Chapter 5
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Purification, Characterization and Crystallization of Ferritin from *Escherichia coli*

5.1. Introduction

Iron is an essential nutrient in all-living systems. Iron exists in two oxidation states in most of the biological environments, the soluble Fe (II) and insoluble Fe (III). Iron is required for many cellular processes such as, cell growth, electron transfer, DNA synthesis, nitrogen fixation, gene regulation and binding and also in transport of oxygen. It is also involved in protein synthesis, namely hemoglobin and myoglobin. The continuous process of oxidation and reduction of iron in cells are the important mechanisms. On the other hand, the excess of iron leads to the formation of insoluble toxic free radicals such as hydroperoxyl (HOO•), and hydroxyl (HO•) radicals as can be seen from the following equation:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}• + \text{OH}^- \\
\text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{HOO}• + \text{H}^+
\]

The formation of radicals by Fenton and haber-Weiss reactions which induce the toxicity and biological damage to the cells. Major functions of iron, namely oxygen transport and electron transfer, are facilitated in mammalian systems by the following proteins, (i) iron containing non-enzymatic proteins, (ii) haem containing enzymes, (iii) iron-sulfur proteins and (iv) iron containing non-haem & non-sulfur enzymes.

In all the organisms (excluding yeast), a protein controls the mechanism where the iron is stored as non-toxic and soluble forms called “Ferritin”.

5.2. Importance, classification and functional aspects of ferritin

The ferritin proteins are found both in bacteria and animals. The bacterial superfamily of ferritin is classified into subfamilies, namely bacterial ferritin (Ftn), haem containing bacterioferritin (Bfr) and DNA binding ferritin (Dps). The Ftn ferritin is found both in prokaryotes and eukaryotes but Bfr & Dps are found only in prokaryotes. The Dps ferritin has smaller structure but lower iron storage capacity when compared with Ftn and Bfr. Mammalian ferritins are classified as two major types, H and L (heavy and light chains), the availability of both types depends on the organism. Both the chains share 55% identity. H and L chains differ in their functions, the H chain involves in the enzymatic activity by oxidizing Fe (II) to Fe (III) through catalytic site called “ferroxidase center”. Ferroxidase center is mainly composed of glutamic acid residues. L chain does not have ferroxidase center where the iron storage and mineralization of iron happen faster than the H chain. L chain is composed of carboxyl groups. Interesting to note that, L chain has higher stability than H chain even at acidic and reducing conditions.

Ferritin involves in many biochemical processes, starting from iron storage, detoxification, cell proliferation, iron regulation, apoptosis, protein translation, neural disorders and in cancer. Recently ferritin is used as a drug delivery vehicle. The *E. coli* Ftn is an example for general housekeeping role of iron storage. Bfr plays a crucial role in stress response. Dps is reported as DNA binding domain and controls the resistance of oxidative stress.

5.3. Structural architecture of ferritins

Ferritin molecules are ubiqitities in all living systems. The molecular weight of ferritin ranges from 17-21 kDa. Generally ferritin molecules (24mer/12mer) are formed
as hollow shell structure with the large inner cavity. The subunits are arranged as bundle of helices, the first four helices are connecting with short fifth helices through loops. In bacterial ferritin, the N-terminal region has about 30 residues higher than other structures. The eight three-fold channels and six four-fold channels act as a gateway of iron entry and exit processes. The iron entry channel has a tendency of metal binding nature. The inner core of the ferritin molecule is used to store the iron, metal, nano particles and drugs.

5.4. Mammalian ferritin

Mammalian ferritins consist of heteropolymeric subunits by two different compositions of H and L chains. The molecular weight of H chain is ~21 kDa whereas the L chain stands out to be ~19 kDa. The mammalian ferritin structure is formed by 24 subunits by the way of 432 symmetry. Structural organization of both the chains (H and L) is similar but differ mainly in their active site and functions. The literature survey indicates that the maximum storage of iron ions in mammalian ferritin may be due to the presence of heteropolymers (H & L).

5.5. Classical or Bacterial ferritin (Ftn)

The bacterial protein (~19 kDa) is made up of 24 identical subunit arrangements of hollow shell structure. The external diameter (12 nm) of ferritin has an inner core (8 nm). The inner core of Ftn has 80Å diameter cavity, which has the capacity to store of iron and minerals. The 4500 iron ions (Fe^{3+}) are stored within the inner core of ferritin as non-toxic form. The formation of octahedral 24mer spherical structure related to 432 fold symmetry is shown in Fig. 5.1. The four-fold and three-fold channels are responsible for iron entry and exit mechanisms.
Fig. 5.1. The crystal structure of bacterial ferritin (Ftn). The 24mer assembly of *E. coli* bacterial ferritin is shown, the single subunit is represented in enlarged view for better understanding. The di-iron binding center and its interacting residues are shown in ball and stick model.

5.6. Bacterioferritin (Bfr)

Structure of bacterioferritin is almost similar to that of bacterial ferritin, which has 24mer arrangement of identical subunits. The unique nature of haem binding mechanism is observed only in bacterioferritin. The total 12 haem groups bind in between the 24 subunits of Bfr by two fold symmetry (Fig.5.2). The methionine residues in every subunit interact with the haem group. The Bfr protein sequence resembles (Stiefel and Watt, 1979) with H chain subunits. However, some of the species (*P. aeruginosa, P. putida* and *C. Synechocystis*) are reported to have heteropolymeric chains.
Fig. 5.2. The crystal structure of bacterioferritin (Bfr). The subunit assembly and the haem binding regions (between two subunits) are shown on right side. The haem group and its interacting residues are shown in ball and stick model.

5.7. DPS ferritin

The DNA binding Proteins from Starved cells (DPS) is a subfamily of ferritin proteins. The DPS proteins are also known as mini-ferritins. The major roles of these DPS proteins are (i) to protect the cells from oxidative stress (ii) to bind with DNA and (iii) to bind and store the irons.

The DPS protein has the unique capacity to control the oxidation reaction of Fe (II) to Fe (III) through H₂O₂, however the other ferritins use O₂ for oxidation.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{OH}^-
\]

DPS protein was identified in the year 1992 (Almiron et al., 1992) and the first structure from *E. coli* was solved at a resolution of 1.6Å using crystallographic methods (Grant et al., 1998). The DPS molecules are made up of 12 subunits structural arrangement, each subunit has a dinuclear iron binding site except the DPS homolog derived from *Listeria innocua* (Ilari et al., 2005). The dodecameric identical subunits
are arranged as hollow shell-like structure with inner and outer diameter of 9 and 5 nm through 32 tetrahedral symmetry. Inner core is capable of storing 500 iron atoms in non-toxic form. In DPS, the four fold channels are not present unlike other ferritins. The following active site residues mediate the metal ion interaction of ferroxidase center of DPS protein; one histidine, two phenylalanines, two leucines and one tyrosine (Fig.5.3).

![The docemeric assembly of DPS protein is shown in cartoon representation. The metals and its interacting residues are shown in ball and stick model.](image)

**Fig. 5.3.** The docemeric assembly of DPS protein is shown in cartoon representation. The metals and its interacting residues are shown in ball and stick model.

The N-terminal region of DPS ferritin was responsible for DNA binding mechanism, where the lysine residues are responsible for protein-DNA binding (Grant *et al.*, 1998).
The major difference observed in DPS from other ferritins is in its functional mechanism of DNA binding. Till date, a number of DPS ferritins are reported from bacteria. The DPS proteins control the stress resistance by using the following parameters,

1. DNA binding nature
2. Metal binding mechanism
3. Ferroxidase activity and

Based on structural and functional aspects of ferritin, such as iron storage, detoxification, stability, capability of mineralization and storage of variety of metals, drugs and nano particles with in structural interior and drug delivery mechanism, we purified and crystallized the *E. coli* ferritin.

5.8. Purification of ferritin from *E. coli*

The cloned plasmid was transformed into *E. coli* BL21- CodonPlus (DE3)-RIL-X strain. The protein was over expressed at mid-log phase by addition of 1mM IPTG. Cells were harvested by centrifugation at 13000 rpm for 20 minutes. The pellet was dissolved in buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol and sonicated. The total lysate was incubated with DNase I and RNase A with 5 mM of CaCl₂ and 25 mM of MgCl₂ for 30 minutes and the sample undergoes heat treatment at 343 K for about 10 minutes. Afterwards, the sample was centrifuged at 15000 rpm for 10 minutes. The supernatant from centrifugation was applied to ammonium sulfate precipitation (80%). Pellet was dialyzed against buffer containing 20 mM Tris-HCl pH 8.0, 50 mM NaCl.
The protein sample was purified in hydrophobic columns (Resource ISO, Resource PHE1, GE Healthcare Biosciences) using ammonium sulfate gradient method. Finally the sample was concentrated and applied into gel filtration column (Superdex 75, GE Healthcare Biosciences). The expression and final purification results of SDS-PAGE results are shown in Fig. 5.4.

5.9. Mass spectroscopy analysis

The reports indicate that the molecular weight of ferritin varies in different species. To confirm the molecular weight, purified protein sample was characterized by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer using Voyager-DE pro (Applied Bio System). 1 mg of sinapic acid was dissolved in 25 μL of milliQ water, 25 μL of acetonitrile were added and 0.4 μL of trifluoro acidic acid was mixed with above mixture and homogenated thoroughly using the tabletop centrifuge. The protein sample mixed with pre mixture in 1:1 ratio and loaded. The MALDI-TOP spectrum obtained is shown in Fig.5.5. A strong single peak was observed at 19 kDa, suggested the purity of the protein without any contamination.

5.10. Protein sequencing

N-terminal protein sequencing was performed by Edman degradation method, using PROCISE protein sequencer for the purified protein sample. The membrane was cleaned, maintained at wet condition by adding 10 μL methanol. 100 μL of protein sample (1pmol/μL) was added to membrane and loaded. The 20 amino acids in the N-terminal region (MLKPEMIEKLNEMQNLELYS) were identified through sequencing (Fig.5.6) and the complete sequence was predicted by sequence search using BlastP online tool.
Fig. 5.4. Purification of ferritin. **A.** SDS-PAGE analysis of the ferritin expression. Total expression pattern of ferritin after sonication. The standard marker was loaded on lane 1. The supernatant and the pellet were loaded on the gel in different concentration from lane 2-10. **B.** The gel filtration elution of the purified samples was loaded. Marker loaded on lane 1, the different fractions were loaded from lane 2-16. The arrow on right side indicates the expressed protein.
Fig. 5.5. MALDI-TOF spectrum of purified ferritin.
Fig. 5.6. N-terminal sequencing of the ferritin. A. The standard peaks of 20 amino acids obtained from the spectrum are shown. B. The corresponding predicted peaks of N-terminal amino acids.
5.11. Crystallization

Several trials of experiments were carried out to crystallize the ferritin protein. Initial crystal screening experiment was performed by hanging drop vapor diffusion and batch methods using 8 mg/ml protein sample. The crystallization screening kits such as Hampton Crystal screen, Crystal screen II, wizard crystallization kit I, II were used for the initial screening. The micro crystals were obtained in some of the crystal conditions under hanging drop method, moreover the screening inspection indicates the lack of protein concentration.

The protein sample was concentrated upto 12 mg/ml using commercially available protein concentrator for further use. The buffer condition was manually optimized with respect to concentration and pH and the concentrated protein was used for crystallization. The crystallization setup by hanging drop vapor diffusion method using Hampton Natrix kit I and II for 12 mg/ml concentrated protein yielded the crystals. Finally better quality crystals were obtained in buffer containing 10 mM MgCl₂ hexahydrate, 0.05 M MES pH 5.6, 2 M Lithium sulfate condition within 5 days at 20°C. The crystals were reproducible within a week in better shape. The crystal image is shown in Fig. 5.7.

![Fig. 5.7. The crystal image of ferritin protein](image)