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The sedimentation properties of ferritins. New insights and analysis of methods of nanoparticle preparation

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ABSTRACT

Background: Ferritin exhibits complex behavior in the ultracentrifuge due to variability in iron core size among molecules. A comprehensive study was undertaken to develop procedures for obtaining more uniform cores and assessing their homogeneity.
Methods: Analytical ultracentrifugation was used to measure the mineral core size distributions obtained by adding iron under high- and low-flux conditions to horse spleen (apoHoSF) and human H-chain (apoHuHF) apoferritins.
Results: More uniform core sizes are obtained with the homopolymer human H-chain ferritin than with the heteropolymer horse spleen HoSF protein in which subpopulations of HoSF molecules with varying iron content are observed. A binomial probability distribution of H- and L-subunits among protein shells qualitatively accounts for the observed subpopulations. The addition of Fe^{2+} to apoHuHF produces iron core particle size diameters from 3.8 ± 0.3 to 6.2 ± 0.3 nm. Diameters from 3.4 ± 0.6 to 6.5 ± 0.6 nm are obtained with natural HoSF after sucrose gradient fractionation. The change in the sedimentation coefficient as iron accumulates in ferritin suggests that the protein shell contracts ~10% to a more compact structure, a finding consistent with published electron micrographs. The physicochemical parameters for apoHoSF (15%/85% H/L subunits) are $M = 484{,}120$ g/mol, $\overline{\nu} = 0.735$ mL/g, $s_{20,w} = 17.0$ S and $D_{20,w} = 3.21 \times 10^{-7}$ cm^2/s; and for apoHuHF $M = 506{,}266$ g/mol, $\overline{\nu} = 0.724$ mL/g, $s_{20,w} = 18.3$ S and $D_{20,w} = 3.18 \times 10^{-7}$ cm^2/s.
Significance: The methods presented here should prove useful in the synthesis of size controlled nanoparticles of other minerals.

1. Introduction

Ferritin was first isolated from liver and spleen and crystallized by Laufberger in 1937 [1] and identified as a protein containing unusually large amounts of ferric iron. Soon thereafter, Granick and coworkers in a series of papers reported on the iron binding and magnetic properties of ferritin [2–4]. In the decades following this early work, ferritins have become one of the most investigated and widespread classes of proteins known. They are found in cells of nearly all animal tissues and in plants, fungi and bacteria [5–12]. A search in SciFinder Scholar results in over 30,000 articles related to ferritin. All canonical ferritins share the same basic structural features, an approximately spherical protein shell of M_r ~500,000 composed of 24 similar or identical subunits arranged symmetrically with 4:3:2 cubic symmetry about a hollow interior which encapsulates large amounts of iron in the form of a hydrous ferric oxide mineral resembling ferrihydrite [13], the structure of which is much debated [14–19]. In addition to ferrihydrite, iron oxide phases corresponding to hematite, magnetite/maghemite and wüstite have also been observed in lesser amounts as single phases in individual protein molecules [20,21].

Abbreviations: AUC, analytical ultracentrifugation; DTT, dithiothreitol; HoSF, horse spleen ferritin; HuHF, recombinant human H-chain ferritin; MOPS, 3-(N-morpholino) propanesulfonic acid; TEM, transmission electron microscopy; D, diffusion coefficient (cm^2/s) under the specific conditions of protein concentration, buffer and temperature; c, concentration (g/cm^3); $D^0_{20,w}$, diffusion coefficient of infinite dilute protein in pure water at 20 °C (cm^2/s); $D_{20,w}$, diffusion coefficient of the protein at the stated concentration in pure water at 20 °C (cm^2/s); d, Stokes diameter (nm); f, frictional coefficient (g/s); $g(s^*)$, concentration distribution function (see Fig. 2 legend); η, viscosity (poise); ρ, density (g/mL); n_{Fe}, number of iron atoms per protein shell as determined by AUC or ferrozine assays; M, molar mass (g/mol); M_r, relative molar mass (unitless); r, Stokes radius (nm); r_m, radius of meniscus (cm); s, Svedberg constant for the protein at stated concentration and temperature in buffer (10^{-13} s); s_W, weighted average of s over the g(s*) curve; $s^0_{20,w}$, Svedberg constant for infinitely dilute protein in pure water at 20 °C (10^{-13} s); $s_{20,w}$, Svedberg constant of protein at stated concentration in pure water at 20 °C (10^{-13} s); ω, angular velocity (rad/s); σ, linewidth of Gaussian function as defined in the text (svedbergs); $\overline{\nu}$, partial specific volume (mL/g)

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In recent years there has been considerable interest in understanding the superparamagnetism, ferromagnetism, Mössbauer and quantum tunneling properties of the nanoparticle iron core of ferritin as well as the mechanism of water proton relaxation enhancement in magnetic resonance images of iron laden tissues with high concentrations of ferritin and hemosiderin [23–29]. Additionally, the protein cage has proven useful as a reaction vessel, serving as a 3-D template for the synthesis of nanoparticles of magnetite/maghemite (*e.g.*[30]), various iron salts [31] and minerals of other metals such as gadolinium, lead, cadmium, nickel, cobalt, chromium and gold with interesting and useful properties [32–36]. Ferritin is increasingly finding use in nanotechnology as discussed in other chapters of this volume. Control or knowledge of the size of the nanoparticles within ferritin is important in many studies and applications. For example, uniform iron core sizes with a standard deviation within ± 1 nm are needed for the fabrication of memory devices [37]. In the case of metallic cobalt cores of ferritin, self capacitance is directly proportional to the diameter of the core particle [38] whereas the superparamagnetic relaxation time τ_s of the ferrihydrite nanoparticles depends exponentially on the volume of the particle [24,27,28].

While it is evident that good particle size control is desirable in most applications, this is not always easily achieved. The capacity of the protein cage in principle limits the maximum size of the nanoparticle but, in the case of iron, it is usually difficult to fill the cavity to capacity *in vitro*. Partially filled ferritin 24mer shells contain cores of varying size about some average which reflects the overall iron content of the protein. The subunit composition of the ferritin used strongly influences the size distribution which is obtained. Mammalian ferritins are composed of two types of subunits, H and L, with $M_r \sim 21{,}000$ and 20,000, respectively, which are structurally interchangeable within in the protein shell. Because these subunits have different functions, the H-subunit containing a ferroxidase site [39,40] and the L-subunit mineral nucleation sites [41], the protein shell is inherently polydisperse in subunit composition, resulting in polydisperse iron mineral cores as well (*vide infra*).

The mineral core is formed by three iron oxidation reactions [Eqs. (1)–(3)] [42]:

$$2Fe^{2+} + O_2 + 4H_2O \rightarrow 2Fe^{III}OOH_{(core)} + H_2O_2 + 4H^+ \quad (1)$$

$$2Fe^{2+} + H_2O_2 + 2H_2O \rightarrow 2Fe^{III}OOH_{(core)} + 4H^+ \quad (2)$$

$$4Fe^{2+} + O_2 + 6H_2O \rightarrow 4Fe^{III}OOH_{(core)} + 8H^+ \quad (3)$$

which further contribute to its polydispersity in size. Depending upon how iron is added to the protein and the subunit composition of the ferritin, one or more of these reactions may dominate. When iron is added in small increments (low-flux ~ 50 Fe per addition), two irons are oxidized per O_2 at the ferroxidase center through the ferroxidase reaction [Eq. (1)]. When a large flux of iron is presented to the protein (1000 Fe per addition), the reaction largely occurs on the mineral surface and the stoichiometry of the reaction approaches 4 Fe(II) oxidized per O_2 consumed [Eq. (3)]. At intermediate fluxes, the H_2O_2 produced at the ferroxidase sites [Eq. (1)] oxidizes further Fe(II) by the detoxification reaction [Eq. (2)]. Each of these reactions is important under different experimental conditions of iron loading the protein. Essentially identical mineral cores are produced with either O_2 or H_2O_2 as the oxidant [(Eqs. (1) and (3) vs. Eq. (2)] under the same flux of iron into the protein (50 Fe/shell per addition) as demonstrated by Mössbauer spectroscopy [24].

Ferritins isolated from tissue or apoferritins reconstituted *in vitro* show that a significant fraction (10–30%) of the sample remains relatively iron-free while another fraction is more fully loaded (approaching 4500 Fe/shell) [43–46]. The basis for these unusual iron distributions has been uncertain. Mineral growth models have related oxidation through Eq. (3) to the surface area of the growing mineral [44]. As the core grows in size, additional sites for oxidation and mineralization are created, allowing for increased capacity for deposition of additional iron by an autocatalytic mechanism. Under this scenario, those ferritin molecules that were able to first form a small core would have a greater likelihood of acquiring more iron than proteins that had not, leaving the latter proteins largely iron free. However, this model excludes any role for the protein in iron oxidation other than, perhaps, the formation of the initial iron cluster. An oxidation pathway directed purely by the ferroxidase site would seemingly allow for more uniform distribution of core sizes because all iron would be oxidized by the enzyme site and, therefore, in the same manner at the same rate. Such would be the case if all the protein shells in heteropolymer mammalian ferritins had the same number of H-subunits, i.e. they were uniformly distributed among the protein shells, which is not the case [5].

Analytical ultracentrifugation is well suited for studies of the heterogeneity of ferritin. The first detailed study was reported by Rothen in 1944 in his seminal work on horse, dog and human ferritins [46]. While the instrumentation available then was primitive compared to that today, the main conclusions of Rothen's work still hold. Apoferritins were observed to be relatively homogeneous whereas iron containing horse spleen ferritin exhibited marked heterogeneity and broad sedimentation patterns in which both apoferritin (or low iron ferritin) and ferritin components were present.

Rothen [46] made the first molecular weight determination of apoferritin using sedimentation velocity measurements. The molar mass was calculated from the Svedberg equation [Eq. (4)]

$$M = sRT / D(1-\overline{v}\rho) \quad (4)$$

where $s, R, D, \overline{v}$ and ρ are the sedimentation coefficient, the gas constant, the diffusion constant, the partial specific volume of the protein and the density of the solution, respectively. The sedimentation coefficient of apoHoSF was determined to be $s^o_{20,w} = 17.6$ in Svedberg units (10^{-13} s). ($s^o_{20,w}$ corresponds to the sedimentation coefficient in pure water at 20 °C extrapolated to infinite dilution.) Rothen measured the diffusion coefficient $D^o_{20} = 3.61 \times 10^{-7}$ cm^2/s using a Tiselius apparatus and the partial specific volume $\overline{v}^o_{20} = 0.747$ mL/g of the protein using a quartz pycnometer. Given $\rho^o_{20} = 0.99823$ g/cm^3 for pure water, he calculated a molecular weight of 467,000. In 1973, Crichton and coworkers reported a value of $M = 443{,}000$ based on their measured value of $s^o_{20,w} = 17.12$ S for the apoprotein and using a value of $\overline{v}^o_{20} = 0.731$ mL/g from the amino acid composition known at that time [47]. A more recent value of $M_r \sim 450{,}000$ by sedimentation equilibrium measurements has been reported [48]. Weight average molecular weights of 460,000, 480,000 and 515,000 have been published for horse spleen, liver and heart ferritins from sedimentation velocity measurements [49]. Other values reported for the horse spleen protein have ranged from 430,000 to 480,000 and are summarized in [50]. However, from the molecular weights of 19,978 and 21,269 from the cDNA sequences of the H- and L-subunits, respectively, a heteropolymer of 15% H and 85% L-subunit (a common composition of the horse spleen protein) would have a molecular weight of 484,120 only slightly larger than the molecular weight of 479,472 for the L-chain homopolymer and larger than the values predicted by the above referenced centrifugation measurements.

The Stokes diameter d of spherical particle (of which ferritin is a good example) is related to the diffusion coefficient D and viscosity of the solvent ($\eta = 0.01002$ poise for pure water at 20 °C) through the Stokes-Einstein equation [Eq. (5)]:

$$d = k_B T / 3\pi\eta D \quad (5)$$

where $k_B = 1.3807 \times 10^{-16}$ erg-K^{-1} is the Boltzmann constant and T the absolute temperature. D has been measured for horse spleen ferritin and apoferritin by a number of other methods, including