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COMMUNICATION

Ferritin Light-Chain Subunits: Key Elements for the Electron Transfer Across the Protein Cage

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The first specific functionality of the light-chain (L-chain) subunit of the universal iron storage protein ferritin was identified. The electrons released during iron-oxidation were transported across the ferritin cage specifically through the L-chains and the inverted electron transport through the L-chains also accelerated the demineralization of ferritin.

The spherical hollow structure of apoferritin is a convenient biotemplate in nanotechnology.¹ As a ubiquitous protein for iron biomineralization, ferritin is omnipresent throughout microorganisms, plants and animals.² In mammals, two distinct subunits of ferritin have emerged as a result of evolution. The heavy-chain subunit (H-chain) conserves the common di-iron binding site and catalyzes the oxidation of Fe²⁺ to Fe³⁺, being a ferroxidase. Although similar in sequence and size, the light-chain subunit (L-chain) lacks ferroxidase activity as well as further enzymatic activities. In vivo, the cytosolic ferritin is a heteropolymer consisting of 24 protein subunits with a tissue-specific ratio of H:L-chains. The presence of L-chains in the assembled molecule is known to improve both the stability and the iron mineralization ability in general.^{3, 4} An increased L-chain fraction lowers the crystallization tendency of the mineral inside the cavity.⁵ However, the detailed mechanisms of involvement of the L-chains in the mineralization process have not yet been understood.⁶ Although its catalytic activity is not given, the L-chain seems to have important and specific biological roles, since all ferritin gene disorders identified so far involve the L-chain, but not the H-chain.^{3, 4}

Here, we report on a specific function of the L-chain protein, discovered upon investigation of the catalytic activity of homopolymeric L- and H-chain apoferritins. Utilizing oxidized cytochrome c (Cyt-c) as electron acceptor under anaerobic conditions, neither homopolymeric human L- nor H-chain apoferritin (apo-huFL and apo-huFH) alone could enable a complete redox reaction with an iron oxidation occurring in the inner cavity and a Cyt-c reduction occurring at the outer

surface. The ability of natural and reassembled heteropolymeric apoferritin to catalyze the reaction demonstrated the distinct function of two subunits. More detailed investigations of the mechanisms reveal that the H-chain serves only as the electron source by catalyzing the iron oxidation, while the presence of the L-chain is important and specific for the electron transfer across the protein shell of ferritin for the reduction of Cyt-c outside the cavity.

Initially, we evaluated the intrinsic ferroxidase activity of the recombinant apo-huFL or apo-huFH. The apoferritin catalyzed iron oxidation has a characteristic broad absorption peak with a maximum at 246 nm, which enables following the progress of the reaction and thus the ferroxidase activity by UV-vis spectroscopy.^{7, 8} Since the recombinant apo-huFH has ferroxidase active sites on each subunit, the apo-huFH expectedly showed a high ferroxidase activity (Fig. 1a). No ferroxidase activity was observed from apo-huFL, which is also expected, as the L-chains do not contain ferroxidase active sites. For comparison, we measured also the iron-oxidation activity of purified natural horse spleen apoferritin (apo-hoSF). Apo-hoSF is a heteropolymeric and L-chain rich apoferritin with 5-10% H-chain content. With only 1 or 2 ferroxidase-active H-subunits per apoferritin cage, apo-hoSF showed a moderate ferroxidase activity. It oxidized iron gradually with much slower velocity than the pure H-chain apoferritin as shown in Fig. 1a. In aerobic conditions, the oxygen molecule acts as electron acceptor during iron oxidation and is being reduced to water. Oxygen can easily reach the inner cavity and become reduced on-site while Fe²⁺ is oxidized. Besides oxygen, many acceptor molecules may not be able or in need to enter the cavity of apoferritin. For example, under anaerobic conditions, Cyt-c can be reduced in the presence of heteropolymeric apo-hoSF while Fe²⁺ is oxidized.⁹

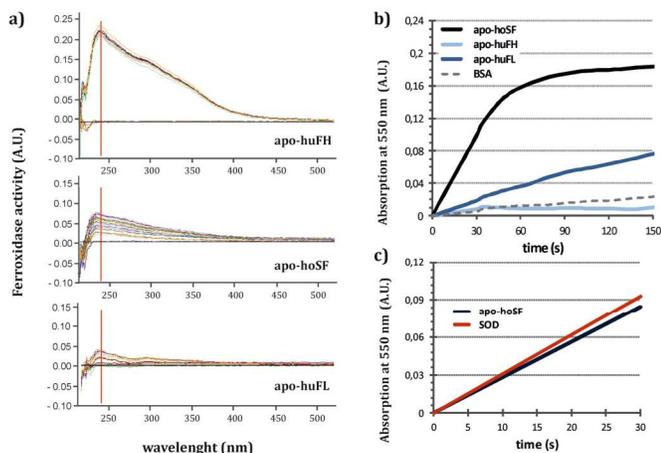


Figure 1. a) Ferroxidase activity characterized by UV-Vis spectroscopy. The reaction was carried out during 5 min. with 0.15 mg/ml protein (apo-hoSF: horse spleen apoferritin; apo-huFH: human H-chain apoferritin; apo-huFL: human L-chain apoferritin). b) Formation of reduced Cyt-c measured through changes in absorption at 550 nm over time. The reaction was carried out with 0.30 mg/ml protein, 10 μ M oxidized Cyt-c and 50 μ M Fe^{2+} . c) Cyt-c reduction with apo-hoSF with or without addition of 10 μ g/ml superoxide dismutase (SOD).

Cyt-c is a small heme protein and an important participant in the electron transport chain in mitochondria. With a molecular weight of around 12 kDa it is too large to pass through the channels of apoferritin and in this way enter the cavity. Since both oxidized and reduced Cyt-c show characteristic UV-vis absorption peaks, the absorption at 550 nm is suitable to characterize the reduction reaction and was thus used in this study (Fig. S1 and S2).¹⁰ In agreement with previously published data, apo-hoSF catalyzed the redox reaction of Cyt-c with Fe^{2+} under anaerobic conditions (Fig. 1b). The development of the UV-vis spectra between 250-300 nm indicates the iron oxidation during the Cyt-c reduction (Fig. S1). As expected, the reduction of Cyt-c was much slower with the homopolymeric ferroxidase-inactive apo-huFL. Surprisingly, however, the strongly ferroxidase-active apo-huFH did not show the ability to reduce Cyt-c either (Fig. 1b). Its activity was similar to that of bovine serum albumin (BSA) protein that served as the negative control. We considered the possibility that binding of Cyt c may be a potential reason for the different activities of the L- and H-subunits. However, no specific protein-protein interactions of Cyt c with either H- or L-subunits were detected upon application of 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC) or formaldehyde as protein crosslinkers (Fig. S3).

In order to confirm that the different activities of the three types of apoferritin are due to the distinct properties of the two involved types of subunits, it was necessary to rule out the possibility that the observed Cyt-c reduction was caused by superoxide, which may be produced during the apoferritin-catalyzed iron oxidation.^{11, 12} Upon use of Dojindo's tetrazolium salt as indicator,¹³ no superoxide production could be detected during the anaerobic iron oxidation under our experimental conditions. This was further confirmed by

following the kinetics of the apo-hoSF-catalyzed Cyt-c reduction with addition of superoxide dismutase (SOD) that can efficiently inhibit the superoxide-induced reduction of Cyt-c.¹¹ The reduction of Cyt-c by apo-hoSF was not inhibited in the presence of SOD (Fig. 1c). Therefore, the superoxide was not involved in the Cyt-c reduction in our experiments and the L- and H-chains obviously play distinct roles in the electron transfer between iron and Cyt-c.

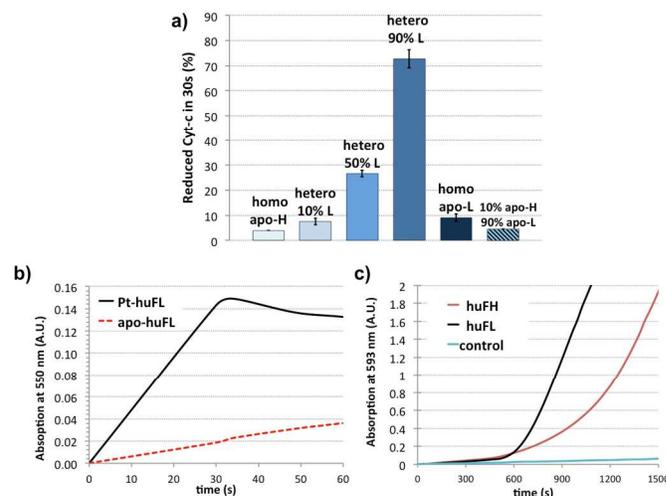


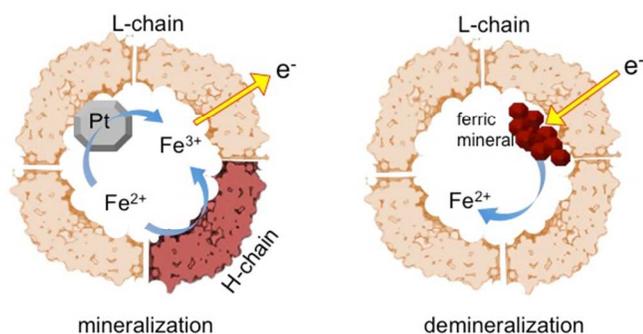
Figure 2. a) Percentage of reduced cytochrome c (Cyt-c) after 30 s of reaction with 0.15 mg/ml protein (H- or L-chain apoferritin (apo-H or apo-L) and heteropolymeric apoferritin with the indicated L-chain content). 10 μ M oxidized Cyt-c was added to the reaction solution. b) Comparison of the Cyt-c reduction by human L-chain apoferritin (apo-huFL) with or without Pt nanoparticles in its cavity (Pt-huFL). c) Comparison of the demineralization of human L- and H-ferritin (huFL and huFH) with the identical iron mineral quantity. The reaction without the addition of FMN serves as control (control).

The stronger activity of the heteropolymeric apo-hoSF in the Cyt-c reduction compared to that of the homopolymeric apoferritin implies that only the combination of the two types of protein subunits makes the assembled apoferritin seriously active. The involved redox reaction can be simplistically seen as: 1) the oxidation of Fe^{2+} to Fe^{3+} ; 2) the transport of the electrons from iron across the protein shell and reduction of Cyt-c. The inability of apo-huFH to reduce Cyt-c indicated the ferroxidase activity of the H-chain can carry out the first step only, thus it can be assumed that the L-chains in the heteropolymer are responsible for the electron transfer.

In order to verify the assumption, homopolymeric apo-huFL and apo-huFH were disassembled and again reassembled in distinct proportions following the previously described method.⁶ In this way a variety of heteropolymeric apoferritins containing 10, 50 and 90% L-chains on the average was obtained. Fig. 2a shows the measured Cyt-c reduction activities of these new heteropolymeric apoferritins given as the percentage of reduced Cyt-c after 30 s reaction time. Upon increased percentage of L-chains in the apoferritin molecules, the activity increased considerably. The heteropolymer with 50% L-chains had a sixfold activity compared to the H-chain

homopolymer. A 18-fold increase was achieved once the apoferritin contained 90% L-chains, which roughly corresponds to the mixture in the natural heteropolymeric apo-hoSF. Note that the activity enhancement could only be achieved with variation of the L:H proportion within the heteropolymer. A simple coexistence of H- and L- homopolymers in 1:9 ratio in the solution did not yield any activity increase (Fig. 2a). Thus, for beneficial interactions of the L- and H-chains one molecule of apoferritin needs to contain L-chains to transfer the electrons evolving during the iron oxidation.

The question occurs whether or not the interactions between H- and L-chains are necessary for the electron transport. To confirm that the L-chains are the only responsible entity for the electron transfer, it was necessary to find a substitute for the H-chains as the electron source. Previous work of our group showed that Pt nanoparticles synthesized within apoferritin can act as active sites for the Fe^{2+} oxidation.¹⁴ Once synthesized within L-chain apoferritin, such artificial ferroxidases make the need for H-chains obsolete. We evaluated the activity of such L-chain apoferritins with Pt nanoparticles embedded in their cavity (Pt-huFL) for the reduction of Cyt-c. Indeed, the Pt-huFL was catalytically active (Fig. 2b). The result demonstrates that the H-chain proteins are not required for the electron transfer and the electron transfer is a specific property of the L-chains.



Scheme 1. Schematic presentation of the electron transfer through the L-chain subunits during the iron-mineralization and demineralization of ferritin.

The Cyt-c reduction provides the evidence of outward electron transport from the interior iron oxidation site and facilitates the iron mineralization in ferritin (Fig. S4). In natural circumstances this may be important for the newly synthesized apoferritin to mineralize and store excess iron. Even upon iron oxidation under aerobic conditions, the possibility for the electrons to be transferred across the protein shell may speed up the reaction while circumventing the rate determining step, the diffusion of reactants such as O_2 into the ferritin cavity.¹⁵ But for the mineral containing ferritin the reverse electron transport is necessary in order to reduce the interior Fe^{3+} and demineralize iron where needed. In order to verify whether or not the electron transport through the L-chain is bi-directional, NADH/FMN was used as the electron donor for the demineralization of ferritin.¹⁶ We compared the

demineralization of the human homopolymeric L- and H-chain ferritin with a similar iron mineral content. As shown in Fig. 2c, the demineralization of L-chain ferritin took place much earlier and with higher velocity than that of the H-chain ferritin. Since NADH/FMN molecules are too large to penetrate the ferritin shell, the reduction of the ferritin mineral through the direct contact with the reducing agent was avoided. The differences of the reaction velocity reflect the serious differences between the L- and H-chains in terms of electron transport ability and consequently for the demineralization of ferritin.

Our experiments clearly evidence that the electron transport is a specific function of the L-chain subunit. This represents the first identified function of this type of protein, which is obviously a result of evolutionary optimization of functionalities. The presented results seriously contribute to the understanding of the biological functions of L-chain in ferritin. Two protein subunits with distinct and complementary functionality provide the organisms flexibility for organ-specific adjustments of ferritin to meet the varying requirements and supply of iron. As shown in aforementioned results, the iron mineralization capacity of ferritin can be adjusted simply by changing the ratio of the two subunits in the molecule. This may be one important reason for the existence of the ferroxidase-inactive L-chains in mammals, besides the ferroxidase-active H-chain. In this light, the previously described observation of a few L-chains being enough for altering the capacity of the ferritin for iron incorporation becomes meaningful.^{3,4}

The electron transport across the protein shell as a specific functionality of the L-chain is another piece of the puzzle in understanding the biological and medical significance of the ferritin subunits. The biological role of this protein seems to be important, since a decreased L-chain level or L/H-ratio has been observed in Parkinson's and Alzheimer diseases.¹⁷ However, further and more detailed investigations of the transfer mechanism through the L-chain will be important from the biochemical and biotechnical perspectives.¹⁸

As ferritin has demonstrated application potential in various forms in many biomedical or nanotechnological branches, the electron transfer activity of the L-chains identified here may enable further optimization and adaptation of the various applications through the use of further electron acceptors and donors outside of the protein shell, which will accelerate the reactions and increase the overall speed of iron (or other incorporated minerals) turnover within ferritin (Scheme 1). Furthermore, the tailoring of the functionality of ferritin with the distinct chemical properties of L- and H-chains is highly interesting for further functionalization of nanomaterials in apoferritin-templated synthesis and engineering.^{2, 14, 19}

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