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Oxidative Damage Caused by Iron Loading into Ferritin

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Abstract

Ferritin is the iron storage protein found in humans, animals, plants, fungi and bacteria. We are interested in how iron is loaded and stored in mammalian ferritin. Ferrous iron must be oxidized to ferric iron in order to be stored in ferritin. It is generally believed that ferritin does the loading itself, dependant upon a "ferroxidase activity." Oxidation of iron can result in the production of the hydroxyl radical which can cause oxidative damage to surrounding proteins and other biomolecules. An indicator of oxidative damage to proteins is the formation of carbonyl groups. Using only the H subunit of human ferritin expressed in *Escherichia coli* to make H homomers, we studied the amount of oxidative damage to ferritin based on the amount of iron available for loading into ferritin. We are interested to see if the amount of oxidative damage to ferritin increases as the amount of available iron increases. The carbonyl assay has yet to yield any results so no conclusions can be made at this time. There is another carbonyl group assay that has yet to be used.

Introduction

Ferritin is a ubiquitous intracellular iron-storage protein that is involved in the regulation of iron homeostasis. It is found in animals, plants, fungi and bacteria. Ferritin is composed of 24-subunits, which are either H (~21,000 Da) or L (~20,000 Da) subunits. The subunits combine in variable ratios with a total of 25 possible subunit ratios possible, ranging from L_0H_{24} to $L_{24}H_0$. The subunits combine to form heteromers with a core space that is approximately 80-90 Å in diameter [1,2]. This core is used to store iron in the form of a ferric-oxyhydroxyphosphate complex. In the ferritin core, up to 2,500 atoms of ferric iron can be stored [2].

Ferritins from human tissues have different ratios of H and L subunits that vary based on where they are found in the body (i.e. spleen, liver, pancreas, kidney, heart). It is still unknown exactly what roles each of the subunits are, but there is some idea. The human H ferritin subunit is required for iron loading into ferritin and it is believed that the H subunit is responsible for the "ferroxidase" activity exhibited by ferritin, oxidizing ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}). The L ferritin subunit is believed to be involved with the initiation/stabilization of the ferritin iron core and in regulating the amount of

iron incorporated into the ferritin. It is generally believed that iron is loaded into ferritin by itself, dependant upon the ferroxidase activity of the H subunit. The stoichiometry of iron oxidation by this system is two moles of iron oxidized per mole of molecular oxygen [3]. This indicates that H_2O_2 is generated as a result of this oxidation. Hydrogen peroxide in the presence of ferrous iron can result in the production of the hydroxyl radical (·OH) by reducing hydrogen peroxide further [4,5]. The hydroxyl radical is a non-specific oxidant of biomolecules that can cause oxidative damage to nearby biomolecules, including the ferritin molecule. Therefore, this form of iron loading could damage the ferritin.

Iron has been loaded into ferritin enzymatically using ceruloplasmin, a copper-containing enzyme that oxidizes four moles of iron with the reduction of molecular oxygen to water with no release of partially reduced oxygen species. Reilly et. Al. [2] has demonstrated that a specific protein-protein complex forms between ceruloplasmin and ferritin during iron loading. In addition, deficiencies in active ceruloplasmin have a profound effect on iron metabolism. This set of data suggests that ceruloplasmin, or a similar copper-containing protein, may be involved in maintaining iron homeostasis.

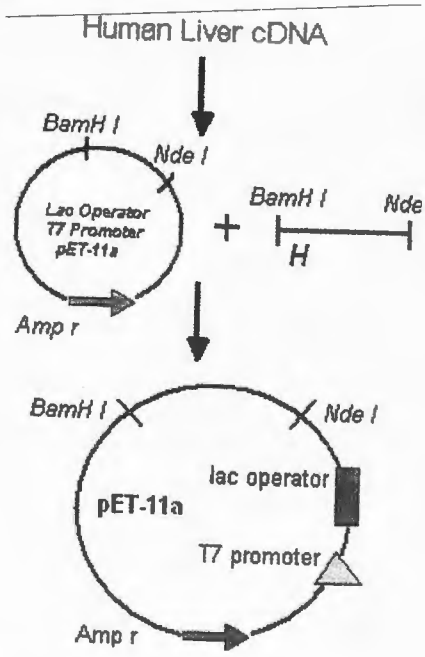
This study was conducted to determine the effect on ferritin of iron loading without a copper-containing ferroxidase. It has been found that recombinant human ferritin loaded with iron via its own ferroxidase activity did not resemble native human ferritin [2]. In this study we wished to determine if the amount of oxidative damage done to ferritin as a result of iron loading increased as the amount of available iron in the media increased. Using an H human homomer, L_0H_{24}, expressed in *E. coli* and growing it in media with increasing amounts of added iron (no added iron, 25μM and 100μM) we studied how ferritin was affected by various concentrations of iron present in the growth media.

Experimental

Expression of recombinant H human ferritin:

The H ferritin gene was amplified directly from human liver cDNA by PCR. The restriction enzyme recognition sites used were *BamH I* and *Nde I*. The final PCR volume was 100- μl containing 0.2 μM dNTP's, 2.0 μM primers, 2.5 U of *Pfu* DNA polymerase,

1.0 ng of DNA template, 2.0 mM $MgCl_2$ and 1x PCR reaction buffer. The temperature cycle were set as follows: initial denaturation at 94°C for 5 minutes, Denaturation at 94°C for 40 seconds, annealing at 48°C for 40 seconds, and elongation at 72°C for 3 minutes for 30 cycles. A final elongation step was performed at 72°C for 7 minutes.



The gene for human H ferritin was inserted downstream of the T7 lac promoter into the pET-11a expression vector. Restriction enzyme digestion and PCR analyses were used to verify the construct. The expression vector was transformed into *E. coli* B834-DE3.

Production of Ferritin in E. coli Cells:

Three separate media were made to grow the bacteria. All were M9 media, made with 970 mL of ddH_2O, 0.4 g Amino Acid Mixture (The mixture contained 0.2 grams of each amino acid per liter), 10 g M9 minimal salt from Sigma, 25 mL of 20% glucose solution, 1 mL of 1 M $MgSO_4 \cdot 7H_2O$, 1 mL 0.13 M $CaCl_2$, 1 mL 3 mM Thiamine-HCl and 1 mL of the "Trace Elements" solution for each liter of media. The "Trace Elements" solution contained the following components at the indicated concentrations. The "Trace Elements" solution was made up to have a final volume of 250 mL.

- $ZnSO_4 \cdot 7H_2O$ 8 μM
- $MnSO_4 \cdot H_2O$ 10 μM
- $CuSO_4 \cdot 5H_2O$ 2 μM
- H_3BO_4 1 μM
- $Na_2MoO_4 \cdot 2H_2O$ 1 μM

A total of nine liters of media were made, three liters for each concentration of iron. The first three liters of media had 25 μM/liter of iron and the second three liters had 100 μM/liter. The final three liters had no iron added. Antibiotics (1000x chloramphenical and 2000x ampicillin) were added to all nine liters.

Cells were added to a 50 mL starter culture of M9 minimal medium with ampicillin and chloramphenical and incubated at 37°C for 8 hours at 225 rpm. A volume

of 10 mL of the starter culture was then removed and centrifuged at max rpm in a table-top centrifuge in the cold room. The supernatant was removed and the cells were resuspended in M9 medium. Next, 5 mL of the resuspended cells solution was added to each liter of the M9 medium containing the three different concentrations of iron (0, 25 and 100uL).

Cultures were incubated at 37°C while shaking at 180 rpm until the absorbance at 600 nm was about 0.8. Protein (ferritin) synthesis was then induced by adding 0.119 grams/liter of IPTG. After one hour 0.8 grams/liter of Rifampin dissolved in methanol was added to each flask. The Rifampin was used to inhibit the bacterial DNA polymerase in the *E. coli* so that only the vector would be expressed, making a excess amount of H ferritin. The cultures were allowed to grow for 5 more hours and then centrifuged at 5000 xg at 4°C for 12 minutes.

Purification of Ferritin:

Those supernatants were discarded and the pellets (i.e. cells) resuspended in Lysis Buffer (50 mL of 50 mM Tris pH 7.0, 500 μL of 1 mg/mL DNase, 500 μL of 1 mg/mL RNase, and 0.5 grams of lysozyme). The resuspended cells were aliquoted into several 50 mL falcon centrifuge tubes and freeze/thawed three times using liquid N_2 and a 42°C water bath.

The crude lysates were centrifuged at 10,000 rpm for 20 minutes. The supernatants were centrifuged again at 19,000 rpm for 20 minutes and saved. The supernatants were then concentrated to around 15 mL using Amicon XM 300 membranes. The supernatants were applied to DEAE columns.

The DEAE columns were washed with 1 bed volume of 1 M NaOH, 1 bed volume of 2 N NaCl and 6 bed volumes of 50 mM Tris, pH 7.0. The supernatants were loaded onto the column and eluted as follows:

1) (Load)- filled to one bed volume (~250 mL) with 50 mM Tris, pH 7.0
2) (Wash)- 2 bed volumes of 50 mM Tris, pH 7.0
3) 1 bed volume of 50 mM NaCl/50 mM Tris, pH 7.0
4) 1 bed volume of 100 mM NaCl/50 mM Tris, pH 7.0
5) 1 bed volume of 150 mM NaCl/50 mM Tris, pH 7.0
6) 1 bed volume of 200 mM NaCl/50 mM Tris, pH 7.0

7) 1 bed volume of 2 M NaCl/50 mM Tris, pH 7.0

Each fraction of effluent was subjected to SDS-PAGE to identify the ferritin-rich fractions. These were pooled and concentrated to 1-5 mL using YM-100 filter membranes (100 kDa molecular weight cutoff). The concentrated fractions were heat-treated at 65°C for 15 minutes in a water bath. The solutions were then centrifuged at 8,000 rpm for 20 minutes in a table-top centrifuge to remove any denatured proteins. The supernatants were loaded onto a size-exclusion column at 4°C using a Chelex-100 (Sigma)- treated 50 mM NaCl (pH 7.0) at a flow rate of 0.3 mL/min.

The Bradford protein assay was used to identify the protein rich regions and then SDS-PAGE was used to identify the ferritin. Fractions containing ferritin were pooled and concentrated to 1-2 mL. The Bradford assay was used on these concentrates, along with protein standards, to determine the protein concentration of the isolated ferritin samples.

Protein Carbonyl Assay:

A volume of each corresponding to 1 mg of ferritin were put into small centrifuge tubes and put into a vacuum centrifuge until dry and then 500 μL of 10 mM 2,4-dinitrophenyl hydrazine in 2 M HCl was added to the dried pellets. The resuspended pellets were left at room temperature for one hour, with vortexing every 10-15 minutes. Trichloroacetic acid (500 μL of 20%) was added and centrifuged for 3 minutes at 11,000g. The supernatants were discarded and the pellets washed with 1 mL of ethanol-ethyl acetate (1:1), allowed to sit for 10 minutes and then centrifuged for 3 minutes. This was done a total of three times.

The precipitated proteins were redissolved by adding 600 μL of guanidine solution and setting the tubes in a 37°C water bath for 15 minutes. Any remaining insoluble material was removed by centrifuging for three minutes. We then obtained spectra for the samples, read against a blank of guanidine solution. The λ_{max} was 360-390 with 22,000 $M^{-1}cm^{-1}$.

Results / Discussion

All three separate *E. coli* cultures (no iron, 25 mg/mL iron, 100 mg/mL iron), grew successfully and ferritin was isolated from all three cultures. Unfortunately assays

for the protein carbonyl were not successful so the amount of oxidative damage in relationship to the iron concentration in the media could not be determined. The reason that the assay failed repeatedly has yet to be determined. There is literature that shows that not only has this assay been used successfully before, but it has been successfully used with ferritin. There is another carbonyl assay, one that uses an ELISA [6], that is available and will probably be our next step in determining the amount of oxidative damage in each sample if the assay that we are currently using continues to yield no data.

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