymer, enhanced the ferroxidase activity of ceruloplasmin. Since we already showed that native ferritin and rH-Ft homopolymer do not have ferroxidase activity, the increase of iron oxidation is apparently by ceruloplasmin, but not by ferritins. Therefore, this enhancement of ferroxidase activity of
ceruloplasmin may be due to the association of ferritin and ceruloplasmin. The association may alter the conformation of ceruloplasmin, increasing its ferroxidase activity. We believe that this association is definitely required for the iron-loading reaction, because without association, iron oxidized by ceruloplasmin may be released into solution without incorporation into ferritin. A conceivable example is tyrosinase, which has ferroxidase activity but is not capable of loading iron into ferritin (39). The feature may imply a functional role of ceruloplasmin to load iron into ferritin in vivo. Further investigation of ferritin and ceruloplasmin association is underway.

REFERENCES


CHAPTER IV

SUPPRESSION OF CELL GROWTH BY HEAVY CHAIN FERRITIN

ABSTRACT

While producing recombinant rat liver H and L chain ferritin homopolymers using the baculovirus expression system, we noticed that rat liver H chain ferritin, but not L chain ferritin, had a suppressive effect on the growth of Spodoptera frugiperda (Sf-21) cells. Suppression was observed immediately after infection with recombinant H chain ferritin baculovirus prepared from lysed infected cells. Immediate suppression was observed when purified with either recombinant H chain apoferritin or various holoferritins (loaded with 1,970 ± 50 or 2,520 ± 90 atoms of iron/ferritin) indicating that suppression was not due to sequestration of iron required for cell growth. Suppression by H chain ferritin was also observed upon attempting to express the protein in

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Escherichia coli. Strategies for expression of recombinant rat liver H and L ferritin homopolymers in both prokaryotic and eukaryotic expression systems were developed.

INTRODUCTION

Ferritin is a ubiquitous protein composed of 24 subunits of H and L chains and is considered to be the intracellular storage protein for iron (1). The 24 subunits form a protein shell that sequesters and stores up to 2,500 atoms of iron per molecule of ferritin (2). We have cloned the genes of H and L chains of rat liver ferritin into expression vectors to investigate the functions of the two chains. In the process we noticed that H chain had a strong suppressive effect on Escherichia coli growth. The yield of recombinant H chain ferritin (rH-Ft) was poor due to the limitation of cell growth when rH-Ft was present. This was not observed when recombinant L chain ferritin (rL-Ft) was expressed.
Our first attempt involved expression of rH-Ft in \textit{E. coli} using expression vector pDR540 (from Pharmacia) which contains a very strong fusion promoter, the \textit{tac} promoter, for expression of inserted genes. The growth of \textit{E. coli} stopped upon transformation with the recombinant pDR540 vector containing rat liver H chain ferritin gene. The \textit{tac} promoter of pDR540 may be so strong such that some rH-Ft protein was expressed, even though the inducer, isopropyl-\textit{\beta}-D-thiogalactoside (IPTG), had not been applied. Under this situation, the inhibition of cell growth by the rH-Ft resulted in very low yield of protein. This prompted us to consider other expression systems which may effectively postpone the time of expression so that the host can grow before rH-Ft expression is initiated. The baculovirus expression system was also used to investigate the suppression of eukaryotic cell growth during expression of rH-Ft. The results provide important information regarding expression strategy for rH-Ft production in both prokaryotic and eukaryotic expression systems.
MATERIALS AND METHODS

Materials. The baculovirus expression system was purchased from PharMingen (San Diego, CA) and insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS). The plasmid pBR322 was obtained from Pharmacia Biotech (Alameda, CA). The genes for T7 promoter and T7 terminator were obtained from plasmid pET-14b, which was from Novagen, Inc. (Madison, WI). The E. coli hosts, JM109 and JM109(DE3), were purchased from Promega Corporation (Madison, WI). The expression vector pET-11a and the E. coli host B834(DE3)pLysS were purchased from Novagen, Inc. (Madison, WI). The pfu DNA polymerase and polymerase chain reaction (PCR) reagents were from Stratagene. Other enzymes used for DNA manipulations were purchased from Stratagene Cloning Systems (La Jolla, CA), Boehringer Mannheim Biochemicals (Indianapolis, IN), or United States Biochemicals (Cleveland, OH) and used according to manufacturers' instructions. Other chemicals were of reagent grade and obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI).
Baculovirus expression system for rH-Ft and rL-Ft.

Construction of recombinant baculovirus and expression of rH-Ft and rL-Ft homopolymers have been previously described (3). Briefly, rat liver ferritin H and L chain cDNA were inserted individually at BglII site on the pAcUW21, a baculovirus transfer vector containing a p10 promoter upstream of the cloning site. The inserts were sequenced by the dideoxy chain termination method using Sequenase (United States Biochemicals, Cleveland, OH). Sf-21 cells maintained in EX-CELL 401 medium were transfected with recombinant pAcUW21 containing rat liver H or L chain gene and wild type baculovirus using Lipofectin® (Gibco BRL, Gaithersburg, MD). Recombinant virus (Occ+) was purified by three rounds of plaque screening. Confirmation of rat liver ferritin H or L chain gene inserts was corroborated by PCR analysis. Cultured Sf-21 insect cells were then infected with isolated rH-Ft recombinant baculovirus (H-virus) or rL-Ft recombinant baculovirus (L-virus). Cell medium supernatants harvested 2 days postinfection, at which time the recombinant ferritins had not been expressed
Recombinant baculovirus stocks of H-virus and L-virus were also prepared 7 days postinfection, at which time all the infected cells were lysed and the expressed recombinant proteins were released to the medium. The concentration of virus stocks from the non-lysed cells (2 days postinfection) or the lysed cells (7 days postinfection) were adjusted to $2.3 \times 10^8$ virus/ml by dilution with insect cell medium EX-CELL 401.

**Suppression of insect Sf-21 cells during expression of ferritin.** Insect Sf-21 cells, about $(1.1 \pm 0.2) \times 10^6$ cells, were cultured in 6 ml EX-CELL 401 media at 27°C. The high titer virus stocks (approximately 0.1 ml) prepared from the non-lysed 2 days postinfection infected cells or the lysed 7 days postinfection infected cells were added into cell cultures at a multiplicity of infection of 20 per cell. The cells were counted every 24 h using a Hausser Hy-Lite hemocytometer (from Fisher Scientific, Pittsburgh, PA).

**Effect of ferritin on the baculovirus expression system.** The methods for purification of rH-Ft, rL-Ft,
and ceruloplasmin were as described earlier (3). Both
rH-Ft and rL-Ft, examined by total iron assay (4),
contained iron. Holoferritin was prepared by loading
iron into apoferritin using ceruloplasmin (3). Some rH-
Ft was loaded with a similar amount of iron as found in
native rat liver ferritin and other was maximally loaded
with iron. These two holoferritins contained 1970 ± 50
and 2520 ± 90 atoms of iron per molecule, respectively.
To investigate the suppressive effect on the Sf-21 cell
growth, 0.1 mg of rH-Ft, rL-Ft, or holoferritin of rH-Ft,
which contained the different amounts of iron, were added
individually into the 6-ml insect cell cultures. The
number of cells were quantitated every 24 h as described
above.

**Suppression of E. coli during expression of**
ferritin. The plasmids constructed for this study are
diagrammed in Fig. 13. Rat liver H (561 bp) and L (564
bp) chain ferritin genes were amplified from the
recombinant baculovirus transfer vectors, which were
described earlier (3), by PCR using *pfu* DNA polymerase
(5). The T7 promoter (108 bp) and T7 terminator (88 bp)
Sequential PCR synthesis of T7 promoter and T7 terminator

PCR synthesis of rH-Ft or rL-Ft coding region using the recombinant baculovirus transfer vector as a template

Sequential PCR synthesis of T7 promoter and T7 terminator

FIG. 13. Schematic illustration of the E. coli expression vectors constructed for rat liver rH-Ft and rL-Ft expression. The T7 promoter (T7P) and T7 terminator (T7T) were amplified by PCR, using pET-14b as a template, to generate a BglII-HindIII fragment and a EcoRI-BamHI fragment, respectively. Both rat liver H and L chain genes were also amplified by PCR, using the previous recombinant baculovirus transfer vectors (3), to generate a BamHI-HindIII fragment. The T7 promoter and the T7 terminator were sequentially ligated onto the 5'-and 3'-end, respectively, of both ferritin genes. The gene fragments of rat liver rH-Ft and rL-Ft containing T7 promoter and terminator were inserted into EcoRI + HindIII cut pBR322 to produce recombinant rH-Ft and rL-Ft plasmids, respectively.
genes were also amplified from a plasmid pET-14b using the same PCR polymerase. The T7 promoter was ligated upstream and the T7 terminator downstream of ferritin H chain and L chain genes. The DNA products were then cloned into the plasmid pBR322. The sequences of the cloned DNAs were confirmed by DNA restriction mapping and sequencing.

The expression of rat liver H or L chain ferritin in this system is driven by T7 RNA polymerase. The polymerase is highly selective for its own promoters, the gene for which does not exist naturally in *E. coli* (6) or in pBR322 (7). The host, *E. coli* strain JM109 (DE3), derived from JM109, contains a chromosomal copy of the gene for T7 RNA polymerase (6). Expression of T7 RNA polymerase by the host therefore can regulate expression of inserted ferritin genes on the derived pBR322. The gene for T7 RNA polymerase in the host chromosome has been placed under the control of lac UV5 promoter, which can be induced by IPTG. In the absence of IPTG, little or no T7 RNA polymerase should be produced by JM109 (DE3) therefore preventing expression of ferritin genes.
Seed cultures of JM109 or JM109(DE3), containing either H or L chain ferritin expression vector, were grown in 100-ml Luria-Bertani (LB) medium containing 100 μg/ml ampicillin overnight (16 h) at 37°C and 200 rpm in an orbital incubator shaker. The seed cultures were transferred to 100 ml fresh LB medium containing 100 μg/ml ampicillin to a final cell density of approximate 0.035 OD₆₀₀ nm and incubated at 37°C and 200 rpm in an orbital incubator shaker. Cell densities were quantitated spectrophotometrically at 600 nm every 30 min. IPTG was added to the cell cultures to a final concentration of 5 mM at an early stage (2 h after the seed culture transfer) or at a late stage (4 h after the seed culture transfer) of exponential growth.

Construction and expression of ferritin H chain homopolymer using E. coli B834(DE3)pLysS. The rat liver ferritin H chain gene described above was cloned into a pET-11a vector which contained a Lac operator between the T7 promoter and the cloned gene for additional regulation of gene expression. E. coli B834(DE3)pLysS, which contains the T7 RNA polymerase gene in its genome
under control of lacUV5 and a plasmid containing the gene for T7 lysozyme, was transformed with the recombinant pET-11a vector for expression of rat liver ferritin H chain homopolymer. The T7 lysozyme is a natural inhibitor of T7 RNA polymerase (8) which should suppress any basal expressed T7 RNA polymerase.

Expression of ferritin H chain homopolymer using the E. coli B834(DE3)pLysS cells was conducted similarly as for E. coli JM109(DE3), as described above.

RESULTS

Suppression of insect Sf-21 cells during expression of ferritin. The average doubling time for the growth of Sf-21 cells was approximately 18 h (Fig. 14). Upon addition of medium from lysed H-virus infected cells (7 days postinfection), the growth of Sf-21 was suppressed from the first day of infection (Fig. 14). The suppression of cell growth was not observed with media lysed cells (7 days postinfection) infected with either from wild-type baculovirus or L-virus (the doubling time of cell growth was about 19 ± 1 h). When media prepared
FIG. 14. Suppression of Sf-21 cell growth during the expression of rH-Ft. Sf-21 cells (1.1 × 10⁶) were infected with various recombinant baculoviruses prepared from non-lysed infected cells (2 d p.i.) or lysed infected cells (7 d p.i.) and incubated at 27°C. The viable cells were counted using a hemocytometer and the trypan blue exclusion method. Each point and bar represents the mean and SD of three experiments done in triplicate. The growth curve of Sf-21 cells infected with 7 d p.i. H-virus (■) was significantly different from that of Sf-21 cells alone (○) (p < 0.01). No statistically significant difference (p < 0.05) was shown among the growth curves of Sf-21 cells alone (○), cells infected with wild-type baculovirus (●), and cells infected with 2 d p.i. L-virus (▲) or 7 d p.i. L-virus (▲). The asterisk shows the time of which a statistically significant difference (p < 0.01) occurred for cells infected with 2 d p.i. H-virus (□). The data for 96-h (4 d p.i.) were not included for statistical analyses because the cells were lysing at this time.
from non-lysed cells (2 days postinfection) infected with wild-type baculovirus, H-virus, or L-virus were used for infection, the growth of Sf-21 cells during the first 2 days postinfection was similar to noninfected cells. The expression and accumulation of polyhedrin in the cell nuclei was observed on the third day postinfection, indicating expression of the inserted ferritin gene. At that time the growth of cells infected with H-virus ceased (Fig. 14). Cells infected with either wild-type baculovirus or L-virus prepared from the non-lysed infected cells (2 days postinfection) did not exhibit the suppressive effect (Fig. 14). All infected cells started to lyse on the fourth day postinfection.

Direct addition of rL-Ft into the Sf-21 cultures caused no suppressive effect on the cell growth (the doubling time of cell growth was about 18 ± 1 h) (Fig. 15). However, the addition of rH-Ft showed strong suppression of the cell growth (the doubling time of cell growth was increased to 64 h) (Fig. 15). The suppressive effect on cell growth was also observed,
FIG. 15. The effect of various ferritins on Sf-21 cell growth. Sf-21 cells \((1.1 \times 10^6 \text{ in } 6 \text{ ml})\) were incubated with 1 mg of various ferritins at 27°C and viable cells counted daily using a hemocytometer and the trypan blue exclusion method. Each point and bar represents the mean and SD of three experiments. The growth curves for Sf-21 cells grown with rH-Ft \((\bigcirc)\), or with rH-Ft containing 1970 \((\blacktriangle)\) or 2520 \((\blacktriangledown)\) atoms of iron were significantly different from that of Sf-21 cells alone \((\bigcirc)\) \((p < 0.01)\). The curves for cells grown with rH-Ft containing 1970 \((\blacktriangle)\) or 2520 \((\blacktriangledown)\) atoms of iron were also significantly different from that of rH-Ft \((\bigcirc)\) \((p < 0.01)\). No statistically significant difference was found between the growth curves of Sf-21 cells alone \((\bigcirc)\) and cells grown with rL-Ft \((\square)\) \((p < 0.01)\) or between the growth curves of cells grown with rH-Ft containing 1970 \((\blacktriangle)\) and 2520 \((\blacktriangledown)\) atoms of iron \((p < 0.05)\).
although to a lesser extent, when holoferritins of rH-Ft containing 1,970 atoms of iron or 2,520 atoms of iron were added (the doubling times were 39 h and 38 h, respectively) (Fig. 15).

Construction of E. coli expression system for rH-Ft and rL-Ft. Construction of expression vectors containing the genes of rH-Ft and rL-Ft was accomplished as described under Materials and Methods. The cloning orientation and DNA sequences for both were correct (data not shown). Preliminary analyses confirmed that the recombinant ferritin homopolymers were only expressed by the host JM109(DE3), which was induced with 5 mM IPTG. Expression of recombinant ferritin homopolymers was not observed if IPTG was not added. Additionally, no ferritin was expressed when the expression vector was carried by a host, such as JM109, lacking the T7 polymerase gene (data not shown).

Suppression of E. coli during expression of ferritin. The doubling time for JM109(DE3) was 35 min, only slightly longer than for JM109 (31 min). When JM109(DE3) was transformed with the expression vector
containing the rL-Ft gene and induced with IPTG, the
growth rate remained the same as wild type JM109(DE3).
However, the doubling time of JM109(DE3) containing the
rH-Ft expression vector approximately doubled (71 min)
(Fig. 16). When 5 mM IPTG was added to this culture
during the early stage of exponential growth (OD600 was
0.10 ± 0.04), expression of rH-Ft was observed within 30
min (data not shown) and cell growth was limited. When 5
mM IPTG was added during the late stage of exponential
growth (OD600 was 0.40 ± 0.07), rH-Ft was expressed after
30 min (Fig. 17). Similar to when IPTG was added
earlier, cell growth rate declined after 1 h (Fig. 16).
The suppressive effect on the cell growth was not
observed with the host containing the rL-Ft expression
vector (Fig. 16). Cultures transformed with the
expression vector containing the rH-Ft gene formed
massive clumps of cells soon after the addition of IPTG.
Therefore, the OD600 readings after addition of IPTG was
not a reliable determination of E. coli growth. The E.
coli precipitated out of the medium completely if the
culture were left unshaken for an hour.
FIG. 16. Suppression of E. coli growth during the expression of rH-Ft. Recombinant E. coli JM109(DE3) (OD$_{600}$ = 0.035) containing rH-Ft or rL-Ft plasmid were grown in 100 ml of LB medium shaken at 37°C. The inducer IPTG (5 mM) was added at 2 h or 4 h after incubation. The cell density was monitored spectrophotometrically at 600 nm every 30 min. Each point and bar represents the mean and SD of three experiments done in triplicate. The growth curves for JM109, JM109(DE3), and JM109(DE3) with the rL-Ft plasmid were essentially identical. Only the curve for the latter (Δ) is shown. The growth curve for JM109(DE3) with the rH-Ft plasmid (Φ) was statistically (p < 0.01) lower than the host without the plasmid. The asterisks show data points which were statistically significant lower (p < 0.01) when IPTG was added at 2 h (▲) or 4 h (▼).
FIG. 17. Time course of expression of rat liver rH-Ft in recombinant E. coli JM109(DE3) upon induction with IPTG. IPTG was added 4 h after seed culture transfer. Samples (1 ml) were taken every 30 min after adding the IPTG and centrifuged at 3,000 g for 10 min. The resulting pellet was resuspended in 0.5 ml 50 mM Tris (pH 7.0) and 10 µl was subjected to denaturing polyacrylamide (12%) electrophoresis gel. After electrophoresis the gels were stained with Coomassie blue. Lanes 0, 0.5, 1, 1.5, 2, 2.5, and 3 represent the hours after adding the IPTG; lane Rat represents partially purified native rat liver ferritin.
Expression of ferritin H chain homopolymer using E. coli B834(DE3)pLysS cells. E. coli B834(DE3)pLysS cells containing the LH-Ft expression vector was grown in LB medium and 5 mM IPTG was added after 4 h of incubation. No suppression of cell growth was observed before IPTG was added (data not shown). The induction of ferritin H chain expression was observed 30 min after IPTG induction and expression was greater (Fig. 18) than in E. coli JM109(DE3) using the recombinant M13 derived T7 promoter driven expression vector as described above (Fig. 17).

DISCUSSION

The suppressive effect of ferritin H chain on the proliferation and functions of high iron-requiring cells, such as lymphocytes, granulocytes, and myeloid progenitor cells, has been observed previously (9-11). These effects were proposed to be associated with the sequestration of iron required by the cells (12). However, we found that rat liver rH-Ft with or without iron exhibited a suppressive effect on cell growth. This phenomenon indicated that the suppression could not be only by sequestration of iron required for growth.
FIG. 18. Time course of expression of rat liver rH-Ft in recombinant E. coli B834(DE3)pLysS upon induction with IPTG. The expression induction and sample preparations were the same as Fig. 5. Ten microliters of the resuspended cells was subjected to denaturing polyacrylamide (12%) electrophoresis gel. After electrophoresis the gels were stained with Coomassie blue. Lanes 0, 0.5, 1, 1.5, 2, 2.5, and 3 represent the hours after adding the IPTG; lane Rat represents partially purified native rat liver ferritin.
Furthermore, the rat liver rH-Ft expressed in the recombinant baculovirus system did not contain any detectable iron. Interestingly, we also found that the expression of human liver rH-Ft was less suppressive than the expression of rat liver rH-Ft in E. coli (unpublished data).

It has been reported that the ferritin H chain may associate with mRNA and inhibit in vitro translation of certain mRNA (13,14). Mulvey et al. (15) demonstrated that Mengo virus infection induced apoferritin, which effectively inhibited the translation of mRNA in reticulocyte lysates. They proposed that this inhibition may turn off the synthesis of certain proteins in the host, which might cause the suppression of cell growth, and liberate translation components for the synthesis of viral proteins. We also observed an inhibitory effect of rat liver rH-Ft on globin mRNA translation in vitro (unpublished data). These observations suggested that rH-Ft might serve other novel functions for regulation of cell growth.
The baculovirus system expresses the viral genes required for the initiation of viral DNA synthesis at an early stage, genes required for the assembly of virus particles at a late stage, and the gene inserted in the recombinant viral genome at a very late stage of the viral cycle (16). This provided a strategy for production of rH-Ft, even though the protein was highly suppressive to cell growth. Baculovirus multiplies and is released as a budding virus into the medium in the first couple days postinfection. Little or no rH-Ft was expressed during this time. Therefore, H-virus produced during the first 48 h of infection can be used for infection with little or no suppression of cell growth. The suppression of insect cell growth occurred approximately 48 h postinfection, at which time the rat liver rH-Ft was expressed (3). The expressed rat liver rH-Ft was present in the 7 days postinfection media due to the lysis of cells. Therefore upon infection of Sf-21 cells with this H-virus media, the suppression of cell growth occurred immediately and the yield of rat liver rH-Ft was very low (less than 0.1 mg per 100 ml of cell
culture). The initial cell density of Sf-21 for infection should be about $5 \times 10^5$ cells/ml. This condition allows the growth of insect cells to reach maximal density of $2 \times 10^6$ cells/ml before the rat liver rH-Ft is expressed. Therefore, the expression system may have enough cells for expression and achieve a higher yield of recombinant protein production.

The strategy for designing the *E. coli* expression system was to construct a vector that was under stringent control for expression of the inserted gene. Regulation of the expression of the RNA polymerase for the promoter of the inserted gene was considered better than to directly control the expression of inserted gene because the former method could lengthen the processing time for expression of the inserted gene if regulation was not stringent. Prior to addition of IPTG, the growth of JM109(DE3) containing the expression vector of rH-Ft was relatively slow when compared to wild type JM109 (DE3) or JM109 (DE3) containing the expression vector of rL-Ft. This observation suggested that the regulation of T7 mRNA polymerase expression was leaking. However, the amount
of rH-Ft expressed was not high enough to be observed upon SDS-PAGE, and was not enough to completely suppress growth of the host JM109(DE3). We were able to induce the expression of rH-Ft by adding the IPTG when the growth of the host JM109(DE3) reached a late stage of exponential growth. In order to obtain a higher yield of recombinant protein, we suggest that the inducer, IPTG, should be added when the OD<sub>600</sub> of cell culture reaches 0.4.

When <i>E. coli</i> B834(DE3)pLysS cells containing a recombinant pET-11a expression vector were used for expression of ferritin H chain homopolymer, no suppression was observed prior to the addition of IPTG and the system produced a greater quantity of recombinant protein than the JM109(DE3) system. Since <i>E. coli</i> B834(DE3)pLysS included two additional constructs, a Lac operator downstream of the T7 promoter and the gene for T7 lysozyme, for stringent regulation of expression of the inserted gene, no suppression of cell growth was observed and the production of ferritin H chain occurred after addition of IPTG. These results demonstrate that
suppression by the ferritin H chain may be avoided in a highly stringent expression system and provide for significant H chain ferritin expression in such an expression system.

In conclusion, the potential significance of the present observations is apparent when regarding the suppressive effect of rH-Ft on cell growth, and when considering the development of a system for the expression of the rH-Ft. This study provided evidence that the suppression of growth of the host, insect cells and *E. coli*, was related to the presence of rat liver rH-Ft, and might be due to inhibition of certain gene expression of host. The suppressive effect of rH-Ft on the host of expression system may be avoided or reduced by controlling the time of expression of rat liver rH-Ft.

REFERENCES


CHAPTER V

STRUCTURAL AND FUNCTIONAL DIFFERENCES OF RECOMBINANT RAT LIVER HEAVY CHAIN FERRITIN HOMOPOLYMERS PRODUCED IN INSECT CELL-BACULOVIRUS AND ESCHERICHIA COLI EXPRESSION SYSTEMS

ABSTRACT

Recombinant H chain rat liver ferritin (rH-Ft) homopolymers were produced in Spodoptera frugiperda cells infected with a recombinant baculovirus and in Escherichia coli JM109(DE3) using a T7 expression vector. The protein expressed in the E. coli system (rH-Ft-E) contained 158 ± 8 atoms of iron per ferritin molecule, whereas no iron was detected in the rH-Ft expressed in an insect cell system (rH-Ft-I). Both were made up of a 21-kDa subunit; however, the electrophoretic migration of the rH-Ft-E homopolymer was faster than that of the rH-Ft-I homopolymer upon nondenaturing PAGE. On a nondenaturing isoelectric focusing polyacrylamide gel, the rH-Ft-E homopolymer resolved into 3-4 species, whereas the rH-Ft-I homopolymer showed only a single
species. All of the rH-Ft-E homopolymers showed relatively greater pIs and carbonyl content than the rH-Ft-I homopolymer. Evidence for protein oxidation (increased carbonyl content) from iron incorporation during expression of the homopolymer in the E. coli system also resulted in a decrease in ordered secondary structures, compared with the rH-Ft-I homopolymer, as analyzed by circular dichroism. Different rates and extent of iron incorporation were also found between the rH-Ft-E homopolymer and the rH-Ft-I homopolymer using a ceruloplasmin loading system. Taken together, these results suggested that the insect cell-baculovirus system, instead of E. coli system, should be used for expression of ferritin H chain homopolymer to obtain physiologically-relevant ferritin.

INTRODUCTION

The expression of recombinant rat liver ferritin H chain homopolymer has been successfully performed in insect Spodoptera frugiperda (Sf-21) cells infected with a recombinant baculovirus (1). However, the yield was
not great due to a suppressive effect of the expressed ferritin H chain on the growth of the Sf-21 cells (2). An alternative *Escherichia coli* JM109(DE3) expression system, under stringent regulation, was developed for a greater yield of the ferritin H chain homopolymer (2). Since expression of rat liver ferritin *in vivo* does not require any post-translational modification, the protein folding and structures of the ferritin H chain homopolymers expressed in eukaryotic and prokaryotic cells were thought to be similar. In fact, *E. coli* expression systems have been frequently utilized for the production of human (3,4) and bullfrog (5) ferritin H and L chain homopolymers. Ferritin homopolymers expressed in the *E. coli* system were mainly used for studying the iron loading functions of ferritin using a Good type buffer system, such as Hepes and Mops buffers. Some researchers demonstrated that when ferritin was incubated with Fe(II) in Hepes buffer, ferritin accelerated the rate of Fe(II) oxidation and iron incorporation (6,7). However, we found that iron deposition into ferritin in the presence of Good buffers was directly related to the rate of iron
autoxidation which produced damaging oxygen radical species and oxidized amino acid residues in the ferritin (8). It seems unlikely that such an iron-loading system would occur in vivo because no oxidative modification of amino acid residues was found on native ferritin isolated from rat liver (8). We have demonstrated that it may be more physiological to load ferritin with iron using ceruloplasmin as a ferroxidase (8,9).

When the recombinant ferritin H chain homopolymer (rH-Ft) expressed in an E. coli system (rH-Ft-E) was purified, we found that it, but not the rH-Ft expressed in an insect cell system (rH-Ft-I), already contained a certain amount of iron. When ceruloplasmin was used to load iron into native rat liver ferritin, rH-Ft-I, and rH-Ft-E, the loading rate and extent of the rH-Ft-E were different from those of native rat liver ferritin and the rH-Ft-I. We suspected that the structures of the rH-Ft homopolymer had been varied during the expression in the E. coli system. The aim of this work was to investigate this aspect of the rH-Ft-E homopolymer. In addition, a possible reason for oxidative damage on the rH-Ft-E
homopolymer and the role of ceruloplasmin for loading iron into ferritin are discussed.

MATERIALS AND METHODS

Materials. Rat livers and serum were purchased from Pel-Freez Biologicals (Roger, AR). *Spodoptera frugiperda* (Sf-21) cells, baculovirus transfer vector pAcUW21, and the linearized baculovirus BaculoGold were purchased from PharMingen (San Diego, CA) and insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS). The *E. coli* host JM109(DE3) was purchased from Promega Corporation (Madison, WI). The plasmid pBR322 was obtained from Pharmacia Biotech, Inc. (Alameda, CA). The genes for the T7 promoter and the T7 terminator were obtained from plasmid pET-14b, which was purchased from Novagen, Inc. (Madison, WI). The enzymes used for DNA manipulations were purchased from Stratagene Cloning Systems (La Jolla, CA), Boehringer-Mannheim Biochemicals (Indianapolis, IN) or United States Biochemicals (Cleveland, OH) and used according to the manufacturers' instructions. Other enzymes and chemicals were of
reagent grade and obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI).

Preparation of native rat liver ferritin and apoferritin. Ferritin was isolated from rat liver tissue using a method as described previously (1). Apoferritin was prepared by using thioglycolic acid and $\alpha,\alpha'$-dipyridyl, as described by Dognin and Crichton (10). Protein concentration was determined by the method of Bradford (11) with a Bio-Rad protein assay kit using BSA as a standard. Total iron assays were performed as described by Brumby and Massey (12).

Expression of the rH-Ft homopolymer in insect Sf-21 cells. Manipulation of recombinant baculovirus for expression of rat liver rH-Ft homopolymer in insect cells was described previously (1). Sf-21 cells were grown at 27°C in serum-free EX-CELL 401 insect cell medium. Cultures of Sf-21 cells at a density of approximate $2 \times 10^6$ cells/ml were infected with the recombinant baculovirus containing the rH-Ft gene at a multiplicity of 20 and harvested 4 days postinfection. The cells were harvested by centrifugation at 1,000 $g$ and resuspended in
50 mM phosphate buffer (pH 7.0). The cells were subjected to three cycles of freeze-and-thaw and cleared by centrifugation at 100,000 g for 30 min. The two-step, nonheated ferritin purification procedure was as follows. The supernatant was applied to a 10-ml DEAE Sepharose Fast Flow column equilibrated in 50 mM Tris-HCl (pH 7.0). The column was eluted using a discontinuous gradient of 50, 100, 150, 200, and 250 mM NaCl in 50 mM Tris-HCl (pH 7.0). The rH-Ft homopolymer eluted around 150-200 mM NaCl. The fractions containing the rH-Ft homopolymer were concentrated and subjected to a Sepharose CL-6B size exclusion chromatography and eluted using 50 mM phosphate buffer (pH 7.0). The fractions containing ferritin were pooled and concentrated with an Amicon concentrator (Centriprep-100, 100-kDa cutoff) (Amicon, Beverly, MA).

Expression of the rH-Ft homopolymer in E. coli. The details for the expression of rH-Ft homopolymer were described previously (2). E. coli cultures (1 liter) were harvested, centrifuged at 5,000 g, and resuspended in 5 ml of 50 mM phosphate buffer (pH 7.0). Cell free extracts were prepared by incubation of the 5-ml cell
suspension of E. coli with 0.1 g of lysozyme at 37°C for 30 min, followed by three cycles of freeze-and-thaw. The preparation was cleared by centrifugation at 100,000 g for 30 min and the rH-Ft-E homopolymer purified by the two-step, nonheated procedure described above.

**Gel electrophoresis.** The molecule mass of the subunits of rH-Ft-E and rH-Ft-I were determined by SDS-PAGE using a 12% Ready Gel (Bio-Rad Laboratories, Hercules, CA). The assembly of rH-Ft-E and rH-Ft-I homopolymers were determined by PAGE using a 7.5% Ready Gel (Bio-Rad Laboratories) without SDS. Isoelectrofocusing was performed on a Hoeffer 600 series vertical unit using 7.5% nondenaturing polyacrylamide gel (4.5% stacking gel) with a linear gradient, pH 3.5-10.0, according to the instructions of the manufacturer, Pharmacia Biotech, Inc. The isoforms of the rH-Ft-E and rH-Ft-I were visualized by staining of Coomassie blue R (Sigma).

**Loading iron into ferritins in HEPES buffer.** Ferritins (1.1 nmol) were incubated with 0.55 µmol of Fe(NH₄)₂(SO₄)₂ in 1 ml of 50 mM HEPES buffer (pH 7.0) for
30 min. Ferrozine (1 mM) and desferrioxamine (1 mM) were added to terminate the reaction. The nonincorporated iron was removed by passing the preparation through an Econo-Pac 10 DG desalting column. The iron in ferritins was quantitated by protein assay and total iron assay as described above.

**Loading iron into ferritins using ceruloplasmin.**

The method for loading iron into ferritin using ceruloplasmin has been described previously (1). Ceruloplasmin was prepared from rat serum as described by Ryan et al. (13). The iron found in the rH-Ft-E homopolymer was released by using thioglycolic acid and $\alpha,\alpha'$-dipyridyl as described above to provide apoferritin. Iron loading reactions were conducted by incubation of 0.22 nmol of rat liver ceruloplasmin and 0.22 nmol of various apoferritin in 1 ml of 50 mM NaCl under 37°C. Histidine (550 nmol) chelated Fe(II) (110 nmol) was added and iron oxidation was monitored by optical density at 380 nm. The reactions were terminated by adding 1 mM ferrozine and 1 mM desferrioxamine. The nonincorporated iron was removed by an Econo-Pac 10 DG desalting column.
Iron content of ferritin was analyzed by protein assay and total iron assay as described above.

Protein carbonyl content. The carbonyl content of the various ferritin preparations was determined by the 2,4-dinitrophenylhydrazine method described by Levine et al. (14). Iron, if any, was removed from ferritin before analysis (10). The carbonyl content was determined by the absorbance spectrophotometrically at 375 nm using a molar absorption coefficient of 22,000 M⁻¹ cm⁻¹ (15).

Circular dichroism spectra. Circular dichroism (CD) spectra were obtained with an Aviv Circular Dichroism Spectrometer (model 62DS), using a 0.1-cm path length quartz cuvette. Spectra were averaged over five scans at a 30 nm/min, and the buffer baselines were subtracted. Protein concentration was 0.15 mg/ml. Only the far UV region from 200 to 260 nm was analyzed due to increased noise at shorter wavelengths.

RESULTS

Expression of the rH-Ft homopolymers. The yields of the rH-Ft-E and the rH-Ft-I homopolymers after
purification were approximately the same, 1.5 mg/100 ml of cell culture. However, the times required for expression of the rH-Ft-E and the rH-Ft-I homopolymers were significantly different, i.e., 8 h and 4 days, respectively. When the recombinant ferritins were analyzed for total iron and protein, the rH-Ft-E homopolymer contained 158 ± 8 atoms of iron per ferritin molecule, but the rH-Ft-I homopolymer contained no detectable iron. Both the rH-Ft-E and the rH-Ft-E homopolymers were soluble and able to be maintained under 4°C for weeks without significant change.

The purified rH-Ft-E and rH-Ft-I were seen as a single band in an SDS-PAGE with an apparent molecular mass of 21.6 kDa, corresponding to the molecular mass of the H chain of native rat liver ferritin (Fig. 19). The electrophoretic mobilities of the rH-Ft-E and rH-Ft-I homopolymers on a nondenaturing 7.5% polyacrylamide gel indicated that both the rH-Ft-E and the rH-Ft-I formed multi-subunit complexes (Fig. 20). However, the electrophoretic mobility of the rH-Ft-E homopolymer was somewhat greater than that of the rH-Ft-I homopolymer.
FIG. 19. SDS-PAGE of various ferritins. Approximately 10 μg of the purified rH-Ft-E (lane E), rH-Ft-I (lane I), and native rat liver ferritin heteropolymer (lane R) were subjected to SDS-PAGE on a 12% gel. The lane M is molecular mass standards.
FIG. 20. Nondenaturing PAGE of various ferritins. Approximately 10 μg of the purified rH-Ft-E (lane E), rH-Ft-I (lane I) homopolymers, and native rat liver heteropolymer (lane R) were subjected to PAGE on a 7.5% non-denaturing gel. Both monomers and dimers of various ferritins are shown on the gel.
The results suggested that the rH-Ft-E homopolymer might be more acidic and/or less molecular mass than the rH-Ft-I homopolymer. Upon isoelectrofocusing, a single band was observed with the purified rH-Ft-I homopolymer with an apparent pI of 5.19, whereas the rH-Ft-E homopolymer was resolved into several bands with apparent pIs between 5.12 and 5.35 (Fig. 21).

Carbonyl content of ferritin. The carbonyl contents of native rat liver ferritin, the rH-Ft-I, and the rH-Ft-E homopolymers were 4.5 ± 2.2, 3.5 ± 0.9, and 23.4 ± 2.6 nmol per nmol of protein, respectively. When the ferritins were incubated with 500 molar equivalents of Fe(NH$_4$)$_2$(SO$_4$)$_2$ in 50 mM HEPES buffer (pH 7.0) for 30 min, the carbonyl contents of native rat liver ferritin, the rH-Ft-I, and the rH-Ft-E homopolymers became 53.1 ± 4.4, 85.6 ± 7.3, and 94.0 ± 6.7 nmol per nmol of protein, respectively.

Loading iron into ferritins by ceruloplasmin. Fig. 22 shows the repeated oxidation of 110 nmol Fe(II) (chelated with 550 nmol histidine) by ceruloplasmin during incubation with 0.22 nmol of various ferritins in
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**FIG. 21.** Isoelectric focusing of various ferritins. The pH range of Ampholine (Pharmacia Biotech) were 3.5 - 10.0. The protein samples applied to the IEF were 10 μg each lane. The gel was stained with Coomassie blue. Native rat liver ferritin (lane R); the rH-Ft-I homopolymer (lane I); the rH-Ft-E homopolymer (lane E); the isoelectric focusing standards (lane M).
FIG. 22. Iron oxidation during incubation of ceruloplasmin with various ferritins. Ceruloplasmin (0.22 nmol) and apoferritin (0.22 nmol) were incubated at 37 °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. Iron oxidation was monitored spectrophotometrically at 380 nm. Data represent measurements from at least three individual experiments. Rat liver ferritin (□), rH-Ft-I homopolymer (○), and rH-Ft-E homopolymer (△).
50 mM NaCl (1 ml). The initial rate and extent of iron oxidation for native rat liver ferritin and the rH-Ft-I homopolymer were similar. When the rH-Ft-E homopolymer was used, iron oxidation was biphasic and the initial rate was approximately 2-fold greater than that for the rH-Ft-I homopolymer and native rat liver ferritin. After iron loading and washing, the rH-Ft-E homopolymer, the rH-Ft-I homopolymer, and native rat liver ferritin all contained approximately 97-98% of the added iron (by total iron assays).

Circular dichroism measurements. As shown in Fig. 23, the near UV (240-300 nm) CD spectra of the apo-proteins of the rH-Ft-E, the rH-Ft-I, and native rat liver ferritin were similar. The far UV (200-240 nm) CD spectra of the rH-Ft-E and the rH-Ft-I homopolymers showed two ellipticity minima at about 210 and 222 nm. However, the spectra suggested that the apo-protein of the rH-Ft-I homopolymer had a greater amount of α-helical structure than the rH-Ft-E homopolymer, which suggested that the rH-Ft-E homopolymer contained less ordered secondary structures than the rH-Ft-I homopolymer. The
FIG. 23. CD spectra of various ferritins. The CD spectra were obtained in 50 mM NaCl, pH 7.0. The protein concentrations were 0.15 mg/ml and a cell of 0.1-cm pathlength was used for measurements. All the ferritins were prepared as an apo-protein. Solid line is rat liver ferritin. Dashed line is the rH-Ft-I homopolymer. Dotted line is the rH-Ft-E homopolymer.
amount of \(\alpha\)-helical structure of the apo-protein of the rH-Ft-I and rH-Ft-E homopolymers appeared to be slightly below that of native rat liver ferritin.

**DISCUSSION**

The rH-Ft homopolymer expressed in the *E. coli* system already contained iron, whereas the homopolymer expressed in the insect cell system did not contain any detectable iron. The question is why the *E. coli* cells, but not the insect cells, provided iron to the expressed rH-Ft homopolymer. It could be possible that some of iron was available in the cytosol of the prokaryotic cells, but not in the insect cells. When the rH-Ft homopolymer was expressed in the *E. coli* cells, this cellular iron may have been oxidized and incorporated. However, *E. coli* cells may not have a ferroxidase-like protein for iron loading into ferritin. Such a protein has been reported in yeast (16) but not in *E. coli*. The mechanism of the oxidation and incorporation of iron into the rH-Ft-E homopolymer in this case might be similar to that using the Good type buffer (6,7). As we reported
previously (8), this kind of iron loading is non-physiological and may oxidize certain amino acid residues to generate carbonyl residues.

The results described here show that the recombinant rat liver ferritin H chain homopolymers expressed in an E. coli and an insect cell system consist of a similar molecular mass subunits and both seemed to form multi-subunit complexes. However, the rH-Ft-E homopolymer showed greater electrophoretic mobility than the rH-Ft-I homopolymer on the nondenaturing gel. The rH-Ft-E homopolymer might be modified and contain more basic surface charge comparing to the rH-Ft-I homopolymer. The reason might be that some of amino acid residues were modified due to a nonphysiological iron oxidation and incorporation into the rH-Ft-E homopolymer during expression in the E. coli cells. In this study, the isoelectrofocusing results indicated that the surface charge of the rH-Ft-E homopolymer was indeed more basic than the rH-Ft-I homopolymer. Additionally, we found that the rH-Ft-E homopolymer resolved on the isoelectrofocusing gel showed several different pIs,
whereas the rH-Ft-I homopolymer showed only one.

Compared to the unique pI value of the rH-Ft-I homopolymer, all the pIs of the rH-Ft-E homopolymer were more basic. We have reported that nonphysiological incorporation of iron into ferritin in HEPES buffer may cause the damage of histidine and lysine residue of native rat liver ferritin (17). This kind of damage would make the rH-Ft-E homopolymer more acidic, instead of basic. However, if we consider that the protein damage may occur as the iron is deposited into the core of ferritin, the net surface charge of damaged protein may possibly appear more basic simply because the more acidic amino acid residues in the protein are not exposed to the surface. We believe that may be the reason why we observed the rH-Ft-E homopolymer showing more basic surface charge than the rH-Ft-I homopolymer on the non-denaturing PAGE and isoelectrofocusing gel.

The carbonyl content of the rH-Ft-E homopolymer was 6 times higher than that of native ferritin and the rH-Ft-I homopolymer. Carbonyl formation was also observed upon loading the various ferritins with iron in HEPES
buffer. These results suggest that some amino acid residues, especially histidine (which is converted to glutamyl semialdehyde) (13), of the rH-Ft-E homopolymer were oxidatively modified during the nonphysiological iron loading. This kind of modification of rH-Ft-E amino acid residues might also cause the protein conformation changes of the homopolymer and result in changes in its surface charge.

Circular dichroism was also utilized to investigate possible structural differences between the rH-Ft-E and the rH-Ft-I homopolymers. The results suggest that both the rH-Ft-E and the rH-Ft-I homopolymers have a typical CD spectrum and contain a high degree of α-helical structure. However, the circular dichroism analysis indicated that the rH-Ft-E homopolymer contains a less ordered secondary structure, compared with the rH-Ft-I homopolymer. In other words, the secondary structure of the rH-Ft-E homopolymer might partially unfold when some of the amino acid residues were modified. Although the differences of CD spectra between the rH-Ft-E and the rH-Ft-I homopolymers were not great, a minor change might
potentially affect the property of the protein. This effect may particularly be seen upon the iron loading into the rH-Ft-E homopolymer using ceruloplasmin as a ferroxidase.

When ceruloplasmin was used to load iron into rH-Ft-E, rH-Ft-I, and native rat liver ferritin, the rH-Ft-I, but not the rH-Ft-E, homopolymer behaved similar to native rat liver ferritin. We reported previously that ceruloplasmin loading iron into ferritin requires an association between proteins (18). This association may enhance the ferroxidase activity of ceruloplasmin. Since the rH-Ft-E homopolymer was modified during expression, its protein conformation might be changed. This alteration might also affect the association of the rH-Ft-E homopolymer and ceruloplasmin and thus result in a biphasic iron oxidation by ceruloplasmin.

The results of the protein structure studies indicate that the protein shell of the ferritin H chain expressed in the E. coli system was modified, perhaps due to nonphysiological oxidation of iron in E. coli. This protein modification resulted in the ferritin H chain
homopolymer having different protein structure, surface charges, and an uncommon iron-loading pattern. The results suggest that the recombinant rat liver ferritin H chain homopolymer expressed in the E. coli system, which is quite commonly used, may be not appropriate for iron-loading studies. The results suggest that the expression of the ferritin H chain homopolymer should not be done in an E. coli system or more care must be used to avoid damage to the expressed protein. The insect cell-baculovirus expression system may be a better alternative for expression of recombinant ferritin.

REFERENCES


CHAPTER VI

MUTATIONAL ANALYSIS OF THE FOUR-α-HELIX BUNDLE

IRON-LOADING CHANNEL OF RAT LIVER FERRITIN1

ABSTRACT

We previously reported that the heavy chain of ferritin was required for loading it with iron using ceruloplasmin as a ferroxidase [J.-H. Guo, M. Abedi, and S.D. Aust (1996) Arch. Biochem. Biophys. 335, 197-204]. Site-directed mutagenesis, K58E and G61H, on recombinant rat liver L chain ferritin (rL-Ft) was performed to construct a proposed iron-loading channel in the α-helix bundle similar to rat liver H chain ferritin (rH-Ft). Conversely, the channel in rH-Ft was closed by mutations E62K and H65G to form a K62 to E107 salt bridge, which is believed to exist in the L chain. Both variants were expressed in insect cells and were soluble and able to form multi-subunit homopolymers. The rH-Ft mutant homopolymer could not be loaded, whereas the rL-Ft mutant

homopolymer could be loaded with iron by ceruloplasmin. However, we found that the initial rate of iron loading into the rL-Ft mutant homopolymer by ceruloplasmin was less than that into the rH-Ft homopolymer. When 500 atoms of iron per ferritin were used for loading, 98% was loaded into the rH-Ft homopolymer by ceruloplasmin in 15 min, but only 30% was loaded into the rL-Ft mutant homopolymer in the same time. Moreover, the ferroxidase activity of ceruloplasmin was enhanced in the presence of the rH-Ft and the rH-Ft mutant homopolymers, but not in the presence of the rL-Ft or the rL-Ft mutant homopolymers. These observations suggested that the four-α-helix bundle channel of ferritin is required for iron loading, but an additional factor, i.e., a site that stimulates the ferroxidase activity of ceruloplasmin, is also essential.

INTRODUCTION

Ferritin is an iron storage protein which plays a key role in the regulation of iron homeostasis in vivo. It sequesters iron in a nontoxic form and also serves as an iron reservoir that may supply iron for the synthesis
of iron-containing proteins (1,2). Since the reactivity of iron in an aerobic environment may produce toxic oxygen radical species (3,4), ferritin ubiquitously distributes among living species to prevent iron toxicity (5). The synthesis of ferritin is mainly regulated by iron. Higher eukaryotes have evolved a system which may quickly respond to incoming iron primarily by post-translational regulation (6-8); however, transcriptional induction also plays a role under certain conditions (9,10). The increase of ferritin synthesis ensures that cells have more storage space for iron (11).

The loading of iron into ferritin needs to be initiated with Fe(II), with oxygen as an oxidant, to deposit iron in the core of ferritin as ferric oxyhydroxides. We reported that ceruloplasmin, a copper-containing ferroxidase, can accomplish these reactions and reduce O₂ directly to water without generating any oxygen radical intermediates (12-15). We later reported that ceruloplasmin could only load iron into the ferritin H chain homopolymer, but not the ferritin L chain homopolymer (16). Furthermore, we found that an
association occurs between ceruloplasmin and ferritin during the iron-loading reaction (17). We also found that the ceruloplasmin association site is present on the ferritin H chain, but not on the ferritin L chain (18). The ratio of ceruloplasmin to the H chain of ferritin for optimal iron loading was 1 (18).

Ferritin is made of 24 subunits in various proportions of H and L chains which assemble as with 4, 3, and 2 symmetry (19). The internal cavity of ferritin with an 80-Å diameter is capable of storing up to 2,300 Fe(III) atoms as a polynuclear ferric oxyhydroxide-phosphate complex (20). Each subunit is folded into a four-α-helix bundle with a fifth short helix at the C-terminal. Several channels have been proposed for iron to enter and exit the core of ferritin. By the interaction of three subunits, eight hydrophilic channels are formed in the ferritin shell, through which iron was proposed to enter and exit (21). By the interaction of four subunits, six hydrophobic channels are formed, through which iron chelators and iron-chelator complexes were proposed to enter and exit (22). However, ferritin
H and L chains have essentially the same structural architecture. The homopolymers of both chains have similar arrangements of amino acid residues in these hydrophilic and hydrophobic channels (23). The amino acid sequence identities between ferritin H and L chains can be up to 55% (24). However, we demonstrated that only the ferritin H-chain homopolymer could be loaded with iron by ceruloplasmin, but not ferritin L-chain homopolymer (16). It seems that the iron-loading channel of ferritin is only present within the H chain.

Although the ferritin H and L chains have similar folding in the four-α-helix bundle, the ferritin H chain has a narrow 1.0-Å channel through the four-α-helix bundle, whereas this channel is blocked by the presence of a salt bridge (Lys 62 vs Glu 107) inside the four-α-helix bundle of L chain (25). Some investigators proposed that a ferroxidase center is located at the four-α-helix bundle of the ferritin H chain (26,27). However, our laboratory could not demonstrate ferroxidase activity in native ferritin (13,14), nor in the recombinant ferritin H-chain homopolymer (16). However,
the ability of ceruloplasmin to load iron into ferritin may require the iron-loading channel in the four-α-helix bundle of the ferritin H chain.

Levi et al. (28) attempted to open the iron-loading channel of the recombinant ferritin L chain but the protein was insoluble when expressed by an *Escherichia coli* system. However, we have successfully obtained a recombinant mutant L-chain homopolymer in which the salt bridge was removed, as well as a recombinant mutant H-chain homopolymer in which the salt bridge was included, using an insect cell-baculovirus expression system. Both proteins are soluble and form a multiple subunit complex. Here, we report the effects of these mutations on loading of iron into ferritin using ceruloplasmin as a ferroxidase. Additionally, we demonstrate the importance of a ceruloplasmin recognition site on the ferritin H chain which, in concert with the iron loading-channel, results in highly efficient loading of iron into ferritin.
MATERIALS AND METHODS

Materials. Rat livers and serum were purchased from Pel-Freez Biologicals (Roger, AR). The baculovirus transfer vectors, pAcUW21 and pAcUW51, linearized wildtype baculovirus BaculoGold™, and insect cells Spodoptera frugiperda (Sf-21) were purchased from PharMingen (San Diego, CA), and the insect cell medium EX-CELL 401 was purchased from JRH Biochemicals (Lenexa, KS). AmpliTaq polymerase and polymerase chain reaction (PCR) reagents were purchased from Perkin-Elmer Cetus (Foster City, CA). Other enzymes used for DNA manipulations were purchased from Stratagene's Cloning Systems (La Jolla, CA), Boehringer-Mannheim Biochemicals (Indianapolis, IN), or United States Biochemicals (Cleveland, OH) and used according to the manufacturers' instructions. Other chemicals were of reagent grade and obtained from the following suppliers: phenylmethylsulfonyl fluoride, e-amino-n-caproic acid, ferrous chloride, and ferrozine from Sigma (St. Louis, MO); potassium phosphate monobasic, potassium phosphate dibasic, and ammonium sulfate from Mallinckrodt Chemical,
Inc. (Paris, KY); sodium acetate from Fisher Scientific (Fair Lawn, NJ); and L-histidine from Eastman Kodak Co. (Rochester, NY).

Purification of native rat liver ferritin and aApo ferritin. Ferritin was isolated from rat liver tissue using the method described by Thomas et al. (29) with some minor modifications. All purification steps were carried out at 4°C. Washed rat livers (about 600 g as wet weight) were blended in 300 ml of 50 mM phosphate buffer (including 1 mM phenylmethylsulfonyl fluoride and 20 mM e-amino-n-caproic acid; pH 7.0), using a Hamilton Beach blender (Hamilton Beach Inc., Glen Allen, VA), and then homogenized, using a Potter-Elvehjem Teflon-glass homogenizer (Kontes Glass Co., Vineland, NJ). The homogenate was heated to 65°C for 15 min and centrifuged at 10,000 g for 30 min to remove denatured protein. The pellet was discarded, and 5% (v/v) of 0.2 M sodium acetate was added to the supernatant. The supernatant was then acidified to pH 4.8 using glacial acetic acid. After stirring for 1 h, the mixture was centrifuged at 10,000 g for 30 min. Solid ammonium sulfate was added
slowly to the supernatant to give 55% saturation (31% w/v). After stirring for 16 h, the precipitate was obtained by centrifugation at 6,000 g for 30 min and dissolved in 5 ml of 50 mM phosphate buffer followed by dialysis against the same buffer. Insoluble material was removed by centrifugation at 10,000 g for 30 min. The supernatant was then applied to a Bio-Gel A-1.5m Gel column (2.5 x 90 cm) equilibrated with 50 mM phosphate buffer and eluted with the same buffer. Fractions containing ferritin were pooled and concentrated with an Amicon Diaflow ultrafiltration using a PM10 membrane (Amicon, Beverly, MA). The concentrate was applied to a Bio-Gel A-0.5m Gel column (2.5 x 110 cm) equilibrated with 50 mM phosphate buffer and eluted with the same buffer. The purity of ferritin was analyzed by SDS-PAGE carried out according to Laemmli (30). Fractions containing pure ferritin were combined. Protein concentration was determined by the Bradford's method (31) with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Apoferritin was prepared by using
thioglycolic acid and ferrous iron chelator, α, α'-dipyridyl, as described previously (16).

Construction of recombinant baculovirus transfer vector with the rat liver H or L chain ferritin genes. The rat liver ferritin H chain gene was cloned into baculovirus transfer vector pAcUW21 under the control of p10 promoter as described previously (16). The rat liver ferritin L-chain gene was similarly cloned into another baculovirus transfer vector pAcUW51 in the BamHI site under the control of the polyhedrin promoter.

Site-directed mutagenesis of ferritin H- and L-chain homopolymers. Site-directed mutagenesis was performed by oligonucleotide in vitro mutagenesis as described by Kunkel et al. (32) using the Bio-Rad Muta-Gene Phagemid In Vitro Mutagenesis Version 2 kit according to the manufacturer's instructions. Single-stranded recombinant baculovirus transfer vectors (pAcUW21 containing the ferritin H-chain gene and pAcUW51 containing the ferritin L-chain gene) were used as the template for the site-directed mutagenesis. The synthetic oligonucleotides (30-mers) used for the generation of the point mutations
were 5'-TCT CAT GAA **AAG AGG GAG** GCT GAG AAA -3' (for ferritin H-chain mutant, E62K and H65G) and 5'-GCC GAG GAG CGC GAG CAC GCC GAG CGT -3' (for ferritin L-chain mutant; K58E and G61H), respectively, in which the double-underlined codons indicated the location of the mutation. The oligonucleotides for the mutagenesis of the rH-Ft and the rL-Ft genes were designed such that the restriction endonuclease recognition sites, **Nsp I** and **Nar I**, respectively, were altered (the single-underlined regions). These designs allowed screening of mutant genes by DNA restriction endonuclease mapping. *E. coli* strain XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) was transformed with the mutated recombinant baculovirus transfer vectors. Both ferritin H- and L-chain mutants were screened by restriction endonuclease analysis and then confirmed by sequencing the entire coding region of the modified DNA.

**DNA sequence determinations.** DNA sequencing for the rH-Ft and rH-Ft mutant genes cloned in baculovirus transfer vector pAcUW21 was carried out by the dideoxy chain termination method of Sanger et al. (33) using
Sequenase T7 DNA polymerase (United States Biochemical) according to the manufacturer's instructions. The rL-Ft and rL-Ft mutant genes cloned in baculovirus transfer vector pAcUW51 were sequenced by an automated DNA sequencing machine (Applied Biosystems, Foster City, CA) in the Utah State University, Biotechnology Center Service Laboratory.

**Expression and purification of recombinant ferritin homopolymers in insect cell-baculovirus system.** Sf-21 cells were grown at 27°C in EX-CELL 401 serum-free medium. Recombinant baculovirus for expression of the recombinant H-chain ferritin (rH-Ft), the rH-Ft mutant, the recombinant L-chain ferritin (rL-Ft), and the rL-Ft mutant were generated, screened, purified, and propagated using methods described in (16). Monolayers of Sf-21 cells were transfected with mixtures of the recombinant transfer vector and linearized wildtype baculovirus DNA BaculoGold™. After 3 days incubation, recombinant baculoviruses were plaque-purified from media of these transfected cells. The viruses were amplified by the infection of Sf-21 cells and high-titer virus stocks were
obtained from medium at the third day of infection and stored at 4°C. For expression of rH-Ft, rH-Ft mutant, rL-Ft, and rL-Ft mutant, suspension cultures of exponential growing Sf-21 cells (approximately $2 \times 10^6$ cells/ml) were infected with recombinant baculovirus at a multiplicity of 20. The infected cells were harvested 4 days after infection. Cell-free extracts were prepared as described previously (16). Expressed ferritin homopolymers were purified by chromatography on a DEAE-Sepharose Fast Flow and Sepharose CL-6B columns. The characteristics of rH-Ft and rL-Ft have been described by Guo et al. (16) and are analogous to native rat liver ferritin.

**Gel electrophoresis.** Purity and assembly of expressed ferritin homopolymers were monitored by SDS denaturing and nondenaturing gel electrophoresis, respectively, carried out as described previously (16). The ferritin homopolymers loaded with iron were analyzed by nondenaturing gel electrophoresis and stained with 0.1% potassium ferrocyanide in 1 N HCl for 30 min as described previously (16).
Preparation of rat ceruloplasmin. Rat ceruloplasmin was prepared as described by Ryan et al. (34). The preparation was kept in 50 mM phosphate buffer (pH 7.0) under 4°C and used for iron-loading studies within 2 weeks. Ceruloplasmin used in the iron-loading studies was desalted into Chelex-100-treated 50 mM NaCl (pH 7.0) using an Econo-Pac 10 DG column.

Loading iron into ferritin by ceruloplasmin. Loading iron into ferritin by ceruloplasmin was studied as described in (16) using a molar ratio of iron:ferritin of 500:1. Anaerobically prepared 11 mM ferrous chloride (chelated with 55 mM histidine, pH 7.0) (10 μl) was added to 0.22 nmole ferritin in 50 mM NaCl, pH 7.0, at 37°C to a final volume of 1 ml. Iron oxidation by ceruloplasmin in the presence of various ferritins was monitored by optical density at 380 nm. The reactions were terminated by adding 1 mM ferrozine and the nonoxidized Fe(II) remaining quantified at 564 nm \( (E_{564} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}) \), after which 1 mM desferrioxamine was added. The nonincorporated iron was removed by an Econo-Pac 10 DG desalting column. Protein concentration was determined
with bicinchoninic acid assay kits (Pierce, Rockford, IL) using bovine serum albumin as a standard. The amount of iron loaded in the ferritins was assayed using the method as described by Brumby and Massey (35).

**Monitoring the ferroxidase activity of ceruloplasmin.** The ferroxidase activity of ceruloplasmin was quantitated as the disappearance of Fe(II) using a discontinuous assay using the same conditions as described above for the loading of iron into ferritin. Nonoxidized Fe(II) was measured as a ferrozine complex by transferring, at 4-min intervals, 0.1 ml of the reaction solution into 0.9 ml of 1 mM ferrozine. The Fe(II) remaining was determined using $E_{564} = 27.9 \text{ mM}^{-1}\text{cm}^{-1}$.

**RESULTS**

*Site-directed mutagenesis of the rH-Ft and rL-Ft.* Recombinant baculovirus transfer vectors containing the desired mutations were obtained and screened by restriction endonuclease analysis (data not shown). Results indicated that the restriction endonuclease recognition sites *NspI* and *NarI* of the mutated rH-Ft and
rL-Ft genes, respectively, were deleted. The mutations were then verified by DNA sequencing (data not shown).

Expression and purification of rH-Ft, rH-Ft mutant, rL-Ft, and rL-Ft mutant. Analysis of insect cell extracts by the SDS-PAGE (Fig. 24) indicated that the rH-Ft mutant and the rL-Ft mutant were maximally expressed at the fourth day after infection by the recombinant baculovirus-infected cells. Wildtype rH-Ft and rL-Ft were also produced by a similar method as described previously (16). All the purified recombinant proteins consisted of a single band of protein with the same molecular masses as the corresponding subunit of native rat liver ferritin (Fig. 25). The electrophoretic mobilities of the rH-ft, rH-Ft mutant, rL-Ft, and rL-Ft mutant homopolymers on a nondenaturing 7.5% polyacrylamide gel agreed with the relative molecular masses and surface charges of native rat liver ferritin (Fig. 26). The results indicated that the rH-Ft mutant and the rL-Ft mutant formed multi-subunit complex similar to wildtype rH-Ft, rL-Ft, and native rat liver ferritin. Both rH-Ft mutant and rL-Ft mutant appeared soluble in 50
FIG. 24. Expression of mutant recombinant rat liver ferritin H and L chains. SDS-polyacrylamide gel electrophoresis analysis of lysate (10 μl) of 3 × 10⁴ Sf-21 cells 72 h (lane A and C) or 96 h (lane B and D) after infection with either recombinant baculovirus of rat liver L (lane A and B) or H (lane C and D) chain mutant. Coomassie blue-stained 12% polyacrylamide gel is shown. Lane Rat is native rat liver ferritin.
FIG. 25. SDS-polyacrylamide gel of purified recombinant rat liver ferritin H and L chains and their mutants. Purified recombinant rat liver ferritin H mutant (lane mH, 5 μg), H (lane H, 5 μg), L chain (lane L, 5 μg), L chain mutant (lane mL, 5 μg), and native rat liver ferritin (lane Rat, 5 μg) were subjected to electrophoresis on a 12% SDS-polyacrylamide gel. Coomassie blue-stained gel is shown. Relative molecular weight (kDa) of standard proteins (lane M) are given.
FIG. 26. Nondenaturing polyacrylamide gel of purified recombinant rat liver H- and L-chain ferritin homopolymers and their mutants. Approximately 10 µg of the purified recombinant rat liver L-chain mutant homopolymer (lane mL), L-chain ferritin homopolymer (lane L), H-chain ferritin homopolymer (lane H), H-chain ferritin mutant homopolymer (lane mH), and native rat liver ferritin heteropolymer (lane Rat) were electrophoresed on a 7.5% nondenaturing polyacrylamide gel. The gel was stained for protein with Coomassie blue dye.
mM phosphate buffer (pH 7.0) after purification.

However, the rL-Ft mutant slowly formed a precipitate during 1 week of storage at 4°C, whereas the rH-Ft, rL-Ft, and rH-Ft mutant did not. All the purified recombinant proteins were promptly used for iron-loading studies within 1 week after purification.

Analysis of iron loading of rH-Ft, rH-Ft mutant, rL-Ft, and rL-Ft mutant homopolymers. Neither the rH-Ft, the rL-Ft, nor their mutants were found to have detectable iron within the protein after purification. However, the native rat liver apoferritin, which was prepared using thioglycolic acid and α,α'-dipyridyl, contained 45 ± 4 atoms of iron per ferritin molecule (Table II).

The time course for iron oxidation, assayed at 380 nm, when ceruloplasmin was incubated with the various ferritins is shown in Fig. 27. Iron oxidation was observed with rH-Ft homopolymer and rL-Ft mutant homopolymer, but not with rL-Ft homopolymer. The initial rates of iron oxidation with native rat liver ferritin, rH-Ft homopolymer, and rL-Ft mutant homopolymer by
TABLE II

Incorporation of Iron into Ferritin by Ceruloplasmin

<table>
<thead>
<tr>
<th>Ferritins</th>
<th>Initial iron atoms/ferritin</th>
<th>Incorporated iron atoms/ferritin</th>
<th>Nonoxidized Fe(II) nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td>45 ± 4</td>
<td>492 ± 8</td>
<td>NDa</td>
</tr>
<tr>
<td>rH-Ft</td>
<td>NDa</td>
<td>490 ± 5</td>
<td>NDa</td>
</tr>
<tr>
<td>rH-Ft mutant</td>
<td>NDa</td>
<td>5 ± 7</td>
<td>NDa</td>
</tr>
<tr>
<td>rL-Ft</td>
<td>NDa</td>
<td>3 ± 4</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>rL-Ft mutant</td>
<td>NDa</td>
<td>151 ± 15</td>
<td>43 ± 4</td>
</tr>
</tbody>
</table>

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 under Materials and Methods.
FIG. 27. Iron oxidation during incubation of ceruloplasmin with various ferritins. Ceruloplasmin (0.22 nmol) and apoferritin (0.22 nmol) were incubated at 37°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 oxidation was monitored spectrophotometrically at 380 nm. Data are representative measurements from at least three individual experiments. Rat liver ferritin (□), rH-Ft (○), rH-Ft mutant (●), rL-Ft (▼), and rL-Ft mutant (▲).
ceruloplasmin were 66 ± 4, 69 ± 5, and 34 ± 4 atoms of iron per ferritin molecule per minute, respectively. Iron oxidation by ceruloplasmin in the presence of native rat liver ferritin and rH-Ft homopolymer reached a plateau after 15 min incubation. The reactions were then terminated by adding 1 mM ferrozine and quantified by the nonoxidized Fe(II) remaining quantitated at 564 nm. The nonincorporated iron was later removed from the reactions by passing them through a desalting column and the amount of iron incorporated into the ferritins was determined by the total iron assay. The total iron assay results showed that the native rat liver ferritin, rH-Ft homopolymer, rH-Ft mutant homopolymer, rL-Ft homopolymer, and rL-Ft mutant homopolymer were loaded with 492 ± 8, 490 ± 5, 5 ± 7, 3 ± 4, and 151 ± 15 atoms per molecule, respectively (Table I). After incubation with iron and ceruloplasmin, the wildtype rH-Ft and rL-Ft mutant homopolymers were also subjected to 7.5% non-denaturing polyacrylamide gel and stained for iron before washing (Fig. 28). The results showed that the iron had been incorporated into rH-Ft and rL-Ft mutant homopolymers by
FIG. 28. Iron staining of various ferritins on non-denaturing polyacrylamide gel. Recombinant rH-Ft homopolymer and rL-Ft mutant homopolymer before and after loading with iron by ceruloplasmin were electrophoresed on a 7.5% non-denaturing polyacrylamide gel. The details of preparation of iron-loaded rH-Ft homopolymer and rL-Ft mutant homopolymer and the iron staining gel were described under Materials and Methods and Results. The gel was stained for iron with Prussian blue dye. rH-Ft before iron loading (lane A), rH-Ft loaded with iron by ceruloplasmin (lane B), rL-Ft mutant before iron loading (lane C), rL-Ft mutant loaded with iron by ceruloplasmin (lane D), and native rat liver holoferritin (lane E).
ceruloplasmin, although the intensity of the Prussian blue stain of the rL-Ft mutant homopolymer was less than that of the rH-Ft homopolymer. In addition, the Prussian blue staining gel showed that the nonloaded iron formed a long smear in the lane of the rL-Ft mutant homopolymer, but not in the lane of the wildtype rH-Ft homopolymer (Fig. 28). No iron was observed in the rL-Ft or the rH-Ft mutant homopolymer using Prussian blue staining (data not shown).

Enhancement of the ferroxidase activity of ceruloplasmin by the rH-Ft and the rH-Ft mutant. The initial rate of oxidation of 110 nmol of Fe(II) by 0.22 nmol ceruloplasmin was 1.9 ± 0.4 nmol/min (Fig. 29). Native rat liver ferritin, the rH-Ft, or the rH-ft mutant enhanced the initial rate of Fe(II) oxidation by ceruloplasmin to 11 ± 2, 12 ± 4, 11 ±1 nmol/min, respectively. However, the Fe(II) oxidation rate was not enhanced by the rL-Ft or the rL-Ft mutant, and the initial Fe(II) oxidation rates were 2.4 ± 0.3 and 2.3 ± 0.3 nmol/min, respectively.
FIG. 29. Enhancement of the ferroxidase activity of ceruloplasmin by ferritin. Ceruloplasmin (0.22 nmol) was preincubated at 37°C in Chelex-treated 50 mM NaCl (pH 7.0) in the absence or presence of ferritins (0.22 nmol) for 1 min (1 ml final volume). The reaction was initiated by addition of histidine:Fe(II) (5:1) to a final iron concentration of 11 μM. Aliquots of the reaction (0.1 ml) were removed over time and placed in 1 mM ferrozine (pH 7.0, 0.9 ml). The ferrous iron remaining was determined using $E_{554} = 27.9 \text{mM}^{-1}\text{cm}^{-1}$. Results are expressed as nmol of iron oxidized by ceruloplasmin in the reaction. Data are given as the mean ± SE of at least three individual experiments, each performed in triplicate. Fe(II) alone (○), Fe(II) plus ceruloplasmin (●), Fe(II) plus ceruloplasmin and rH-Ft (○), Fe(II) plus ceruloplasmin and rH-Ft mutant (●), Fe(II) plus ceruloplasmin and rL-Ft (▼), Fe(II) plus ceruloplasmin and rL-Ft mutant (▼), and Fe(II) plus ceruloplasmin and rat liver ferritin (□).
DISCUSSION

We have reported that only the rH-Ft homopolymer, but not the rL-Ft homopolymer, could be loaded with iron by ceruloplasmin (16). We believe there must be a channel on the ferritin H chain for iron loading using ceruloplasmin as a ferroxidase. The α-helix bundle channel of the H-chain ferritin, which has been reported in several ferritin structures (23,25), may likely be used for this purpose. This chapter describes the expression and characterization of the rH-Ft mutant homopolymer and the rL-Ft mutant homopolymers in which the α-helix bundle channel has been closed or opened, respectively, by site-directed mutagenesis, for iron-loading studies using ceruloplasmin. Both proteins were produced in infected insect Sf-21 cells and were purified as soluble multi-subunit complexes. However, the rL-Ft mutant homopolymer showed less stability than the rH-Ft, the rH-Ft mutant, and the rL-Ft homopolymer to storage at 4°C in 50 mM phosphate buffer (pH 7.0). The instability of the rL-Ft homopolymer is somewhat in agreement with the observation of Levi et al. (28) in which the rL-Ft
mutant expressed in E. coli was completely insoluble unless the mutated protein was denatured and then renatured with other types of ferritin subunits, such as wildtype rH-Ft or rL-Ft to form heteropolymers. However, the protein seemed appropriate for studies conducted within 1 week of isolation.

Preliminary data on loading iron into ferritins by ceruloplasmin have shown here that the rH-Ft homopolymer and native rat liver ferritin loaded with iron at a similar rate. However, the rates of loading iron into the rL-Ft mutant using ceruloplasmin were approximately 50% of that for rH-Ft or rat liver ferritin. Also, only 30% of the added iron was loaded into the rL-Ft mutant homopolymer by ceruloplasmin, whereas 98% of the added iron was loaded into the rH-Ft homopolymer and native rat liver ferritin. Furthermore, no iron was found, after iron loading using ceruloplasmin, in the rL-Ft or the rH-Ft mutant homopolymer using a total iron assay and Prussian blue staining gel. These observations indicated that the \( \alpha \)-helix bundle channel is required for iron loading using ceruloplasmin as a ferroxidase. However,
the differences in the initial rates of iron loading and 
the total loaded amounts of iron between the wildtype 
rH-Ft homopolymer and the rL-Ft mutant homopolymer in 
this loading system may be related to a requirement for 
an association between ceruloplasmin and ferritin during 
the iron loading. The ferritin H chain must have an 
iron-loading channel and a ceruloplasmin association 
site. Neither the iron-loading channel nor a 
ceruloplasmin association site seems present in the 
ferritin L chain. Although an iron-loading channel was 
constructed in the ferritin L chain, the ceruloplasmin 
association site was still unavailable. Association 
between ceruloplasmin and the rL-Ft mutant homopolymer 
most likely did not occur and iron loading was not as 
effective as with rH-Ft homopolymer.

We have reported previously (16) that rat liver 
ferritin H chain enhanced the ferroxidase activity of 
ceruloplasmin, which may be due to the association of 
ceruloplasmin and the ferritin H chain. Reilly and Aust 
(17) studied the enhancement of the ferroxidase activity 
of ceruloplasmin by ferritin by observing the oxidation
of reduced ceruloplasmin copper at 610 nm. In this study, we only observed enhanced ferroxidase activity of ceruloplasmin in the presence of native rat liver ferritin, the rH-Ft homopolymer, and the rH-Ft mutant homopolymer, but not in the presence of the rL-Ft homopolymer or the rL-Ft mutant homopolymer. The introduced α-helix bundle channel of the rL-Ft mutant homopolymer did not affect the ferroxidase activity of ceruloplasmin.

In conclusion, we have been able to demonstrate that the rH-Ft mutant homopolymer and rL-Ft mutant homopolymer expressed in an insect cell-baculovirus system will form soluble and multi-subunit complexes. In addition, the α-helix bundle iron-loading channel and the ceruloplasmin association site are both required for loading iron into ferritin using ceruloplasmin as a ferroxidase. Current studies are now aimed at further investigating the ceruloplasmin association site on the ferritin H chain and the association mechanism between the ceruloplasmin and ferritin H chain.
REFERENCES


CHAPTER VII

SUMMARY

Iron often acts as a cofactor for various enzymes because of its utility as a transition metal. However, unregulated transition metals are also capable of catalyzing deleterious oxidation of many biomolecules. Ferritin is ubiquitously distributed in all iron-containing organisms for the handling of cellular iron. The essentiality of ferritin is due to its dual biological functions: to sequester nonused iron, and to provide an immediate iron source for the cell. Iron must be oxidized and taken up through an iron-loading channel in the protein shell of ferritin, and accumulated as an inorganic iron-phosphate complex in the ferritin cavity (1). The work presented in this dissertation demonstrates the roles of the ferritin H and L chains and the channel for iron loading using ceruloplasmin as a ferrooxidase.

Recombinant ferritin H and L chain homopolymers from various species have been expressed in E. coli systems and their structures and functions have been studied
extensively (2-4). Several investigators have proposed that the ferritin H, but not L, chain has ferroxidase activity for iron incorporation (3,4). We do not subscribe to this hypothesis because the ferroxidase activity of ferritin cannot be observed when the HEPES buffer is replaced with saline solution (5). In addition, iron oxidation in the HEPES buffer may generate oxygen radical species which can oxidize certain amino acid residues, such as lysine and histidine, of ferritin (6-8). It is unlikely that these nonphysiological effects occur in vivo because no similar oxidative modification of amino acid residues have been found in native, isolated ferritin (6).

Alternatively, ceruloplasmin may act as a ferroxidase to oxidize Fe(II) and incorporate iron into ferritin (5-11). Since ceruloplasmin is generally considered a serum protein, the physiological significance for ceruloplasmin to load iron into ferritin is questioned. However, ceruloplasmin and its mRNA are detectable in many kinds of tissues (12,13), and ceruloplasmin is capable of directly oxidizing four
Fe(II) to Fe(III) by transferring four electrons to an oxygen molecule to yield two molecules of water without generating any partial reduced oxygen radical species. Using ceruloplasmin to oxidize iron is safe and considered as a physiological way for iron incorporation into ferritin (6-8). Third, ceruloplasmin-loaded ferritin behaves similarly to native ferritin with respect to iron-loading capacity and iron stability. It is very likely that tissue ceruloplasmin also has the ferroxidase activity for loading iron into ferritin intracellularly. In our laboratory, we are still investigating this subject and gathering evidence to support the hypothesis and the mechanism and physiological role of ceruloplasmin for loading ferritin with iron.

In Chapter III, the roles of ferritin H and L chains in iron loading using ceruloplasmin as a ferroxidase were discussed. Recombinant proteins of the rat liver ferritin H and L chains were produced in a baculovirus-insect cell expression system. Both ferritin H and L chains formed a multi-subunit complex similar to native
ferritin isolated from rat liver. The functional differences of the ferritin H and L chain homopolymers had been shown in the iron-loading study using the HEPES buffer system (3,4), but not for the ceruloplasmin loading system. Use of ceruloplasmin as a ferroxidase indicated that iron can only be loaded into the ferritin H, but not L, chain homopolymer. The iron-loading rate and extent of the ferritin H chain homopolymer using ceruloplasmin were similar to those of native rat liver ferritin. Total iron assays and Prussian blue gel staining confirmed that only the ferritin H, but not L, chain homopolymer was loaded with iron by ceruloplasmin. The results somewhat agreed with the hypothesis from other laboratories in which the ferritin H, but not L, chain has a function for the iron loading (2). However, no ferroxidase activity was observed with native rat liver ferritin, the ferritin H, or L chain homopolymer in the absence of ceruloplasmin. Without ceruloplasmin, oxidation of Fe(II) was rather slow in saline solution. When 500 atoms of iron per ferritin was added in the presence of ceruloplasmin, 98-99% of iron was
incorporated into ferritin. The high efficiency of loading iron into ferritin by ceruloplasmin suggested that there should be an association between ceruloplasmin and ferritin because without an association ceruloplasmin-oxidized iron would quickly form a precipitate without incorporation into ferritin. In addition, when the association occurred, it might affect the conformation of ceruloplasmin and alter the enzyme activity of ceruloplasmin.

Indeed, during ceruloplasmin loading of iron into ferritin, the ferroxidase activity of ceruloplasmin was enhanced in the presence of native rat liver ferritin and the ferritin H, but not L, chain homopolymer. Enhancement of ceruloplasmin ferroxidase activity by native rat liver ferritin and the H chain ferritin homopolymer appeared highly relevant to their association. The association site for ceruloplasmin must only be present in the ferritin H, but not L, chain. Reilly and Aust (14) demonstrated that reduced ceruloplasmin had higher affinity for ferritin than oxidized ceruloplasmin. In addition, Juan et al. (15)
reported that the number of ceruloplasmin association sites on ferritin could be determined by titration of ferritin with ceruloplasmin to obtain maximal iron loading. The ratio of ceruloplasmin to the H chain of ferritin for obtaining the maximal iron loading was 1. I believe that the association between ceruloplasmin and ferritin does exist and it may be the way that ceruloplasmin loads iron into ferritin in vivo. In addition, the results also indicate that a correct physiological concentration of ceruloplasmin may be required to properly load into ferritin in vivo.

In Chapter IV, an expression method for rat liver ferritin H chain homopolymer was described. The rat liver ferritin H, but not L, chain homopolymer expressed in insect cells and E. coli cells showed a strong suppressive effect on the growth of the host. Due to the suppression, the yield of the rat liver ferritin H chain homopolymer was poor. This suppressive effect was also observed when the ferritin H chain was loaded with various amounts of iron and added to cultures of insect cells. The mechanism of the suppressive effect of the
ferritin H chain on cell growth was not clear, but could be involved in binding of ferritin H chain to cellular DNA or RNA as described by other investigators (16,17). The suppression of the expression system is therefore probably caused by the same reason. I suspected that the ferritin H chain might be an undefined regulator for cell growth in vivo (18).

Insect cell-baculovirus and E. coli T7 promoter-polymerase expression systems were both constructed for the expression of the rat liver ferritin H chain homopolymer. The insect cell system was less affected by the suppressive effect of the ferritin H chain because the baculovirus generally expresses the cloned gene at a very late stage of the virus generation cycle, which is about 48 h after recombinant baculovirus infection. This time period allows the infected insect cells to grow. When the expression of the ferritin H chain homopolymer in insect cell-baculovirus system was optimized, the production of the recombinant protein reached 1.5 mg per 100 ml of infected cell culture.
The other expression system, an *E. coli* T7 promoter-polymerase expression system, was designed for stringent regulation of expression of the cloned gene in a derived M13 vector. Little expression of the ferritin H chain homopolymer was observed in the absence of the inducer IPTG. Therefore, growth of the host was not affected by the suppressive effects of the ferritin H chain homopolymer. The yield of the recombinant ferritin H chain homopolymer expressed in the *E. coli* T7 promoter-polymerase expression system was about 1.0-1.5 mg per 100 ml of cell cultures, which was similar to that expressed in insect cell-baculovirus system. However, the time for expression the recombinant ferritin H chain homopolymer in *E. coli* system was less than 24 h, whereas the same expression in insect cell-baculovirus system required more than 4 days. It would be much easier to scale up the *E. coli* expression system.

In Chapter V, the structural and functional differences of the ferritin H chain homopolymer expressed in the insect cell-baculovirus and *E. coli* system were investigated. The ferritin H chain homopolymer expressed
in *E. coli*, but not in insect cells, already contained about 150 atoms of iron per protein after purification. The molecular masses of a single subunit of the ferritin H chain expressed in *E. coli* and insect cells were similar. Both homopolymers seemed to form a similar multi-subunit complex as native rat liver ferritin did. However, the ferritin H chain homopolymer expressed in *E. coli* showed multiple surface charges with apparent pIs between 5.12 and 5.35, whereas the ferritin H chain homopolymer expressed in insect cell showed only a unique surface charge with an apparent pI of 5.19. The results suggested that the ferritin H chain homopolymer expressed in *E. coli* might have been damaged. A carbonyl analysis of both homopolymers showed that the ferritin H chain homopolymer expressed in *E. coli* had higher carbonyl content than that expressed in insect cells, which suggested that certain amino acid residues of the ferritin H chain homopolymer expressed in *E. coli* were indeed oxidatively modified. A similar oxidative modification was also obtained when the ferritin H chain homopolymers expressed in *E. coli* and in insect cells
were loaded with iron in HEPES buffer. This oxidative modification appeared to cause the ferritin H chain homopolymer expressed in E. coli to have less ordered secondary structures than that expressed in insect cells. The iron-loading rates and patterns by ceruloplasmin for both homopolymers were also different.

Our data suggest that the eukaryotic insect cells may effectively restrict cellular iron in specific compartments, whereas the prokaryotic E. coli cells may liberate cellular iron in the cytosol. In this condition, the E. coli may not have a ceruloplasmin-like ferroxidase to incorporate the iron into ferritin. When the ferritin H chain homopolymer was expressed in E. coli cells, the cellular iron was oxidized and incorporated in a process which results in oxidation of the ferritin. I found that the medium for E. coli contained about 0.1 mM of iron. When the iron content of the medium was changed by passing through a Chelex-100 column to remove the iron or by adding 1 mM of iron into the medium, the iron content of the expressed ferritin H chain homopolymer in E. coli was also changed to about 5 atoms of iron and 320
atoms of iron, respectively, per ferritin (Guo and Aust, unpublished data). Presumably, the former ferritin H chain homopolymer would have less modification than the latter one. *E. coli* cells may not have a proper ferroxidase-like enzyme for iron loading of rat liver ferritin. The mechanism of the oxidation and incorporation of iron into the expressed ferritin H chain homopolymer in this case was similar to that using HEPES buffer. Therefore, nonphysiological oxidative modification of amino acid residues of both proteins was investigated as shown in Chapter V. The results of this study suggest not only that the *E. coli* expression system may not be proper for production of the ferritin H chain homopolymer, but also that ceruloplasmin may be a better method for the loading of iron into ferritin. Without ceruloplasmin, the loading of iron into ferritin becomes damaging and nonphysiological, which may occur the in test tube and possibly in *E. coli*.

In Chapter VI, the role of the α-helix bundle channel of ferritin for iron loading using ceruloplasmin as a ferroxidase was studied. The α-helix bundle
channel, which is present in ferritin H chain but blocked by a salt bridge (K58 to E103) in ferritin L chain, has been proposed as an iron-loading channel for ferritin (3,4). However, the recombinant ferritin used in these studies was produced in an E. coli expression system and the iron-loading study was performed in the HEPES buffer. By using site-directed mutagenesis, I successfully produced a ferritin H chain mutant (E62K and H65G), in which the α-helix bundle channel was blocked, and a ferritin L chain mutant (K58E and G61H), in which the α-helix bundle channel similar to ferritin H chain was manipulated, using an insect cell-baculovirus expression system. In addition to the nonphysiological oxidation of the expressed ferritin H chain homopolymer, the ferritin L chain mutant homopolymer expressed in E. coli system was reported to be insoluble (19). However, both the mutants of the ferritin H and L chain homopolymers expressed in insect cells were soluble and formed a multi-subunit complex. The successful expression of the ferritin H and L chain mutant homopolymers in this study suggests again that using the insect cell-baculovirus
expression system, instead of the *E. coli* expression system, may be the proper method for production of ferritin H and L chain homopolymers and their mutants.

Ceruloplasmin was capable of loading iron into the ferritin L chain mutant homopolymer, but not the ferritin H chain mutant homopolymer. However, the iron-loading rate and extent for the ferritin L chain mutant homopolymer were significantly less than for native rat liver ferritin and the ferritin H chain homopolymer. Thus it seems evident that the α-helix bundle channel is required for the iron loading using ceruloplasmin as a ferroxidase. However, the reason for which ceruloplasmin cannot rapidly load iron into the ferritin L chain mutant homopolymer may be because the ferritin L chain homopolymer does not have a site for association with ceruloplasmin, as described in Chapter III and VI, and other reports from our laboratory (14,15). Without proper association, the majority of ceruloplasmin-oxidized iron may remain in solution and form a precipitate without incorporating into the ferritin L chain mutant homopolymer.
A similar conclusion also can be obtained from a study of the enhancement of ferroxidase activity of ceruloplasmin. The ferroxidase activity of ceruloplasmin was enhanced in the presence of native rat liver ferritin, the ferritin H chain homopolymer, and its mutant, but not the ferritin L chain homopolymer, or its mutant. The results suggest that the association only occurs between ceruloplasmin and ferritin H chain homopolymer and its mutant, but not between ceruloplasmin and ferritin L chain homopolymer and its mutant. This is possible because the association site of ceruloplasmin must be on the ferritin H, but not L, chain. The presence or absence of the α-helix bundle channel in the ferritin L, or H, chain, respectively, did not affect the ferroxidase activity of ceruloplasmin. The association of ceruloplasmin and ferritin may be relevant to the physiological role of ferroxidase activity of ceruloplasmin for loading iron into ferritin.

In the future, the association sites on ceruloplasmin and, presumably, the ferritin H chain for iron loading should be investigated. Currently, Juan and
Aust (20) are investigating competition binding using ceruloplasmin and a 10-amino acid peptide of a putative association site to the H chain ferritin homopolymer. They are using this method to locate the association site of ceruloplasmin and ferritin and then will use site-directed mutagenesis to identify the amino acid sequences of the association site. A study of the ceruloplasmin and ferritin association site may be able to characterize the form of association.

Secondly, I found, in preliminary investigations, that the association of ceruloplasmin and ferritin was species specific. Apparently, this kind of association is like a "lock and key" type of interaction. A study of the association site may be a way to explore the specific "code" for the association among various species of ceruloplasmin and ferritin. Such a specific "code" for the association might be a significant evidence to support the biological role of ceruloplasmin for iron loading into ferritin. Other investigators (21) who failed to see iron loading into ferritin by ceruloplasmin
did not recognize the species specificity for the association for loading iron into ferritin.

Recently, Reilly and Aust (14) reported that reduced ceruloplasmin has greater affinity for ferritin H chain than oxidized ceruloplasmin. Reduction occurred when Fe(II) was oxidized by ceruloplasmin for incorporation into ferritin. The study provided good evidence for the correlation of ceruloplasmin and ferritin association and iron incorporation into ferritin. In the future, a study of association and dissociation of ceruloplasmin and ferritin, such as the association energy and kinetics, for iron loading might be needed to explore the biochemical and biological characteristics of ceruloplasmin for loading iron into ferritin.

Ceruloplasmin is usually considered as a serum protein. However, it has also been found in cells (22). The biological role and function of intracellular ceruloplasmin is still far from understood. A study of the enzyme activity and properties of intracellular ceruloplasmin for iron loading into ferritin would be interesting and especially important, because the
information from such a study would provide a necessary foundation for the study of iron loading in tissues.

The suppressive effect of ferritin H chain on cell growth is also worth further investigation. Ferritin H chains from rat and human are nearly homologous. They share about 95% identity of their amino acid sequence. However, I found that the suppressive effect of the rat liver ferritin H chain on the cell growth was much greater than that from human. The observation suggested that the 5% of the amino acid sequence of the ferritin H chain from rat and human might be the key residues responsible for the difference of the suppressive effect on the cell growth. Since ferritin H chain has been considered as a potential regulator for the cell growth (18), it would be interesting to investigate the 5% of the amino acid sequence difference of the ferritin H chain to determine the role of this sequence for the cell growth. It could be possible that certain growth hormones may also include a similar sequence for such a regulation.
In conclusion, this study may serve as the start of the understanding of iron loading into ferritin by using ceruloplasmin as a ferroxidase. Here, the expression and structural and functional studies of the ferritin H and L chain homopolymers and their mutants were demonstrated. I have found the suppressive effect of rat liver ferritin H chain homopolymer on cell growth and developed two expression systems, insect cells and E. coli, to overcome this suppression for the production of the ferritin homopolymers. I have discovered the nonphysiological oxidative modification of ferritin H chain homopolymer expressed in an E. coli system, and suggested an alternative expression system, insect cells, for efficient production of ferritin homopolymer. I have also obtained knowledge concerning the role of the ferritin H and L chains and the α-helix bundle iron-loading channel for iron loading using ceruloplasmin. The association of ceruloplasmin with the ferritin H chain may provide evidence for the physiological role of ceruloplasmin to load iron into ferritin. However, many questions remain unanswered with respect to the
biological role and function of tissue ceruloplasmin and its relationship to the loading of iron into ferritin.

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Dear Mr. Guo:

I am responding to your letter of June 19, 1998. You have my permission to use the following articles as part of your Ph.D. dissertation.


Sincerely,

Shu-Hui Juan
CURRICULUM VITAE

Jia-Hsin Guo
(October 1989 - May 1998)

Dissertation: Expression and Iron Loading of Recombinant Ferritin Homopolymer

Major Field: Nutrition and Food Sciences

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Publications:


Conference Proceedings:


Abstracts:


