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Magnetoferritin: In Vitro Synthesis of a Novel Magnetic Protein

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The iron storage protein ferritin consists of a spherical polypeptide shell (apoferritin) surrounding a 6-nanometer inorganic core of the hydrated iron oxide ferrihydrite ($5Fe_2O_3 \cdot 9H_2O$). Previous studies have shown that the in vitro reconstitution of apoferritin yields mineral cores essentially identical to those of the native proteins. A magnetic mineral was synthesized within the nanodimensional cavity of horse spleen ferritin by the use of controlled reconstitution conditions. Transmission electron microscopy and electron diffraction analysis indicate that the entrapped mineral particles are discrete 6-nanometer spherical single crystals of the ferrimagnetic iron oxide magnetite (Fe_3O_4). The resulting magnetic protein, "magnetoferritin," could have uses in biomedical imaging, cell labeling, and separation procedures.

The ability of ferritin to sequester and store iron in a bioavailable form arises from a quaternary structure of 24 polypeptide subunits assembled into a spherical hollow shell that is penetrated by two types of intersubunit channels (*1*). The mechanisms by which iron is accumulated within the 8-nm internal cavity have been elucidated through reconstitution experiments of recombinant apoferritins modified by site-directed mutagenesis (*2*). In brief, two key sites have been implicated: a ferroxidase center residing in the intrahelical domain of H-chain subunits (*3*) and a nucleation site comprising three Glu residues on the cavity surface. These sites appear to act cooperatively in affecting the kinetics of iron oxide deposition such that mineralization occurs specifically within the protein cavity and not in bulk solution. Such precise molecular control of inorganic precipitation within a confined volume could be generally relevant to the synthesis of inorganic clusters and nanoparticles. We have recently shown that Mn(III) oxide cores can be reconstituted in ferritin by incubation of apoferritin molecules with aqueous Mn(II) under controlled reaction conditions (*4*).

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Although we have synthesized iron sulfide cores in ferritin by in situ reaction of native iron oxide cores with gaseous H_2S (*4*), the formation of iron oxide cores other than ferrihydrite has not been achieved (*5*). Indeed, it has generally been considered that the ferrihydrite structure of the biomineral is functionally important in providing a source of relatively labile iron for metabolic use. To determine the extent of structural specificity of iron oxide mineralization in ferritin, we carried out reconstitution experiments under conditions tailored to the synthesis of Fe_3O_4 (Fig. 1) (*6*). Iron was removed from native horse spleen ferritin by dialysis against thioglycolic acid at pH 4.5 (*7*). The resulting apoferritin solution was buffered at pH 8.5 and maintained at a temperature of 55° to 60°C under argon in a water bath. Ten increments of Fe(II) solution were added over 200 min (*8*). Slow oxidation was achieved during this procedure by the introduction of air into the solution with a Pasteur pipette. On completion of the Fe(II) additions, samples of the reconstituted ferritin were taken immediately for transmission electron microscopy (TEM) analysis (*9*). Several samples were left open to the atmosphere for 15 min before TEM investigation to ascertain their stability to possible oxidation in air. Some of these grids were negatively stained with 1% uranyl acetate solution to determine whether any thermal degradation of the protein had occurred and to verify that the crystals had indeed been produced within the protein shell. Protein-free control reactions, involving an analogous procedure in which 0.15 M saline was substituted for apoferritin, were also undertaken (*10*).

Addition of Fe(II) to the apoferritin solutions resulted in an initial increase in turbidity followed by a blackish discoloration after ~1 hour. The final solution remained black, and no bulk precipitation was observed. Exposure to air resulted in a red-brown coloration within a few minutes. Neither the black nor the red-brown protein solutions responded to a bar magnet placed against the side of the sample container. In contrast, a bulk white precipitate [$Fe(OH)_2$] was initially formed in the control reaction, transforming into a green gelatinous deposit and subsequently into a black and magnetic precipitate after 1 hour. The sample remained black under argon and after exposure to the atmosphere for 2 weeks.

Examination of the ferritin and control samples by TEM showed particles of remarkably different size and morphology (Fig. 2). The majority of particles synthesized in the presence of apoferritin were discrete, spherical nanometer-sized crystals (Fig. 2A) of uniform size (mean diameter = 6 nm, σ = 1.2 nm). Negative staining showed that the individual particles were surrounded by a protein shell (Fig. 2B), indicating that the discrete crystals were formed specifically within the apoferritin cavity. In contrast, particles formed in the control preparations were aggregated and heterogeneous in size (range = 4 to 70 nm) and morphology (irregular spheroidal, cubic, and cubo-octahedral) (Fig. 2C). Electron diffraction patterns identified the crys-

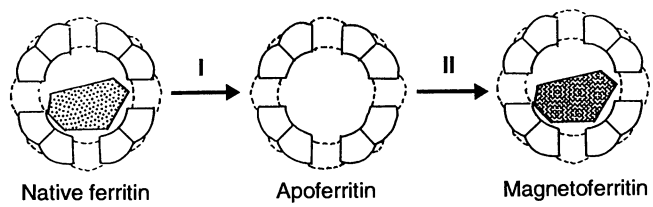

Fig. 1. Schematic showing the synthetic route to magnetoferritin. Step I involves the removal of native ferrihydrite cores from horse spleen by dialysis against thioglycolic acid, under N_2, at pH 4.5. Step II involves the reconstitution of apoferritin with Fe(II) solution under slow oxidative conditions at 60°C and pH 8.5.

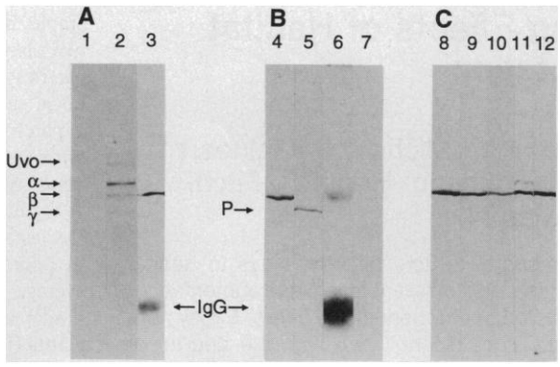

Fig. 2. Transmission electron micrographs of (**A**) an unstained sample of reconstituted ferritin showing discrete magnetite particles (scale bar = 50 nm); (**B**) a stained sample of (A) showing an intact protein shell surrounding the magnetite cores (scale bar = 50 nm); (**C**) magnetite crystals formed in the control reaction (scale bar = 100 nm); the needle-shaped crystals were identified as the mineral goethite (α-FeOOH); and (**D**) a high-resolution lattice image of an individual reconstituted ferritin core showing the single-crystal nature of the particle. Two sets of lattice fringes are observed corresponding to the {111} (interatomic spacing d = 0.485 nm) and {002} (d = 0.4198 nm) planes of magnetite. The angle between these planes is 54°, consistent with a cubic lattice symmetry (scale bar = 5 nm).

tals formed in the reconstituted protein reaction as either of the ferrimagnetic minerals, magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3) (Table 1). Lattice images of the reconstituted ferritin cores (Fig. 2D) were consistent with both of these cubic structures and showed that the imaged cores were well-defined single crystals. The small size of these encapsulated crystals is within the superparamagnetic size range for magnetite (*11*) and accounts for the inability of a bar magnet to induce magnetic separation from solution. Differentiation between magnetite and maghemite was not possible on the basis of electron diffraction studies alone, because the diffraction spacings for these two materials are similar and the intensities of such patterns are too weak to permit one to observe the superlattice reflections required to distinguish between these two phases. However, because the crystals were formed under essentially anaerobic conditions and were black, magnetite may be considered as the most probable structure. Indeed, x-ray diffraction of the control crystals clearly showed that magnetite, and not maghemite, was precipitated under analogous conditions. Exposure of the protein sample to air resulted in transformation to maghemite, as indicated by a color change from black to red-brown, although no changes were observed in the electron diffraction patterns (*12*). Maghemite was detected by x-ray diffraction in the control crystals exposed to the atmosphere.

Table 1. Comparison of electron diffraction data (d spacings) from the protein reconstitution and protein-free (control) experiments with that from stoichiometric magnetite.

d Spacing (Å)			Relative intensity, I/I_o
Control	Ferritin	Magnetite	
4.81		4.85	8
3.016	2.945	2.967	30
2.528	2.558	2.532	100
2.086	2.099	2.099	20
1.722	1.715	1.715	10
1.621	1.636	1.616	30
1.484	1.499	1.485	40
1.286		1.281	10
1.094		1.093	12

Our results establish the use of apoferritin as a confined reaction environment for the synthesis of nanometer-dimension magnetic iron oxides. In this regard, we have taken advantage of the unusual stability of the apoferritin shell to successfully undertake the synthesis of magnetite cores at relatively high temperature (60°C) and pH (8.5). In contrast, established low-temperature methods of magnetite synthesis [for example, the reaction of ferrihydrite with Fe(II) under N_2 (*13*)] failed to produce magnetite in the presence of apoferritin (*14*). Dehydration of intermediate phases such as green rust is a key step in the formation of magnetite (*15*) and is possibly inhibited within the protein cavity under ambient conditions. Although we have not detected any intermediates during magnetite synthesis in ferritin, both green rusts and amorphous hydrated Fe(III) materials were identified in the control experiments at high temperature. It is therefore feasible that the magnetite reaction within the protein cavity proceeds by a similar mechanism; the initial slow oxidation of Fe(II) at the ferroxidase centers after exposure to the atmosphere is followed by migration and subsequent nucleation of Fe(III) in the cavity to form a precursor Fe(III) oxide that subsequently reacts with excess Fe(II) to give magnetite. This process is presumably favored over a competing reaction in bulk solution because of the catalytic oxidation of Fe(II) at the ferroxidase ligands.

The successful utilization of the Fe storage protein ferritin as a novel environment in which to achieve the controlled precipitation of nanometer-dimension magnetic particles is relevant to the current interest in maximizing the ferromagnetic properties of materials used in industrial applications (*16*), the study of magnetic behavior as a function of miniaturization (*17*), and the elucidation of iron oxide biomineralization processes (*18*). Moreover, the production of a biocompatible ferrofluid, "magnetoferritin," could have far-reaching biomedical importance, for example, in the magnetic imaging of biological tissue (*19*) and in separation procedures involving cell and antibody labeling (*20*).

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8. A solution of Fe(II) (24 mM) was prepared by the dissolution of ferrous ammonium sulfate $[(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O]$ in deaerated water. Additions of 0.05 cm^3 of the Fe(II) solution were made every 20 min to 4 cm^3 of a 1 μM apoferritin solution under argon flow, pH 8.5 (0.05 M AMPSO buffer), at 55° to 60°C. The final Fe/protein ratio was estimated as 3000 Fe atoms per ferritin molecule.
9. Electron microscopy was performed with a JEOL 2000FX TEM operating at 100 keV for morphological and electron diffraction studies and at 200 keV for the high-resolution work.
10. Control samples for TEM and x-ray diffraction were collected immediately after completion of the reaction and after exposure to the atmosphere for 2 weeks. In some experiments, samples were collected at different time intervals during the reaction and examined immediately by TEM.
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