Nanophase Cobalt Oxyhydroxide Mineral Synthesized within the Protein Cage of Ferritin

Trevor Douglas* and Victoria T. Stark

Department of Chemistry, Temple University, Philadelphia, Pennsylvania 19122

Received October 27, 1999

Introduction

Ferritins are a class of iron storage proteins that exhibit a high degree of structural similarity across a range of biological species.[1] The protein comprises 24 subunits, of two types (H and L), that self-assemble to form a hollow cage-like structure 12 nm in diameter. In the native ferritin, iron is stored within an 8 nm diameter cavity formed by the protein as a particle of ferric oxyhydroxide (which may also contain phosphate). The mammalian protein has an enzymatic activity and catalyzes the oxidation of Fe(II) at "ferroxidase" sites present only in H subunits.[2] This reaction may proceed through a diferric-μ-peroxo species[3,4] that rapidly decomposes, eventually forming a ferric oxyhydroxide mineral core via an inorganic hydrolysis polymerization that appears to initiate at specific "nucleation" sites on the interior surface of the protein cage.[5] The mineral core, once formed, itself acts as an additional catalytic site for oxidative hydrolysis; thus the ferritin mineralization is an example of an autocatalytic reaction.

The protein cage assembly of ferritin is remarkably stable, able to withstand extremes of pH (2−10) and temperature (up to 70 °C), which has allowed it to be used successfully as a template for constrained material synthesis.[6−11] The use of ferritin to prepare small mineral particles, stabilized in solution, under synthetically mild conditions affords a view into early events in mineral formation as well as rational low temperature synthetic routes to novel materials and precursors. Here we show that horse spleen ferritin (HSFn) can act as a suitable constrained reaction environment for mineralization reactions resulting from the specific oxidative hydrolysis of Co(II). We have adjusted reaction conditions to minimize oxidative hydrolysis in the bulk solution, and we have observed site specific mineralization only within the confines of the ferritin protein cage. This indicates that the protein is able to catalyze and induce mineral growth from a precursor ion that is not ordinarily a substrate for the protein. As has been shown in other protein constrained mineralization reactions, this catalytic effect can be induced by complementary electrostatic interactions between the protein interface and the incipient mineral.[12−14] Ferroxidase-containing subunits are a minor component of the HSFn (85% L, 15% H), and it is therefore possible that this reaction does not involve the ferroxidase activity of the protein.

Experimental Section

Horse spleen ferritin (Sigma) was demineralized with thioglycolic acid[15] to make apo-ferritin, and the protein concentration was determined using the biuret method. An apo-ferritin solution (5 mg, 10^{-5} mmol in 0.1 M NaCl) was adjusted to pH 8.5 with 0.01 M NaOH, and aliquots of $Co(NO_3)_2$ (25 mM, 50 μL) were added, followed by addition of H_2O_2 (3%, 23.3 μL, 0.07 mmol) over a 2 h period with 10 min intervals between additions. Theoretical loading of 2250 Co per ferritin molecule was achieved by 18 addition cycles. The reaction was run unbuffered at pH 8.5, and the H^+ generated during hydrolysis was titrated dynamically with a Metrohm 718 autotitrator (10 mM NaOH).

The protein was isolated by anion exchange chromatography (BioRad, Uno Q) at pH 8.5 (10 mM Tris), eluted with a linear 0−2 M NaCl gradient and monitored by the absorbance at 280 nm (protein) or 350 nm (mineral). The purity and stability of the composite material was assessed by native PAGE, with all reagents adjusted to pH 8.5. Gels were stained for protein using Coomassie Blue and for cobalt using 1-nitroso-2-naphthol[16,17] (50 mM in 1:1 methanol/H_2O). The destaining solution for gels stained for cobalt was 10mM NaOH. Spectrophotometric analysis of the product to determine actual loading factors (Co:ferritin) was performed at 500 nm using 1-nitroso-2-naphthol-3,6-disulfonic acid (25 mM, in acetate buffer, 0.1 M, pH 5) relative to a standard curve.[16,17]

IR spectra (KBr pellet) of the samples were collected using a Nicolet (Protégé 460) spectrometer from vacuum-dried preparations. Samples were prepared for X-ray powder diffraction (Rigaku dMax2) by dialysis against H_2O adjusted to pH 8.5 and subsequently dried under vacuum. Samples for transmission electron microscopy on Formvar coated Cu grids were imaged using a Phillips 400T electron microscope at 120 keV with KEVEX energy-dispersive X-ray analysis system.

Results and Discussion

Demineralized ferritin (apo-ferritin) was treated with aliquots of Co(II) and H_2O_2 at high pH (8.5) and rapidly formed a homogeneous olive-green solution, the result of a spatially constrained oxidative mineralization reaction. The visible absorption spectrum revealed a well-defined shoulder at 350 nm (Figure 1), consistent with the Co(III)−oxyhydroxide Co(O)OH[18] and reminiscent of reported molecular Co−oxyhydroxide bridged cluster species.[19] The reaction progress was monitored either spectroscopically, by the appearance of the absorption band at 350 nm, or by dynamic pH titration (pH-stat), where H^+ generated by the hydrolysis reaction was titrated

* To whom correspondence should be addressed. Phone: (215) 204-7916. Fax: (215) 204-1532. E-mail: tdouglas@nimbus.temple.edu.

(1) Harrison, P. M.; Arosio, P. *Biochim. et Biophys. Acta* **1996**, *1275*, 161.
(2) Lawson, D. M.; Treffry, A.; Artymiuk, P. J.; Harrison, P. M.; Yewdall, S. J.; Luzzago, A.; Cesareni, G.; Levi, S.; Arosio, P. *FEBS Lett.* **1989**, *254*, 207.
(3) Pereira, A. S.; Small, W.; Krebs, C.; Tavares, P.; Edmondson, D. E.; Theil, E. C.; Huynh, B. H. *Biochemistry* **1998**, *37*, 9871.
(4) Hwang, J.; Krebs, C.; Huynh, B. N.; Edmondson, D. E.; Theil, E. C.; Penner-Hahn, J. E. *Science* **2000**, *287*, 122.
(5) Lawson, D. M.; Artymiuk, P. J.; Yewdall, S. J.; Smith, J. M. A.; Livingstone, J. C.; Treffry, A.; Luzzago, A.; Levi, S.; Arosio, P.; Cesareni, G.; Thomas, C. D.; Shaw, W. V.; Harrison, P. M. *Nature* **1991**, *349*, 541.
(6) Meldrum, F. C.; Wade, V. J.; Nimmo, D. L.; Heywood, B. R.; Mann, S. *Nature* **1991**, *349*, 684.
(7) Meldrum, F. C.; Heywood, B. R.; Mann, S. *Science* **1992**, *257*, 522.
(8) Douglas, T.; Dickson, D. P. E.; Betteridge, S.; Charnock, J.; Garner, C. D.; Mann, S. *Science* **1995**, *269*, 54.
(9) Douglas, T. In *Biomimetic Approaches in Materials Science*; Mann, S., Ed.; VCH Publishers: New York, 1996; pp 91−115.
(10) Wong, K. K. W.; Mann, S. *Adv. Mater.* **1996**, *8*, 928.
(11) Wong, K. K. W.; Douglas, T.; Gider, S.; Awschalom, D. D.; Mann, S. *Chem. Mater.* **1998**, *10*, 279−285.
(12) Douglas, T.; Young, M. J. *Nature* **1998**, *393*, 152.
(13) Shenton, W.; Douglas, T.; Young, M.; Stubbs, G.; Mann, S. *Adv. Mater.* **1999**, *11*, 253.
(14) Douglas, T.; Young, M. *Adv. Mater.* **1999**, 679.
(15) Chasteen, N. D.; Theil, E. C. *J. Biol. Chem.* **1982**, *257*, 7672.
(16) Wünsch, G. *Talanta* **1979**, *26*, 177.
(17) Dewey, D. W.; Marston, H. R. *Anal. Chim. Acta* **1971**, *57*, 45.
(18) Varkey, A. J.; Fort, A. F. *Sol. Energy Mater. Sol. Cells* **1993**, *31*, 277.
(19) Dimitrou, K.; Brown, A. D.; Folting, K.; Christou, G. *Inorg. Chem.* **1999**, *38*, 1834.

10.1021/ic991269q CCC: $19.00
Published on Web 03/23/2000

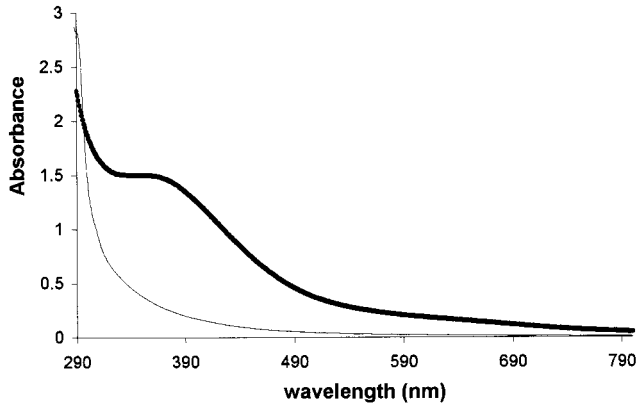

Figure 1. Visible absorption spectrum of apo ferritin (—) and Co(O)OH containing ferritin (···).

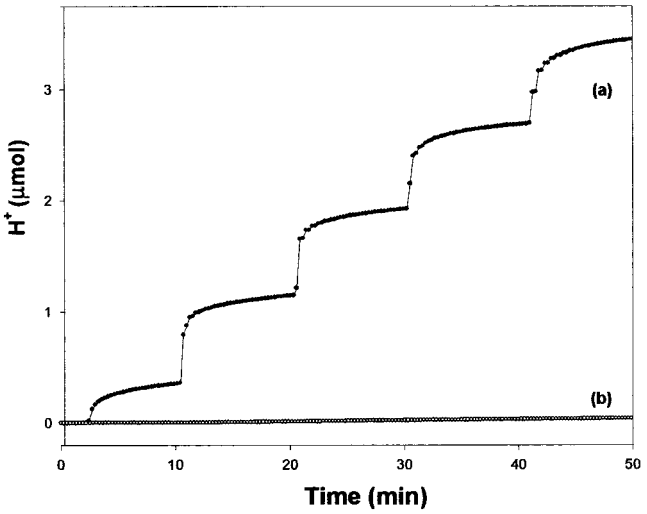

Figure 2. Stepwise reaction of Co(II) with H_2O_2 (—●—) or O_2 (—○—) in the presence of ferritin at pH 8.5 monitored by autotitration of H^+ released by oxidative hydrolysis (reaction 1). Data is shown for five additions of Co(II) and oxidant.

to maintain constant pH at 8.5 (Figure 2). The product solution contained a dispersion of colloidal cobalt oxyhydroxide Co(O)OH encapsulated and stabilized within the protein cage of ferritin. Successive addition of Co(II) followed by oxidation with H_2O_2 allowed us to build up a mineral core comprising over 2000 Co atoms. Control reactions, performed in the absence of the protein cage, resulted in the rapid formation of an extensive green-black bulk precipitate of the Co(O)OH mineral heterogenite.

Curiously, in the presence of the empty apo-ferritin cage the oxidative hydrolysis reaction between Co(II) and H_2O_2 produced a homogeneous product with no detectable bulk precipitation even with a large excess of oxidant. This implies that the protein cage of ferritin played an active role in controlling the oxidation and/or hydrolysis reactions that lead to mineral formation. The spatially specific mineralization within the protein cage appears to be the result of a kinetic bias where protein-catalyzed oxidative hydrolysis within the cage also acts to prevent bulk mineralization. Reaction conditions were designed to minimize the bulk Co(II) concentration, and site specific mineralization within the protein cage further diminishes bulk Co(II) concentration, effectively eliminating bulk mineralization. In the native mineralization of ferric oxyhydroxides, the protein interface provides regions of high charge density (nucleation sites) capable of inducing mineralization through complementary electrostatic interactions between the protein and the incipient mineral

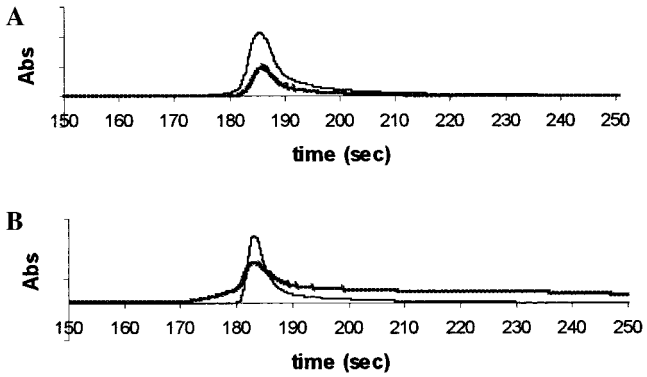

Figure 3. Anion exchange chromatography elution profile of ferritin (A) mineralized with Co(O)OH and monitored at 280 nm (—) and at 350 nm (-•-) and (B) mineralized with Fe(O)OH and monitored at 280 nm (—) and at 420 nm (-•-).

Figure 4. Native polyacrylamide gel electrophoresis stained (A) for protein using Coomassie Blue and (B) for cobalt using 1-nitroso-2-naphthol. Lane 1 is ferritin containing a core of Co(O)OH, lane 2 is apo-ferritin, and lane 3 is native ferritin containing an Fe(O)OH core.

nucleus. We believe that the nucleation sites on the interior interface of ferritin are responsible for the observed catalytic oxidative hydrolysis of Co(II).

The synthetic reaction conditions were maintained at pH 8.5 in a pH-stat autotitration experiment where H^+ generated by oxidative hydrolysis (reaction 1) was titrated dynamically with NaOH. Typical pH-stat data for the synthesis of the mineral are shown in Figure 2, illustrating the successive rapid oxidative hydrolysis reactions in the stepwise synthesis of the mineral core. In this way ferritins with theoretical loading of 2250 Co atoms per protein were synthesized. The resulting composite materials were analyzed spectrophotometrically for protein concentration (Biuret method) and for cobalt content (HNO_3 digestion and complexation of Co(II) with 1-nitroso-2-naphthol-3,6-disulfonic acid OH)[16,17] to determine the actual loading. It was found that the actual average loading corresponded to roughly 2000 Co per ferritin, implying some loss of Co, presumably due to nonspecific mineralization. Attempts to achieve higher loading factors resulted in bulk precipitation, presumably due to having exceeded the capacity of the protein cage.

From the pH-stat titration data we were also able to determine the overall stoichiometry of the reaction where 2.1 H^+ were released per Co(II). Thus, a 2 electron reduction of H_2O_2 by Co(II) would generate Co(III), which could then undergo hydrolysis, consistent with reaction 1, to form the final oxyhydroxide mineral, precipitated within the protein cage of ferritin.

$$2Co^{2+}_{aq} + H_2O_{2aq} \rightarrow 2Co(O)OH_s + 4H^+_{aq} + H_2O_l \quad (1)$$

When the synthesis was attempted with O_2 as oxidant, no reaction was observed over the course of several hours (Figure 2). Continued addition of Co(II) under these conditions resulted in the eventual bulk precipitation of $Co(OH)_2$.

The pH of the reaction was maintained at 8.5 due to the partial solubility of the Co(O)OH mineral at lower pH. However, at

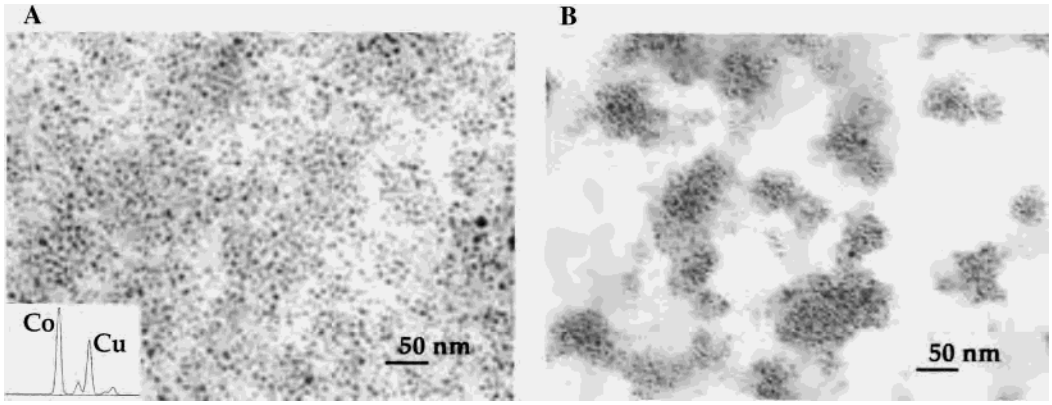

Figure 5. Transmission electron micrographs of (A) CoOOH mineral cores within the protein cage of ferritin (inset) energy-dispersive X-ray analysis (edxa) data indicating the presence of Co and O (Cu signal is due to sample holder) and (B) Co(O)OH ferritin sample stained with uranyl acetate to show the presence of the intact protein surrounding the mineral. Scale bar = 50 nm.

pH 8.5 the mineral−protein composite was extremely stable and was isolated by anion exchange chromatography using a 0−2 M NaCl gradient. The product elution was monitored either at 280 nm (protein) or at 350 nm (mineral), and the coelution of the protein and mineral components from the column (Figure 3A) indicates the composite nature of the material. In addition, the elution profiles of native (Fe_2O_3-containing) ferritin and apoferritin were analyzed (Figure 3B) and found to be identical to that of the Co(O)OH ferritin, indicating that the protein shell remained substantially unchanged and sustained no major alteration during the synthesis. Polyacrylamide gel electrophoresis (PAGE) gels of the Co(O)OH ferritin, under native conditions, were stained for protein (Coomassie) or for the presence of cobalt (1-nitroso-2-naphthol) and revealed comigration of the cobalt-containing mineral and protein (Figure 4), reconfirming the composite nature of the material. Neither apo-ferritin nor native (Fe(O)OH-containing) ferritin was stained by this cobalt specific method.

Analysis of the resulting composite material by transmission electron microscopy revealed homogeneously sized electron dense cores, commensurate in size with the internal dimensions of the ferritin protein cage (av = 70 Å diameter, Figure 5A). We could not measure any electron diffraction from these cores, leading us to the conclusion that the material was largely amorphous. Energy-dispersive X-ray analysis (Figure 5) confirmed the presence of Co (as well as Cu from the TEM grids). Negative stain of the material indicated, as expected, an intact protein shell surrounding the mineral core (Figure 5B). X-ray powder diffraction of bulk precipitated samples from control reactions showed patterns which were broadened and diffuse with *d* spacings of 4.4, 2.4, and 1.82 Å consistent with previously reported Co(O)OH mineral heterogenite.[20,21] IR spectra of Co(O)OH ferritin and bulk precipitate from protein-free control reactions both showed single strong bands at 587 cm^{-1} characteristic of Co(O)OH.[22]

Conclusion

We have shown that the protein ferritin is able to catalyze the oxidation and mineralization of Co(O)OH within the confines of the protein cage structure to yield an inorganic−protein composite. This spatially selective mineralization process is in direct competition with bulk mineralization that occurs rapidly in the absence of the ferritin protein cage. Oxidative mineralization reactions of this kind are autocatalytic, and so the initial formation of a very small amount of mineral inside the protein cage is enough to catalyze the reaction and provide the observed spatial selectivity. Our results suggest that the ferritin protein influences the oxidative hydrolysis of Co(II) through complementary electrostatic interactions at the nucleation site. This is analogous to the protein interaction with its substrate Fe(II), but occurring within different pH and redox windows. We postulate that the high charge density of the nucleation sites on the interior surface of the protein provides the necessary interface for precursor aggregation and nucleation.

Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

IC991269Q

(20) Matsumoto, Y.; Fujisue, M.; Sasaki, T.; Hombo, J. *J. Electroanal. Chem.* **1994**, *369*, 251.
(21) Powder Diffraction File 07-0169 (Joint Committee on Powder Diffraction Standards, Swarthemore, PA).
(22) Matsumoto, Y.; Bando, N.; Hombo, J.; Sasaki, T. *J. Electroanal. Chem.* **1995**, *395*, 45.