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Energetics of surface confined ferritin during iron loading

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ABSTRACT

We report on the first quantitative picture on how iron loading inside ferritin molecules occurs when they are self-assembled onto solid surfaces. Recombinant human ferritin H-chain with ferroxidase activity was adsorbed onto microcantilever beams to form a stable close-packed thin film. The obtained nanomechanical system was used to track in real time the energetics of inter-ferritin surface interactions during incubation with Fe(II) for iron loading. We observed that iron loading is accompanied by increasing attractive in-plane inter-ferritin interactions able to perform a maximum surface work of $6.0 \pm 1.5\ mJ/m^2$, corresponding to a surface energy variation per ferritin of about $40\ k_bT$. Unique to this protein surface transformation, part of the surface work is exerted by the attractive electrostatic forces arising among the new born nanosized iron cores inside the ferritin shells. The remaining work comes from subtle action of steric, bridging and depletion forces. These findings are of fundamental interest and add important information for the rational development of ferritin nanotechnology.

1. Introduction

Application of nanoscience concepts to biological systems has brought increasing attention on ferritins and other biomacromolecules [1]. Ferritins are iron storage proteins that detoxify intracellular free iron and peroxides – reagents that produce toxic free radicals through the Fenton reaction – protecting the cell from oxidative damage [2]. Mammalian ferritins are made of 24 subunits that self-assemble in a 12 nm shell which encloses an aqueous cavity of 8 nm diameter able to accommodate up to about 4500 iron atoms in a mineral form similar to ferrihydrite $[Fe^{III}{}_{10}O_{14}(OH)_2]$ [3] (Fig. 1A). Ferritin catalyzes iron oxidation and its mineralization inside the cavity with a mechanism that has been partly elucidated. The protein shell is pierced by hydrophilic channels along the 3-fold symmetric axes that are permeable to iron. Human ferritin is composed of two subunit types: the H chains (21 kDa), which harbors a di-iron site within the 4-helical bundle that catalyzes iron oxidation, thus named ferroxidase center, and the L chains (19 kDa), which lacks ferroxidase activity. The Fe(III) produced at the catalytic site moves into the cavity where it undergoes hydrolysis and mineralization [4]. The shell structure, self-assembly, high stability and biological properties make ferritins nano-shells with many potential applications in nanotechnology and nanomedicine [1]. For example, they have been used as templates for the synthesis of metallic nanoparticles [5–7], cargos for drug delivery [8,9], and MRI contrast agents [10]. Conjugation of ferritins to inorganic surfaces further widens its potential applications. These novel functional surfaces and nanomaterials [11–13], still require adequate understanding and control of ferritin surface properties, interactions and iron loading function.

Nanomechanical sensors are suited to measure forces, displacements, mass changes and other molecular nanomachinery occurring with biomolecule surface transformations. Such devices can either be dynamic-mode (*viz.* mass) sensors and/or surface-stress (*viz.* static) sensors [14]. We took advantage of the latter ones, which exploit the fact that biomolecular transformations confined on the sensor surface can cumulate and perform an overall surface work in the order of mJ/m^2 [15], that can be probed by tensiometric techniques such as contact angle [16] and microcantilever (MC) beams [17]. In this study, a ferritin-MC assay using recombinant human ferritin H chain (FTH) and a mutant with inactivated ferroxidase activity (Mutant) was designed and prepared following the rationale illustrated in Fig. 1B. A thin film of FTH with ferroxidase activity (green circles) is deposited onto a MC, which responds

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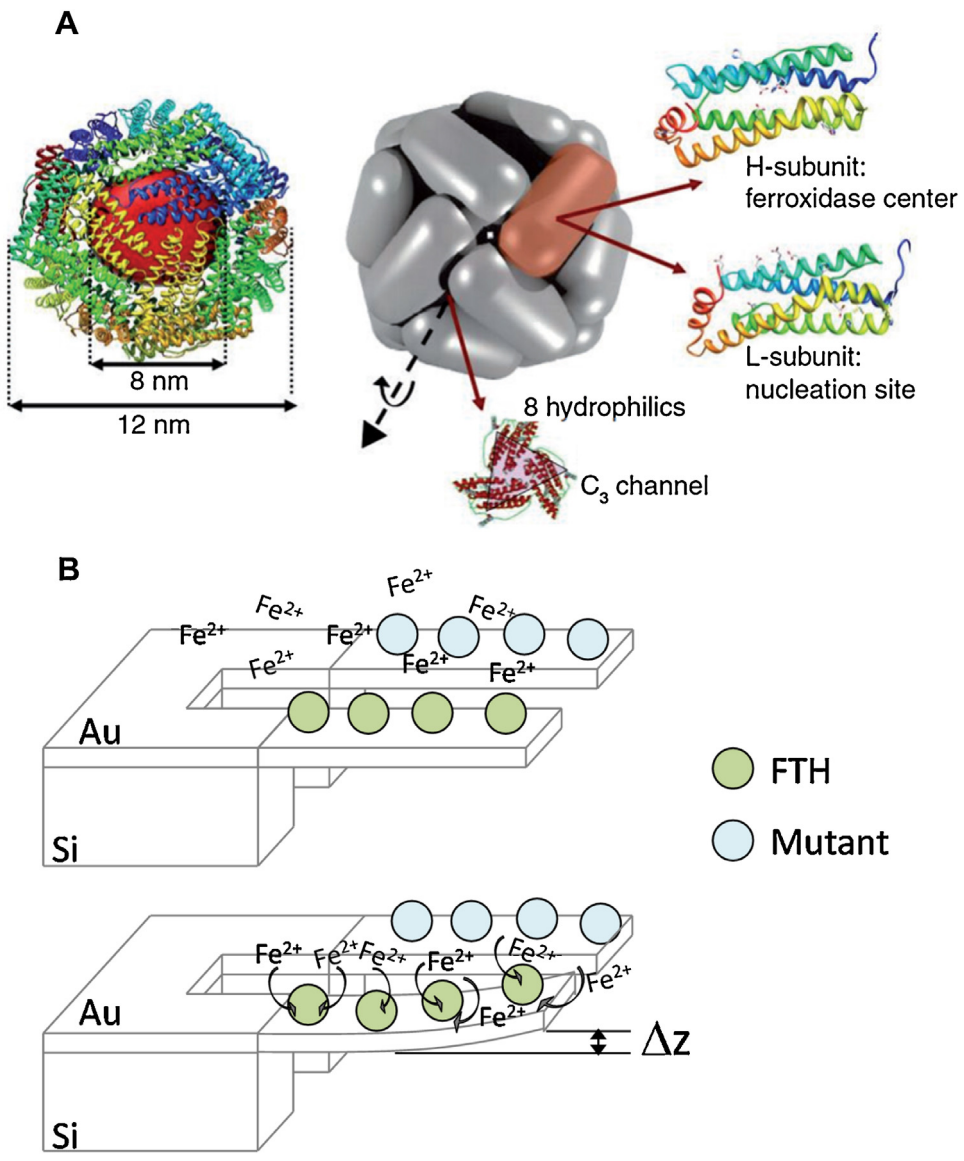

Fig. 1. **(A)** Ferritin is a protein made of 24 subunits that form an almost spherical shell 12 nm across with a cavity of 8 nm. The process of ferritin iron uptake involves Fe(II) entering the cavity through hydrophilic channels and its oxidation at the ferroxidase site followed by a slow hydrolysis and mineralization to form a ferrihydrite core. Adapted from Ref. [9]. (B) Scheme of the ferritin-MC architecture. The surface pressure released after incubation with Fe(II) for iron loading in active ferritin (FTH, green circles) drives MC bending Δz. To rule out "unspecific" contributions to deflection due to Fe(II) and other species adsorption, Δz is referred to a reference MC prepared with mutant ferritin (Mutant, light blue circles) unable to oxidize iron. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by bending to the surface energy variation triggered by iron loading. In other words, the FTH film acts as a molecular switch which drives MC motion fuelled by the Fe(II)/Fe(III) redox reaction [18]. A MC prepared with the Mutant that is not able to take up iron in the experimental conditions (light blue circles) is used as control.

2. Materials and methods

2.1. Chemicals

All the used chemicals, if not otherwise indicated, were obtained from Sigma–Aldrich, Germany.

2.2. Ferritins cloning, expression and purification

The cloning, expression and purification of the human ferritin H chain (FTH) and of the Mutant have been described in Ref. [19]. The Mutant D62K + H65 G alters two non-exposed residues involved in the ferroxidase center and inactivates them [20]. The ferroxidase activity is necessary for ferritin iron incorporation at pH 6.5 and below, but not at pH 7.0 and above, when iron oxidizes spontaneously in aerobic conditions [21]. Ferritins were >98% pure and were used as isolated, FTH contained about 200 Fe atoms per mol, and the mutant less than 20 [19].

Atomic Force experiments were conducted on Si chips both bare and with one face coated with an Au thin film. For ferritin immobilization, both kind of chips were thoroughly cleaned for adsorbed impurities with acetone (30 min) and the deposition face treated by ozone-UV cleaning (30 min at 50 °C). The cleaned chips were than incubated for functionalization in a FTH (or Mutant) solution (1 mg ml^{-1} in TRIS 20 mM buffer at pH 7.4) for 4 h. After incubation the chips were washed with milliQ water and NaOH 10 mM for few seconds and finally gently dried under N_2 flow.

AFM images were performed in air in non-contact mode by using a Jeol (Tokyo, Japan) scanning probe microscope equipped with a conventional piezo-scanner (maximum xy-movement = 28 μm,