The Ferritin Protein Nanocage and Biomineral, from Single Fe Atoms to FeO Nanoparticles: Starting with EXAFS

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Abstract. Ferritins are protein nanocages that use iron and oxygen chemistry to concentrate iron and trap dioxygen or hydrogen peroxide in biominerals of hydrated ferric oxides, 5-8 nm in diameter, inside the cages. The proteins are found in nature from archa to humans. Protein catalytic sites are embedded in the protein cage and initiate mineralization by oxido-reduction of ferrous ions and dioxygen or hydrogen peroxide to couple two iron ions through a peroxo bridge, followed by decay to diferric oxo/hydroxyl mineral precursors; ferritin protein subdomains that fold/unfold independently of the protein cage control recovery of ferrous ions from the mineral. Early EXAFS (1978) was extremely useful in defining the ferritin mineral. More recent use of rapid freeze quench (RFQ) EXAFS spectroscopies, coupled with RFQ Mössbauer, Resonance Raman and rapid mixing UV-vis spectroscopy, have identified and characterized unusual ferritin protein catalytic intermediates and mineral precursors. EXAFS spectroscopy can play an important role in the future understanding of protein catalysis in metalloproteins such as ferritin, ribonucleotide reductase and methane monooxygenases. Needed are instrumentation improvements that will provide rapid-scan fluorescence spectra with high signal/noise ratios.

Keywords: Biominerals, Iron oxy nanoparticles, Ferritin, Protein nanocages, diferric peroxo complexes

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INTRODUCTION

Nanominerals of iron and oxygen are common biological mechanisms for concentrating iron and inactivating damaging oxidants in microorganisms, higher plants, and animals including humans, reviewed in [1]. The minerals form inside self-assembling protein nanocages of 12 or 24 relatively short polypeptides (~170 amino acids, spontaneously folded into 4 α-helix bundles), with internal cavities 5-8 nm of diameter. With or without mineral, the proteins are called ferritins, and the 12 subunit mini-ferritins, limited to bacteria, are also called dps proteins (DNA protection during starvation proteins), for historical reasons. Ferritins can concentrate ferric iron to as much as $10^{11}$ M in solution, more than matched to the iron concentrations in air-living cells ($\approx 10^4$ M) and trillions of times above the concentration of iron in aerated, neutral solutions ($10^{-18}$ M). Iron is concentrated by coupling ferrous ions and dioxygen or peroxide through oxidation/reduction, at multiple (3-24) protein catalytic sites similar to those in di-iron oxygenases, and then translocating, as ferric oxy dimers through the protein cage to the cavity, where hydrated ferric oxides or ferric oxy phosphates form. As many as 4500 Fe atoms can be accommodated in some maxi-ferritins (8 nm cavity), but in Nature, the average mineral size ranges from 500 to 2000 Fe atoms, with a size distribution that relates to unknown factors in tissue biology. Protein subdomains in ferritins control access to electrons and chelators in solution [1], thought to model biological reductants and iron transport proteins in living cells, and resemble the ion channels with gated pores that control ion transport important in cell integrity and environmental control. Whether ferritins evolved to control oxidants in the oxygen-free primordial environment or to minimize the biological dangers of using iron in an oxygen-rich atmosphere, is unknown. However, the importance of using the unique ferritin protein nanosphere to manage iron and oxygen chemistry is clear.
EARLY EXAFS: DISTINGUISHING PROTEIN BOUND-Fe FROM BIOMINERAL-Fe IN FERRITIN

Understanding how ferritin works and manages the transition of iron ions to bulk mineral is still a challenge, met in part by EXAFS studies. The first study, carried out at SSRL in 1978 (Figure 1), answered the question of feasibility, and resulted in the first paper using EXAFS spectroscopy in the Journal of Biological Chemistry. In addition to feasibility, the similarity of ferritin to a well known therapeutic iron source, Imferon® was demonstrated [1]. The physical research environment for EXAFS spectroscopy, at a time when instrumentation dominated experimental design, was alien to a biochemist such as I was at the time, and is likely, even in the contemporary EXAFS spectroscopy environment, to be a similar experience for some scientists studying biological systems.

FIGURE 1. The bulk ferric iron environment in ferritin is compared to a therapeutic iron supplement, Imferon® (Iron-dextran), detected in an early EXAFS experiment (1978). Taken from [2] with permission.

Key to dissecting the steps in the transition of ferrous ions and oxygen to hydrated ferric oxide is the discrimination between Fe bound to protein (catalytic coupling or translocation) sites from Fe sites in the bulk mineral, the multiple ferric Fe atoms are bridged via O. Typically the fraction of the Fe bound to protein in ferritins ranges from 0.05% to 0.2%. However, within the population of ferritin isolated from natural tissue are protein nanocages with only small numbers of Fe atoms (10) compared to the others (average 500 to 2000). Because the density of the ferritin protein nanocage varies with the iron content, resolution by iron content can be achieved by sedimentation through a gradient of sucrose in an ultracentrifuge (100,000 x G). The EXAFS spectrum of a fraction of ferritin (horse spleen) with an average number of 10 ferric-oxy complexes/nanocage, detected a different environment (Fourier transformed data), similar to 2,3 ferric oxalate [3].

Knowing that EXAFS could distinguish between bulk ferric Fe in ferritin (biomineral) and protein-bound ferric Fe, the properties of the ferric oxy multimers and biominal were further studied by Mössbauer spectroscopy and compared to EXAFS on the same samples (Figure 2) [3]. When examined in more detail with more advanced synchrotron sources, the environment of ferric ions in ferritin when there were only 10 Fe/protein nanocage was distinct from either the bulk mineral or models such as ferric citrate or ferric carboxylate, and, based on the Mössbauer analysis, were small ferric oxo-multimers.

FIGURE 2. EXAFS (left) and Mössbauer (right) spectroscopic analyses display differences in the iron environment of bulk ferritin iron (biomineral, ~2000 Fe atoms, 8 nm particle inside protein nanocage) and protein bound-Fe (10 Fe/protein nanocage). The figure is modified from reference [4].

CHARACTERIZING THE INITIAL REACTIONS IN FERRITIN MINERALIZATION - THE CATALYTIC PROTEIN COUPLING SITE

The presence of catalytic sites with highly specific spectral and kinetic properties, embedded in the ferritin protein nanocages, was discovered when abundant amounts of recombinant ferritin became routinely available through advances in
biotechnology (reviewed and annotated in reference 1). Recombinant ferritin proteins could be prepared with essentially no iron, the amount of the two types of substrates (ferrous iron and dioxygen or hydrogen peroxide) controlled, and the properties of the active sites characterized by kinetic analyses, site-directed mutagenesis and protein crystallography. Ferritin catalytic sites are related to those in a family of proteins called di-iron oxygenases that participate in key reactions of synthesizing DNA, unsaturated fatty acids, and in the reduction of methane to methanol. Although the evolutionary relationship of the active sites in ferritin protein nanocages and the di-iron oxygenases is not known, it is notable that the main Fe binding amino acids at ferritin sites can be encoded in DNA that differs in only 2/18 nucleotides of the six required DNA codons. The first reactions of Fe and dioxygen in ferritins and di-iron oxygenases converge on a common intermediate, diferric peroxo (DFP) before diverging [5].

When small amounts of ferrous ions are added to the empty ferritin protein nanocage, the sites are saturated with 2 Fe, which, in the presence of dioxygen, forms a transient blue intermediate DFP on a msec time scale. The intermediate in ferritin was identified by the coincidence, on the kinetic scale of the same intermediate in Mössbauer and Resonance Raman spectroscopies; in the experiments, rapid freeze quench technology was developed and applied to trap DFP. When coupled with rapid mixing UV-vis spectroscopy, an A650nm species was identified as DFP, providing the first specific kinetic assay for ferritin catalytic activity (reviewed in reference 1). DFP decays to a diferric oxo/hydroxo intermediate within a second and appears in “young mineral” after traversing the protein nanocage (10-20 nm) to the biomineral cavity (reviewed and annotated in reference 1).

Three different species in the DFP kinetic cycle of ferritin could be trapped by RFQ in a spectroscopically pure form by synchronizing the multiple catalytic sites with only enough iron to saturate each site and then trapping: (a) 2 ferrous substrates (iron with no dioxygen substrate); or (b) DFP (ferrous substrate + dioxygen, trapped at 25 msec); or (c) diferric oxo/hydroxo product (ferrous substrate + dioxygen, trapped at 1 sec) (Figure 3). Notable is the unusually short Fe-Fe distance in DFP, 2.5 Å, unique among known natural compounds at the time [5].

EXAFS spectroscopy of the catalytic intermediates during the initial steps of ferritin biomineralization (Figure 3) revealed a structure in the diferric peroxo intermediate that had never been observed before in nature or in model peroxo chemistry [4]. The ferrous atoms at the catalytic sites in the ferritin protein nanocages had an Fe-Fe distance of 3.4 Å. After oxidation and coupling in the DFP, the iron atoms had moved closer, with the short Fe-Fe distance of 2.5 Å. Then, during DFP decay, the ferric atoms moved apart and had an Fe-Fe distance of 3.0 Å in the diferric oxo/hydroxo product. EXAFS study of a ribonucleotide reductase analogue confirmed the very short Fe-Fe distance in DFP [6]. How the amino acids at the catalytic sites rearrange to facilitate the changes in site geometry is unknown.

EXAFS spectroscopy has contributed key information to defining and understanding the initial reactions in forming the ferritin biomaterial, formation of diferric oxo/hydroxo mineral precursors, from coupling 2 Fe (II) with O (O2 or H2O2) via a diferric peroxo intermediate, at catalytic protein sites in the ferritin protein nanocages. The steps between the diferric mineral precursor and hydrolysis to form bulk mineral in the cavity remain to be traced (Figure 4), along with the steps from the ferric mineral to the pores after reduction/rehydration. Future combinations of protein engineering and rapid kinetic spectroscopy will define the pathways of iron and oxygen, between solution and solid, within the natural nanoreactors of ferritin, and possibly clarify the process for other biominerals such as bone and teeth. EXAFS spectroscopy can play an important role in the future understanding of protein catalysis in metalloproteins such as ferritin and ribonucleotide reductase. Needed are instrumentation improvements that will provide rapid-scan fluorescence spectra with high signal/noise ratios.
FIGURE 4. The steps in the reversible formation of iron biominerals in ferritin protein nanocages: a partial list shown on graphic provided by M. Matzapetakis. In living cells, a number of proteins, still to be identified, likely participate in delivering iron for entry to the protein nanocage, controlling ferritin pore folding/unfolding, and retrieving iron dissolved from the mineral exposed to cytosolic reductants by the unfolded pores.

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REFERENCES