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# The Fate of Iron Released from Heme by Hemeoxygenase-1

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# THE FATE OF IRON RELEASED FROM HEME BY HEMEOXYGENASE-1

by

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# THE FATE OF IRON RELEASED FROM HEME DEGRADATION BY HEME OXYGENASE-1

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**ABSTRACT**: A strain of *Escherichia coli* was genetically modified to co-express human heme oxygenase-1 and ferritin. The *E. coli* were then grown with varying amounts of hemin to see if the iron released upon degradation of the hemin by heme oxygenase-1 is loaded into ferritin. Following incubation, the ferritin was purified and the amount of iron loaded into ferritin determined. It was found that ferritin purifed from *E. coli* expressing human heme oxygenase-1 contained more iron than *E. coli* that did not contain human heme oxygenase-1. It was concluded that some of the iron released upon degradation of hemin by heme oxygenase-1 can be sequestered by ferritin.

## INTRODUCTION

Recent research has shown that reactive oxygen species (ROS), such as the hydroxyl radical or superoxide, can be the causation of various diseases [1]. The presence of free iron in cellular environments is one source of ROS, which produces the hydroxyl radical according to the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

The hydroxyl radical formed then reacts with surrounding substances, damaging DNA, proteins, or other molecules. Although iron can catalyze the deleterious oxidation of lipids, DNA, proteins, and other biomolecules [2], it is essential for various biochemical

processes. From oxygen carriers to electron transport chain proteins, iron plays many roles essential for life. While the Food and Drug Administration suggests that 18 milligrams of iron should be consumed daily in order to replenish the body's iron, many people (approximately 500 million around the globe) suffer from iron deficiency [3,4]. Conversely, if iron is consumed in excessive amounts, iron concentration rises to toxic levels, a condition called hemochromatosis [3,5,6]. Both conditions can prove fatal. Hence, the uptake, use, and metabolism of iron must be tightly regulated to curb its potentially deleterious biological effects.

*Iron Uptake*

Iron is one of the most abundant elements in the earth's crust. It is widely used both in organic and inorganic processes. One of the biggest problems facing cells when trying to uptake iron is the relative insolubility of ferric hydroxide and other compounds from which iron must be extracted for use. The solubility product of ferric hydroxide is $2.79 \times 10^{-39}$ [7]. The aqueous environment of the cytosol is an inhospitable host for iron extraction from insoluable iron compounds. Of the 18 mg of iron consumed daily, only about one milligram is usually absorbed [8]. This generally is enough to compensate for the small loses of iron from the body, principally in the bile. However, the rate of iron uptake is not static, but varies according to the needs of the body. Iron uptake is greatly increased in pregnant and menstruating women [8]. Ascorbic acid, or Vitamin C, increases iron uptake, as does ingestion of meat [8]. The rate is variable and usually functions, although not perfectly, to maintain the iron balance in the body.

The combination of the hydrophobic plasma membrane and the relative insolubility of iron creates a physiochemical barrier for intracellular iron uptake. In order

to overcome this barrier, iron is chelated and transferred from one organic ligand to another, thus minimizing the amount of free intracellular iron. This process differs in prokaryotic and eukaryotic cells. The uptake of iron by the human body is not fully understood [9-11], but is thought to resembles the uptake of iron by the yeast, *Saccharomyces cervisiae* [12]

*S. cervisiae* has two systems for uptake of iron: a low-affinity system and a high-affinity system [13,14]. The low-affinity system depends on cytoplasmic membrane proteins encoded by genes *FET1, FET2,* and *FET4* [11-13]. The *FET1* and *FET2* genes encode for different reductase proteins and *FET4* encodes for a protein transporter. It seems that these three proteins work together, along with Fet3 copper oxidoreductase, to transport ferrous iron across the plasma membrane, and in the process, oxidizing it to ferric iron. The high-affinity uptake system is more complicated. It involves Fet3 copper oxidoreductase and a permease encoded by *FTR 1* [14-17]. The complete mechanism is still unknown and is being actively researched. It is known, however, that a divalent cation transporter, which is coupled to the membrane proton gradient in the intestinal mucosa, is involved in iron uptake [18,19].

In order for an increased rate in iron absorption, iron is generally reduced to ferrous iron [20]. Ferrous iron is absorbed more rapidly than ferric iron [20]. Once absorbed, iron may be quickly sequestered by transferrin, an iron carrying protein, in the intestinal mucosal cells [3], thus eliminating any possible free iron. In order for the iron to bind to transferrin, the iron must be reoxidized to ferric iron and chelated into an "unreactive" complex [3]. The chelated iron is then transferred to other proteins, such as blood plasma transferrin, and transported throughout the body in a safer, less toxic form.

Although the ultimate source of iron is the diet, 9 billion red blood cells are destroyed daily, yielding 20-25 mg of iron. Most of the iron is either recycled for use in various biochemical processes or stored [8].

*Iron Storage*

Not all iron present in the body is used at the same time. A large portion of the iron is sequestered and stored for later use. A large globular protein called ferritin is the main storage protein for iron. Ferritin consists of an outer protein coat made of 24 subunits. Each subunit forms a four-helix bundle motif. In human ferritin, there are two different subunits: light chain ferritin (molecular weight = 19 kDa) and heavy chain ferritin (molecular weight = 21 kDa). The twenty-four subunits form a cubic array with an empty core [21]. Inside this core is where the iron is stored. The exact mechanism of iron loading into ferritin is still under investigation.

Two mechanisms for iron loading have been proposed. The most widely accepted hypothesis is that initially, a physiological transfer of iron from transferrin to ferritin occurs in which ferric iron is reduced to ferrous iron. The mechanism for this redox reaction is still unknown. It is thought that a diffusible cytoplasmic protein catalyzes this reaction. Once the iron has been reduced to ferrous iron and presented to the ferritin, it has been suggested the iron is reoxidized by molecular oxygen to ferric iron for deposition of iron in the core. This reoxidation is said to be catalyzed by the ferritin's intrinsic ferroxidase activity present in the centers of helical bundles of the heavy chains [22-26]. The proposed reaction for this process is:

$$2\ Fe^{2+} + O_2 + 4\ H_2O \rightarrow 2\ Fe(O)OH_{core} + H_2O_2 + 4\ H^+$$

The biggest problem with this proposed mechanism is that hydrogen peroxide is formed. As noted earlier, iron can catalyze the decomposition of hydrogen peroxide, producing the hydroxyl radical, which will react with the first organic molecule it contacts, which will most likely be the ferritin ferroxidase centers. This will lead to the eventual damage or inactivation of the ferroxidase centers and the ferritin may lose its ability to properly load and store iron. The second hypothesis for iron loading suggests that a cytoplasmic blue-copper oxidase protein, called ceruloplasmin, catalyzes the oxidation of Fe(II) to Fe(III), followed by deposition of the iron in the ferritin core. The proposed reaction for this process is:

$$4\ Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O$$

This mechanism presents iron to the ferritin in such a way as to not produce ROS [27], thus eliminating the possibility of damaging the ferritin. It has been shown that in saline solution at physiological pH, iron presented to apoferritin in the absence of ceruloplasmin was not incorporated into the core [28].

Ferritin is not the only iron storage molecule. Hemosiderin, a protein that seems to consist of ferritin partially degraded by lysosomes, also stores iron. It stores more iron than ferritin [8]. If too much iron is absorbed from the diet, deposits of hemosiderin can accumulate in the liver in toxic amounts.

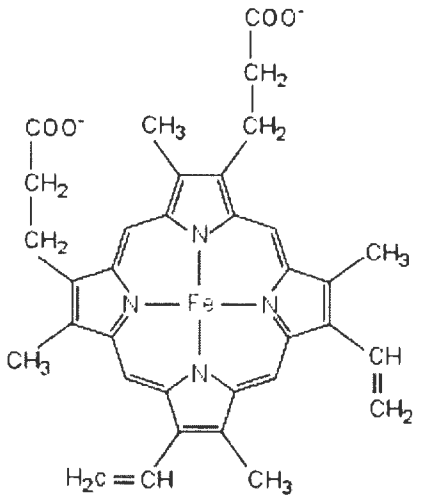


**Figure 1:** Iron Protoporphrin IX (Heme) found in *b*-type cytochromes.

*Iron Usage*

Iron is used in many different proteins throughout the body. The most well known protein is hemoglobin, the four-subunit blood oxygen carrier. Myoglobin, which only contains one subunit, is a similar protein that transports and stores oxygen in the muscle. Each of these proteins contain iron bound in a porphryin ring system. This porphryin-iron ring complex is known as heme (Figure 1). Heme is found in all organisms except anaerobic *Clostridia* and lactic acid bacteria, and can have both beneficial and deleterious effects [29]. In humans, there are many heme-containing proteins including hemoglobin and myoglobin. Hemoglobin and myoglobin are not the only heme containing proteins. Other proteins that include a heme group include, but are not limited to, nitric oxide synthase, cyclooxygenase, and various cytochromes [29].

The details of the process by which iron is incorporated into protoporphryin IX, the porphryin ring used to produce heme, are not known. It is evident, however, that the enzyme ferrochelatase catalyzes the transfer of free ferrous iron into the ring [30]. Another mechanism of iron incorporation also includes the oxidation of ferrous to ferric

iron by ceruloplasmin and be incorporated into heme by direct transfer from ferritin [31]. The study of heme production using recombinant human proteins in *E. coli*, for incorporation of iron into protoporphryin IX has not been successful. Research is currently being done to determine other enzymes that may be needed to assist in this process. It has been shown that there is a docking site between the proteins ferrochelatase and frataxin [32]. It is thought that frataxin serves as an iron chaperone to aid in the insertion of iron into heme.

Heme is not the only form of "usable" iron in the body. Iron also is used in iron-sulfur proteins. This large family of proteins includes proteins such as ferredoxins, rubredoxins, and Rieske iron-sulfur proteins [33-35]. The most simple iron-sulfur complex is the rubredoxin complex. This complex contains iron but no inorganic sulfur. Iron is covalently attached in a distorted tetrahedron to four cysteine residues. Another type of iron-sulfur complex is the 2Fe-2S complex, in which 2 iron atoms are each covalently attached to two cysteine residues and are linked together through bonds to two inorganic sulfur atoms. There are also 4Fe-4S complexes. These complexes are usually used in electron transport reactions. Rieske iron-sulfur proteins are similar in structure. The main difference between them and regular iron-sulfur complexes is that one of the iron atoms is coordinated to 2 histidine residues rather that 2 cysteine residues. This complex is important in the cytochrome $bc_1$ complex (complex III) of the mitochondrial electron transport chain [36].

When complexed into the right proteins, iron plays very crucial roles. However, the proteins wear out with use. When worn out, the proteins are degraded. In the case of hemoglobin, the protein enters a proteosome and is digested into smaller peptide chains and individual amino acids. The proteosome, however, cannot degrade the heme

compontent and releases it to the cytoplasm. Free heme is dangerous to cells because it induces oxidative stress and greatly increases cellular death [1].

*Heme Metabolism*

In order to cope with excess heme, a metabolic pathway evolved that provides for the enzymatic breakdown of heme. The central enzyme of this heme metabolism pathway is heme oxygenase-1 (HO-1) [37]. Human HO-1 is a mitochondrial-membrane bound protein that catalyzes the NADPH- and cytochrome P450 reductase-dependent oxidation and breakdown of heme into carbon monoxide, biliverdin, and ferric iron [37]. The biliverdin produced is then reduced to bilirubin by biliverdin reductase, which is conjugated for secretion [38].

Heme metabolism by heme oxygenase does eliminate heme, however it releases ferrous iron, which can participate in further deleterious oxidations. This redox-active iron must be removed from the cytoplasm in order to prevent such oxidations. Ferritin, a protein with a very high capacity for storing iron [39], could be the protein that sequesters this free iron. Recent research has shown that the up-regulation of heme oxygenase and ferritin in endothelial cells is effective in the protection of endothelium against the damaging effects of exogenous heme and other oxidants [1]. The mechanism behind this phenomenon has not been explained, however.

The hypothesis of this study is that iron released from heme degradation by HO-1 is loaded into ferritin, thus limiting the potential deleterious effects of free iron. In order to test the hypothesis, the genes encoding for human HO-1 and ferritin were simultaneously expressed in *Escherichia coli* strain B834. The strain was then exposed to varying amounts of hemin. Following incubation for 24-36 hours, the ferritin was

purified. The amount of iron loaded into ferritin was determined by a modified version of the reductive release assay determined by Thomas *et al.* [40], utilizing paraquat and bathophenanthroline. If the hypothesis for this study is correct, it should be observed that as the concentration of hemin is increased, more iron should be loaded into ferritin.

## MATERIALS

The heme oxygenase-1 gene, obtained from Dr. Paul R. Ortiz de Montellano (University of California), was inserted into pCDFDuet-1, purchased from Novagen. The plasmid containing a gene for ferritin H-chain homomer and the *E. coli* B834 cell line used for simultaneously expressing ferritin and heme oxygenase-1 were provided by Zane Davis (Utah State University).

M9 minimal medium was prepared using M9 Minimal salts and other chemicals from Sigma. Hemin and chemicals used in ferritin unloading assay were purchased from Sigma.

All water used was doubly deionized water.

## METHODS

*Bacterial Strains. E. coli* strain XL1-Blue was used for initial production of heme oxygenase-1 in pCDFDuet-1 and *E. coli* strain B843 for the dual expression of heme oxygenase-1 and ferritin.

*Construction of modified pCDFDuet-1.* The gene encoding heme oxygenase-1 was amplified by the polymerase chain reaction (PCR) from the pUC18 plasmid provided by Paul Ortiz de Montellano. The 5`-sense oligonucleotide primer (5`-CGCGGATCCA

| Polymerase Chain Reaction (PCR) | | |
|---|---|---|
| | Volume Added(µl) | Final Concentration |
| $ddH_2O$ | 37 | --- |
| dNTPs | 1 | 0.2mM of each |
| 10xBuffer | 5 | 1X |
| $MgCl_2$ | 3 | 1.5mM |
| Template | 1 | 1µg/50µl |
| Forward primer | 1 | 0.2µM |
| Reverse primer | 1 | 0.2µM |
| Taq DNA Polymerase | 1 | 5 units |

**Table 1:** PCR Conditions for Amplification of the gene encoding HO-1.

GAGCGTCCGCAAC-3`) encoded for the *Bam*HI restriction site. The 3`-antisense oligonucleotide primer (5`-CCCAAGCTTTTAAGCCTGGGAGCGG-3`) encoded the termination codon for a truncated form of HO-1 ending at proline 23 amino acids from the C-terminus immediately preceding a *Hind*III site. The sequence encoding the remaining 23 amino acids was not included because they form a signal sequence that directs human heme oxygenase-1 to the mitochondria, which is not needed in this case. PCR was performed using a *Perkins Elmer PCR amplification 9600 Thermocycler System.* The reaction mixture was according to concentrations given in Table 1. The cycle consisted of an initial melting cycle of two minutes at 95°C followed by 30 cycles of denaturation, annealing, and extension, followed by a final ten minute extension. Denaturation was performed at 95°C for one minute, annealing at 55°C for one minute, and extension at 74° C for two minutes (or ten in the last step). The PCR product was purified using the QIAquick PCR Purification kit (Qiagen) and analyzed by agarose gel electrophoresis for purity (Figure 2).

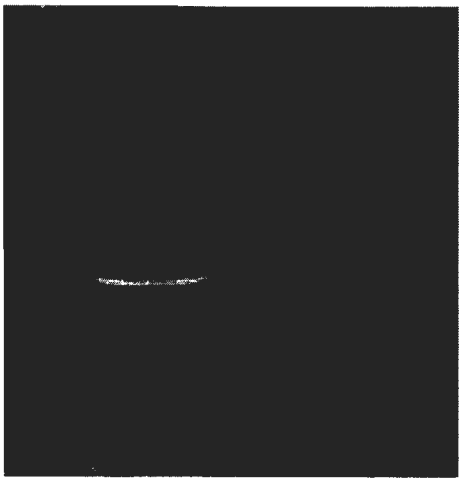

**Figure 2:** Ethidium bromide agarose gel electrophoresis of the gene *HO-1*. The gene *HO-1* was amplified using PCR and analyzed by agarose gel electrophoresis after staining by ethidium bromide. The first column is the *HO-1* gene and the second column is the Fermentas 1kb Gene Ladder.

The pCDFDuet-1 plasmid and gene insert were sequentially digested, first with *Bam*HI, followed by *Hind*III. The initial digestion contained 4 μg of DNA, 10 units* of *Bam*HI, 2 μl of BamHI Buffer (Fermentus), and water, for a final reaction volume of 20 μl. The solution was incubated at 37° C for 2 hours and product purified using QIAquick PCR Purification kit. A second digestion was performed using the total product obtained from the previous step (approx. 2 μg DNA in 30 μl of storage buffer), 10 units of *Hind*III, 4 μl of Buffer R (Fermentus), and water, for a final reaction volume of 40 μl. Analysis by agarose gel electrophoresis of pCDFDuet-1 digested with a single enzyme revealed that both *Hind*III and *Bam*HI were successful at cutting the plasmid.

Ligation of the insert into the digested pCDFDuet-1 plasmid was performed at 1:4 and 1:6 plasmid-to-insert concentrations, using 100 ng of digested pCDFDuet-1 in each ligation mixture. The 1:4 reaction contained 180 ng of digested gene insert and 1:6 contained 240 ng. A negative control contained no gene insert. Each reaction mixture

* 1 unit defined as the amount of enzyme required to completely digest 1 μg of substrate DNA in a 50 μl reaction in 60 minutes.

contained 2.5 units[†] of T4DNA ligase. Reactions were incubated at 16° C overnight (about 16 hours). T4DNA ligase was then denatured by incubating the reaction mixtures at 65° C for 10 minutes.

Ligation products were directly used for transformation. Transformation using *E. coli* strain XL1-Blue was performed as outlined by Stratagene for XL1-Blue Supercompetent Cells. Four separate transformations were performed, each containing 100 µl of XL1-Blue cells. Each was incubated on ice with 1.7 µl of β-mercaptoethanol for 10 minutes. Following incubation, 3 µl of each ligation reaction was added to three of the transformation mixtures. To the fourth was added 50 ng of undigested pCDFDuet-1 to act as a positive control. The transformation mixtures were then incubated on ice for 30 minutes, followed by a heat shock step at 42° C for 30 seconds. The mixtures were then incubated on ice for an additional 2 minutes. Following incubation, 500 µl of SOC medium heated to 42° C was added to each transformation mixture, followed by additional incubation in a rotating shaker at 37° C at 225-250 rpm for 45 minutes. Following incubation, 100 µl of each transformation volume was plated separately on agar plates containing streptomycin and incubated for 24 hours at 37° C.

Colonies were harvested from the 1:6 dilution plate and grown in 3 ml of LB medium containing streptomycin. Following incubation for 10 hours, 1 ml was used to analyze for successful ligation and transformation. Plasmid DNA was purified using Multipore Purification System and analyzed via PCR for incorporation of HO-1 gene. Further confirmation of gene incorporation was obtained by DNA sequencing. Colonies showing successful incorporation of HO-1 were then grown in 100 ml cultures and their plasmid DNA purified.

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[†] 1 unit of enzyme catalyzes the incorporation of 10 nmoles of deoxyribonucleotides into a polynucleotide in 30min at 70°C.

*Simultaneous Expression of HO-1 and ferritin in E. coli.* The plasmid containing the gene for HO-1 was transformed into a B834 strain already containing the gene encoding ferritin H-chain homomer according to the procedure given previously for transformation into XL1-Blue cells. Transformed cells were plated on agar plates containing ampicillin, streptomycin, and chloramphenicol and incubated at 37° C for 24 hours. All colonies that grew were cultured and analyzed for incorporation of both ferritin and HO-1 genes using PCR.

LB medium containing ampicillin, streptomycin, and chloramphenicol was inoculated with cells containing both ferritin and HO-1 genes. Cultures were incubated for about 8 hours at 37° C while shaking at 225 rpm. Ten milliliters were then removed and spun at 7100 rpm (6000 x *g*) in a Sorval SS-34 rotor at 4° C for 20 minutes. The supernatant was decanted and cells resuspended in 5 ml of M9 medium containing ampicillin, streptomycin, and chloramphenicol. Larger cultures of M9 medium (500 ml) were inoculated with this suspension and incubated at 37° C while shaking at 180 rpm until they reached an absorbance $A_{600}$= ~0.8. Each culture was then induced by addition of 0.119 g of isopropylthiogalactopyranoside (IPTG). Incubation was continued for six more hours. Some cells from each culture were removed, lysed, and ran on SDS-PAGE to check for production of both ferritin and HO-1.

*Degradation of Hemin.* Four cultures were grown in M9 medium as described above. Three cultures contained the B834s with both HO-1 and ferritin genes. The fourth culture only had ferritin to serve as a control. Hemin, dissolved in 1 ml of 1 M NaOH, was added to the four cultures according to Table 2. Each culture was incubated for another 24 to 36 hours at 37°C while shaking at 180 rpm.

| Addition of Hemin to Cells containing HO-1 and Ferritin | | |
|---|---|---|
| Culture | Proteins present | Hemin Final Concentration (μM) |
| 1 | HO-1 & Ferritin | 15 |
| 2 | HO-1 & Ferritin | 50 |
| 3 | HO-1 & Ferritin | 100 |
| Control | Ferritin | 15 |

**Table 2:** Addition of Hemin. Three cultures and a control were incubated with differing amounts of hemin to test for the incorporation of iron into ferritin.

*Purification of Ferritin.* Cells from each culture were harvested by centrifugation for 12 minutes at 5500 rpm (5000 x *g*) at 4° C using a GS-3 rotor. Cells were resuspended in 5 ml of Lysis Buffer (DNase at 10μg/ml, RNase at 10μg/ml, and lysozyme at 1mg/ml in 50 ml of 50mM Tris, pH 7.0). The cells were then lysed by freezing in liquid $N_2$ followed by subsequent thawing in a 42° C water bath. This freeze/thaw process was repeated three times to ensure complete lysis. The lysed cells were then centrifuged at 10,000 x *g* (9,000 rpm) for 20 minutes using a SS-34 rotor in a Sorval RC-3B centrifuge to remove unwanted cellular debris. The supernatant was decanted and recentrifuged at 43,000 x *g* (19,000 rpm) for 20 minutes using a SS-34 rotor. The supernatant was saved and pellet discarded. The supernatant was then added to a pre-equilibrated[‡] DEAE-Sepharose column. The column was sequentially washed according to the sequence in Table 3 and fractions from each wash collected. Protein-rich fractions were identified using SDS-PAGE and pooled. The pooled fractions were then concentrated to 1 ml using a YM-100 filter membrane (100 kDa molecular weight cut-off). The concentrated solution was then heat-treated at 65° C for 15 minutes in a

[‡] Pre-equilibrated with 1 bed volume of 1 N NaOH, 1 bed volume of 2 N NaCl at pH 7.0, and 6 bed volumes of 50 mM Tris at pH 7.0.

| DEAE Column Elution | |
|---|---|
| Eluate | 1 bed volume at pH 7.0 |
| 1 | 50 mM Tris |
| 2 | 50 mM NaCl/50 mM Tris |
| 3 | 100 mM NaCl/50 mM Tris |
| 4 | 150 mM NaCl/50 mM Tris |
| 5 | 200 mM NaCl/50 mM Tris |
| 6 | 2 M NaCl |

**Table 3:** Purification of Ferritin by DEAE-Sepharose anion exchange chromatography. The eluate from each step was analyzed by SDS-PAGE for the presence of ferritin. It was found that eluates 4 and 5 contained the ferritin.

water bath, followed by centrifugation at 5,000 x *g* (8,000 rpm) for 20 minutes in a table top centrifuge to remove denatured proteins. The supernatant was chromatographed over a Sepharose CL6B size exclusion column at 4° C using Chelex-100 (Sigma) treated 50 mM NaCl (pH 7.0) at a flow rate of 0.3 ml per minute. A Bradford assay was done to identify protein-rich fractions, followed by SDS-PAGE to identify fractions containing pure ferritin [41]. These were pooled and concentrated to 1 ml and another Bradford assay performed to determine protein concentration.

*Determination of Iron Content.* The amount of iron loaded into ferritin for each sample was determined using an iron unloading assay described by Thomas and Aust [40] with some modifications. This assay utilizes the iron chelator, bathophen-anthroline sulfonate ($\varepsilon$=0.002214 $\mu M^{-1}cm^{-1}$), to quantitatively determine the amount of iron that is reductively released from ferritin. The assay was conducted in a 1 ml volume. To conduct the assay, a mixture of 0.25 mM paraquat, 1.0 mM bathophenanthroline sulfonate, 5 mM glucose in 50 mM NaCl was purged with argon for 5 minutes. Ferritin was then added (concentrations ranging from 20 to 40 ng), along with 0.1 units of P450

reductase, 10 units of glucose oxidase, and 500 units of catalase. This was then allowed to sit for 5 minutes to ensure that the incubation mixture was anaerobic. The reaction was started by adding NADPH (0.3 mM) to the mixture and taking a continuous absorbance using a Shimadzu UV-2101PC UV-VIS Scanning Spectrophotometer at 535 nm. The maximum $A_{535}$ was recorded for each run and concentration of iron-bathophenanthroline determined by $A = \varepsilon l c$, where l equals 1 cm, $\varepsilon$=0.002214 $\mu M^{-1}cm^{-1}$, and c is the concentration of reacted bathophenanthroline, which is directly proportional to the amount of iron released. The amount of iron released per ferritin molecule was then determined algebraically.

**RESULTS**

*Construction of modified pCDFDuet-1 and simultaneous production of ferritin and HO-1.* Successful incorporation of HO-1 into pCDFDuet-1 was verified using PCR and gene sequencing. When incubated in LB medium and induced by addition of IPTG, the medium turned green due to the accumulation of biliverdin. This is consistent with the results of Ortiz de Montellano in which he stated that *E. coli* "have a reductase activity capable of supporting the catalytic turnover of [HO-1]. [42]"

When the modified pCDFDuet-1 was transformed into B834's already containing the gene for ferritin, one colony grew. The colony was harvested, cultured, and the presence of both genes was confirmed by PCR.

Growth of the culture in M9 medium followed by induction by IPTG addition did not lead to a change in color, as it did LB medium. However, SDS-PAGE revealed that both proteins were produced.

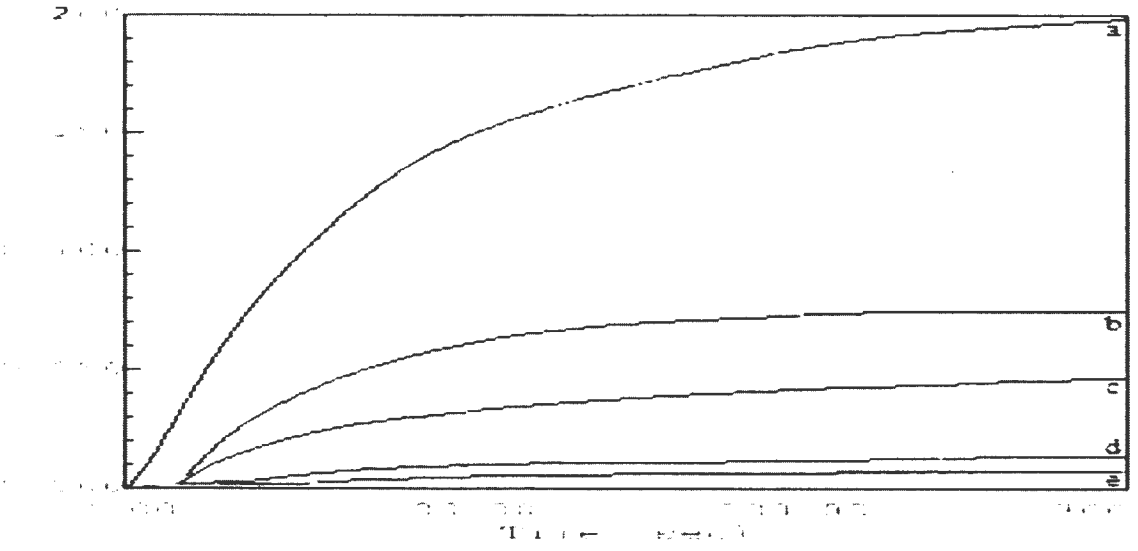


**Figure 3: Total Iron Assay for Iron in Ferritin.** Ferritin was purified from bacteria grown on differing concentrations of hemin. Iron was released from ferritin by paraquat in the presence of bathophenanthroline. Absorbance of bathophenanthroline-iron complex was measured at 535nm. The concentration of hemin in the media is as follows: b) 100 μM hemin, c) 50 μM hemin, d) 15 μM hemin, e) Negative Control (15 μM hemin, no HO-1 gene). Trace a is th release of iron from control ferritin, loaded with iron *in* vitro. The data were obtained in triplicate but the data shown is from one assay.

*Degradation of Hemin.* Hemin, a dark red brown solid, was dissolved in NaOH, and added to the samples as outlined in Table 4. Upon addition of the hemin, the cultures turned different shades of brown, the darker brown corresponding to the higher concentrations of hemin. Incubation of the cultures for 24 to 36 hours led to a slight change in color, with each culture containing HO-1 becoming more green. The control, which did not contain HO-1, showed no noticeable color changes.

*Purification of Ferritin and Determination of Iron Loaded into Ferritin.* Ferritin was purified from each sample and the protein concentrations determined by the Bradford method [41]. The iron was released reductively from ferritin by the cation free radical of paraquat and the absorbance of bathophenanthroline-iron measured at 535 nm (Figure 3). Each sample, excluding the positive control, was analyzed in triplicate. Average iron content per ferritin molecule was then calculated and results tabulated (Table 4).

| Amount of Iron in Ferritin Determined by Reductive Release of Iron from Ferritin by The Cation Free Radicals of Paraquat | | |
|---|---|---|
| Sample | Hemin conc. (μM) | Iron (atoms Fe/Ferritin) |
| -- control | 15 | 2.9 (±0.3) |
| 1 | 15 | 6.7 (±0.4) |
| 2 | 50 | 22.4(±0.4) |
| 3 | 100 | 36.3(±0.6) |
| + control | NA | 329 |

**Table 4:** Iron content in from ferritin purified fom bacterial cells containing heme oxygenase-1 and grown on media containing hemin. The average amount of iron atoms unloaded per ferritin H-chain homomer was determined using the total iron content and the protein concentration of each sample. Each sample assay was performed in triplicate, except for the + control. The + control was a sample of loaded ferritin H-chain homomer. The data are reported as the average with standard deviation.

## DISCUSSION

The results of this study indicate that iron released during the degradation of heme by heme oxygenase-1 is loaded into ferritin. As the concentration of heme increased in the medium, the amount of iron loaded into ferritin increased. This is especially evident when comparing Sample 1 with Control 1. Control 1 does not contain the human heme oxygenase-1 gene. Sample 1, which contained heme oxygenase-1, showed a 2.3-fold increase for iron loaded into ferritin. This increase suggests that the iron released following heme degradation is sequestered by the cell into ferritin in order to eliminate the potential deleterious effects of free iron. This cytoprotective mechanism explains why up-regulation of heme oxygenase-1 and ferritin has proven effective in shielding cells from deleterious effects of free heme [1]. The iron released by heme degradation is loaded into ferritin, thus limiting the potential deleterious effects of free iron.

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