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Photochemical Reactivity of Ferritin for Cr(VI) Reduction

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The iron storage protein ferritin was used to catalyze the photoreduction of aqueous Cr(VI) species to Cr(III). Ferritin is a 24 subunit protein of roughly spherical shape with outer and inner diameters of approximately 12 and 8 nm, respectively. The native mineral core of ferritin is the ferric oxyhydroxide ferrihydrite (Fe(O)OH). Fe(O)OH particles that were used in these experiments ranged from 5 to 7.5 nm in diameter. The ferritin protein without the Fe(O)OH core (i.e., apoferritin) was inactive toward Cr(VI) reduction under our experimental conditions, suggesting that the Fe(O)OH provided the active catalytic sites in the redox chemistry. Experiments using photon band-pass filters suggested that the reaction occurred out of a photoinduced electron−hole pair and the optical band gap for the Fe(O)OH semiconductor was determined to be in the range 2.5−3.5 eV. Comparison of ferritin and protein-free Fe(O)OH mineral nanoparticles indicated that ferritin provided a photocatalyst with significantly more stability to aggregation and the loss of catalytic activity.

Introduction

Biological systems offer an exquisite degree of control in the formation of mineral structures with differing size, morphology, and compositions.[1,2] One such example of this chemistry, and the focus of this contribution, is the iron storage protein ferritin. This particular protein plays a central role in the sequestration and storage of iron in biological systems[3] and there is a remarkable degree of structural conservation among ferritin proteins from different sources.[3,4] Ferritins are comprised of 24 structurally similar polypeptide subunits that self-assemble to form a protein cage structure. The outside diameter of the cage is 12 nm, and the cage surrounds a hollow cavity roughly 8 nm in diameter. Up to 4500 Fe atoms are mineralized and stored within this protein cage as a nanoparticle of the ferric oxyhydroxide, ferrihydrite, (Fe(O)OH),[5] which can in certain organisms also contain phosphate. Reactions to form the mineral particle include the oxidation of Fe(II) and its subsequent hydrolytic polymerization to form ferrihydrite.[6,7]

The mineralization process to form the iron oxide nanoparticles in ferritin is spatially constrained by the reaction volume of the cage and by diffusion of species through the ≈0.5-nm-diameter channels of the protein shell.[3,8] In vitro, the mineralization reaction can be controlled to a high degree, and ferritins with Fe loadings between 500 and 4500 (5- and 7.5-nm-diameter particles, respectively) can be routinely synthesized.[9] Prior research has shown that the ferritin protein cage provides a versatile constrained reaction environment[10] and metal oxyhydroxide nanoparticles such as Mn(O)OH,[11,12] Fe_3O_4,[13] and Co(O)OH[14] can be synthesized within the protein cage. For all synthetic nanoparticles encapsulated within the protein cage, the size of the particle can be controlled by manipulating the metal ion-to-protein ratio (loading factor).

The present study investigated the reactivity of the native metal oxide core of ferritin, Fe(O)OH, and showed that in the presence of visible light the mineral core facilitates the reduction of Cr(VI) to Cr(III). It is mentioned that the reduction of Cr(VI) is an important environmental remediation step[15,16] because of the high

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solubility and toxicity[17] of Cr(VI). The photoreduction of Cr(VI) was studied with ferritins having four different loading factors; 100, 500, 1000, and ≈3000 (native).

Experimental Section

The preparation of ferritin, with an Fe loading of 100, 500, 1000, and 3000, was performed as previously described.[9] For the synthesis of ferritin with 1000 Fe loading, deaerated solutions of $(NH_4)_2Fe(SO_4)\cdot 6H_2O$ (10 mg/mL) were added to 5-mg apoferritin (2.5×10^{-6} M, Sigma) in 20 mL of MES at pH 6.5 (0.1 M) in two equal aliquots (0.4 mL total) and allowed to air oxidize, with a 1-h time interval between additions. For loading factors of 500 or 100 a single aliquot of ferrous ammonium sulfate was added at a stoichiometric Fe:protein ratio of either 500 (0.2 mL) or 100 (0.040 mL). The reminerализed ferritin was dialyzed into 0.1 M Tris buffer at a pH of 8.5 and purity was assessed by size exclusion chromatography, dynamic light scattering, and transmission electron microscopy.

Photolysis reactions were performed in a 4.0-mL quartz cuvette. Typical final concentrations of all species in the reaction (3.1-mL total volume) consisted of (apo)ferritin (1.6×10^{-7} M), Cr(VI) as $Na_2Cr_2O_7$ (ranging from 1.6×10^{-5} to 4.0×10^{-4} M), sodium tartrate (3.2×10^{-2} M), and Tris buffer (0.1 M). Sodium tartrate was used as a reductant in these experiments to increase the rate of Cr(VI) reduction. Experiments were carried out at a pH of 8.5 or 7 (indicated in the figure captions). All solutions were prepared using purified water (18 MΩ resistivity). In control reactions the (apo)ferritin, tartrate, or chromate solutions were substituted by an equivalent volume of Tris buffer.

UV−vis spectra (200−800 nm) were measured every 5 min for 25 min using a Perkin-Elmer Lambda 20. In photolysis experiments, the samples were illuminated with a 150-W Xe arc lamp (Oriel) for 1-min intervals before recording the full spectrum (200−800 nm). The photolysis setup was equipped with a water filter and the temperature of the samples during irradiation showed a maximum temperature rise of 3 K. The decrease in $[CrO_4^{2-}]$ was monitored by the loss of the charge-transfer band at 372 nm and independently confirmed by standard colorimetric analysis for CrO_4^{2-} using 1,5-diphenylcarbazide.[18]

Particle size analysis was undertaken using dynamic light scattering and was performed on the protein before and after all reactions (synthesis of the Fe(O)OH core and reduction of CrO_4^{2-}). Scattering was measured at 90° with a 661-nm diode laser and the correlation function was fit using a non-negatively-constrained least-squares analysis (Brookhaven 90Plus particle size analyzer). Particle size was independently measured by size exclusion chromatography (Superose 6,) on a BioRad QuadTec system. The flow rates for all experiments was 0.5 mL/min (25 mM Tris, 100 mM NaCl, pH 8.5) and the elution was monitored simultaneously at 280 nm for the protein and 405 nm for the Fe(O)OH mineral. The column was calibrated using gel filtration standard (BioRad), which consists of a mixture of five proteins: Thyroglobulin (670 kD), Gamma globulin (158 kD), Ovalbumin (44 kD), Myoglobin (17 kD) and Vitamin B-12 (1350 D).

Transmission electron microscopy data was collected on a Zeiss 100C operating at 80 keV or a JEOL JEM 1010 instrument at 120 kV. XPS data presented in this paper were acquired with unmonochromatized Mg Kα (1253.6 eV) radiation in a vacuum chamber with a working base pressure of 1×10^{-9} Torr. A cylindrical mirror analyzer was used to obtain the energy distribution of the photoemitted electrons at a pass energy of 25 eV. The binding energy scale for the XPS was obtained by calibrating the spectrometer against a series of metals with known binding energies. Samples were washed and dried for approximately 24 h before introduction into the UHV apparatus. Bombardment of the sample with Ar^+ was carried out with an ion gun operating at 500 eV with an Ar back pressure of 5×10^{-5} Torr in the vacuum chamber. Data presented in this contribution were obtained after 2-min bombardment times.

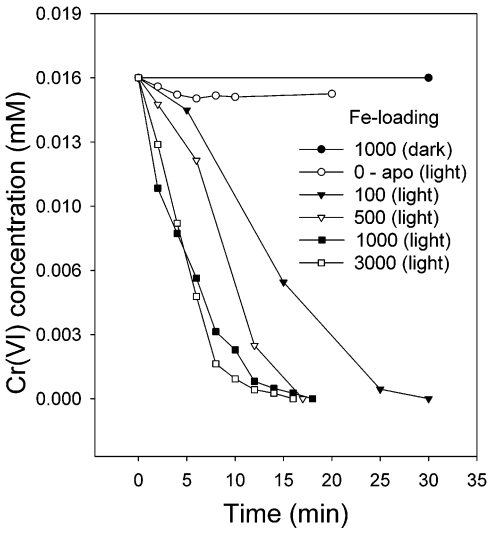

Figure 1. Cr(VI) concentration vs time data for the ferritin-facilitated reduction of Cr(VI) to Cr(III), as a function of Fe loading in the ferritin. Apoferritin is the protein lacking the Fe(O)OH inorganic core. The initial Cr:Fe concentration ratio ranged from 1:30 (for 3000 loading) to 1:1 (for 100 loading). Significant reduction of Cr(VI) by ferritin was only experimentally observed in the presence of light. All these data were obtained in solutions (pH of 8.5) at the same protein concentration.

Results and Discussion

In the presence of light, ferritin showed a remarkable ability to reduce Cr(VI) as compared to reactions in the dark and reactions with apoferritin, as shown in Figure 1. The reduction of Cr(VI) was monitored using UV−vis spectroscopy by the decrease in charge-transfer absorbance at 372 nm. While the photoreduction of Cr(VI) was significantly enhanced in the presence of ferritin, with a range of loading factors, there was no observed reaction under dark conditions. In addition, apoferritin exhibited no activity toward the reduction of Cr(VI) in either the presence or the absence of light. These specific reactions reported in Figure 1 were performed at pH 8.5, and while the source of Cr(VI) was $Na_2Cr_2O_7$, the predominant species in solution at this pH are CrO_4^{2-} in equilibrium with $Cr_2O_7^{2-}$.[19]

Further analysis of data presented in Figure 1 shows that the rate of Cr(VI) reduction showed significant variations with the Fe loading factor. At higher loading factors, faster reduction of Cr(VI) was observed, although the variance in rate between the different loading factors is not well understood. For example, ferritin with Fe loadings of 1000 or 3000 (i.e., native) exhibited similar reduction rates, and the rates are approximately 25 and 55% faster than those exhibited by 500 and 100 Fe-loaded ferritin, respectively. Before

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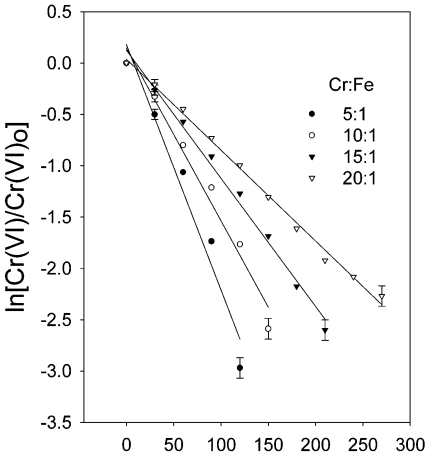

Figure 2. Data showing the stability of the ferritin catalyst (1000 Fe loading) as a function of the Cr:Fe ratio in the presence of light at pH 7. The experimental data were obtained by adding reactant aliquots with fixed concentrations of Cr(VI) to the ferritin solution. After each Cr(VI) aliquot was completely reduced, a subsequent aliquot was added. Each aliquot contained an amount of Cr(VI) that was five times greater than the total amount of Fe within the ferritin. In these specific experiments the ferritin reduced a quantity of Cr(VI) that was 20 times greater than the total Fe. These results strongly suggest that the ferritin is behaving as a catalyst, although there is some degradation of the ferritin activity as Cr(VI) is reduced.

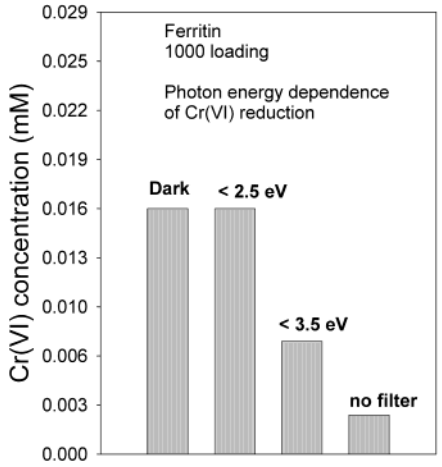

Figure 3. The photon energy dependence of the photochemical ferritin-facilitated reduction of Cr(VI). These data were obtained with an Fe loading of 2500 and an initial Fe:Cr concentration ratio of 1:1. The solution pH was 8.5. Irradiation was carried out for 10 min for each determination. No Cr(VI) reduction was experimentally observed with photon energies less than 2.5 eV, but substantial photocatalytic activity was observed when photon energies between 2.5 and 3.5 eV irradiated the solution. These data suggest that the band gap of the inorganic core material of ferritin lies between 2.5 and 3.5 eV.

possible reasons for this rate dependence are presented, we first present additional experimental results.

Data presented in Figure 2 suggests that ferritin acts as a catalyst during the Cr(VI) reaction. These particular data show that ferritin (1000 Fe loading) reduced at least 20 times more Cr(VI) than there was Fe within the protein cage, suggesting that the reaction was not stoichiometric, but catalytic, in nature. The data do show that there was some degradation of the catalytic performance of the ferritin over the course of the reaction. In particular, the rate of reduction decreased by roughly 10% as the reduced Cr:Fe ratio changed from 5:1 to 20:1.

We probed the dependence of the ferritin-catalyzed reduction of Cr(VI) on the wavelength of the incident radiation using band-pass filters (Figure 3). With filters transmitting photons with energies <2.5 eV (λ > 500 nm), no reaction was observed. However, significant Cr(VI) reduction was observed when the ferritin-containing solution was exposed to photon energies up to 3.5 eV. This difference was presumably due to the increased flux of photons having energies above the band gap of the ferritin. On the basis of these particular measurements, the band gap of ferritin (1000 loading) can be placed in the range 2.5−3.5 eV, which is consistent with prior studies showing band gaps in this same range for ferric oxide semiconductors.[20,21] Examination of data in Figure 3 also shows that the extent of Cr(VI) reduction in the presence of band-pass filters was always less than reactions with no filter. This experimental observation is presumably due to the cutoff by the filter (with a band-pass < 3.5 eV) of a significant number of photons emitted by the Xe arc lamp (>3.5 eV), which had energies capable of photoexciting the Fe(O)OH core.

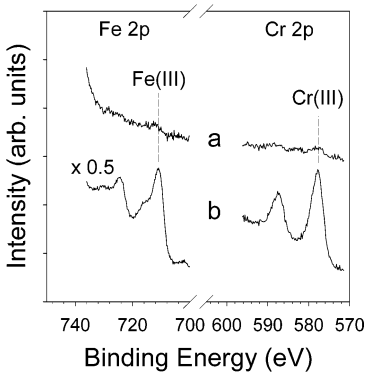

Figure 4. Fe 2p and Cr 2p XPS data (each exhibiting $2p_{3/2}$ and $2p_{1/2}$ spectral features) for ferritin that was removed from solution after catalyzing Cr(VI) reduction (Cr:Fe ratio of 20:1) at pH 7. Examination of the dried sample showed minimal Fe(III) and Cr(III) intensity due to the attenuation of photoelectrons by the protein shell. Ion bombardment with Ar^+, which removed some of the protein shell, led to large Fe 2p and Cr 2p signals associated with Fe(III) and Cr(III). The lack of Cr 2p signal prior to ion bombardment suggests that there is no Cr(III) bound to the protein shell after the sample had been extensively washed with H_2O. The majority of the Cr(III) remains in solution.

In separate experiments, X-ray photoelectron spectroscopy (XPS) was used to determine the location of the Cr(III) product after a solution of ferritin (Cr:Fe ratio of 20:1) completely reduced Cr(VI) to Cr(III) in the presence of light. After the reaction, ferritin was separated from the solution by ultrafiltration and washed two times with water. XPS data presented in Figure 4 show that the dried ferritin sample exhibited only a small Fe 2p and Cr 2p peak. After ion bombardment with 500 eV of Ar^+, however, the Fe and Cr signals increased by a factor of 10. The binding energy position of the Cr $2p_{3/2}$ feature at 577.7 eV was in excellent

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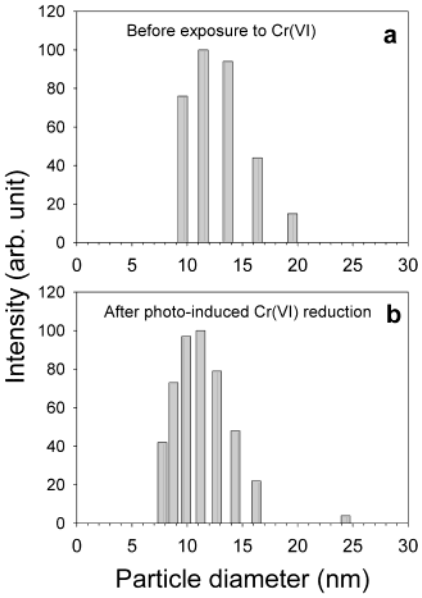

Figure 5. Dynamic light scattering of ferritin (a) before exposure to Cr(VI) and (b) after it facilitated the reduction of Cr(VI) to Cr(III) [Cr:Fe of 20:1] at pH 7. These data show that the size of the nanocatalysts do not change significantly after facilitating Cr(VI) reduction, suggesting that the Cr(III) product does not bind to the protein shell or cause the aggregation of the ferritin.

agreement with published data for Cr(III) solids,[22] supporting our prior presumption from absorption data that Cr(VI) was reduced to Cr(III). The low Fe and Cr 2p intensity prior to ion bombardment was due to the attenuation of Fe 2p and Cr 2p photoelectrons by the 2.0-nm-thick protein shell. This result taken by itself indicates that there was insignificant amounts of Cr(III), the product of the reduction, adsorbed onto the outside of the protein shell. The high Fe $2p_{3/2}$ intensity at 711.1 eV after bombardment was consistent with the presence of Fe(III),[22] suggesting that the Fe(O)OH particle(s) were still intact within the protein cage after the photoreduction process. The stability of the Fe(O)OH core, inferred from this XPS result, is consistent with the absence of Fe(II) in solution after irradiation (using the Fe(II)-ferrozine method).[23] The appearance of Cr 2p intensity after bombardment indicated that a fraction of the Cr(III) product resided within the ferritin protein cage. It is emphasized, however, that the amount of Cr(III) found within the protein was only a small fraction of the total Cr(III) product, suggesting that the majority of the Cr(III) remained in solution. Consistent with this contention was the experimental observation that the solutions in these experiments after irradiation appeared green, a characteristic color of aqueous Cr(III). Ultrafiltration of the sample, through a 100 000 M_w cutoff membrane, indicated that the soluble Cr(III) species were small and molecular and passed freely through the filter while the ferritin sample was completely retained. Dynamic light scattering of ferritin samples before and after the photoreduction showed essentially no increase in the particle size. As shown in Figure 5 the particle size distribution in both cases is centered at roughly 11.5 nm, consistent with the crystallographic dimensions of ferritin (12 nm). Hence, significant amounts of Cr(III) product were not accumulating on the exterior of the ferritin catalyst.

Two potential mechanisms for ferritin-catalyzed photoreduction can be envisioned: The first involves CrO_4^{2-} uptake into the protein cavity, where direct contact with the Fe(O)OH core can occur. However, there is an unresolved debate in the ferritin literature as to the accessibility of anions to the interior of the ferritin cage. Uptake kinetics with negatively charged spin labels have suggested that anion uptake is extremely slow. However, it is well-known that phosphate can enter the protein cage and in certain bacterial and plant ferritins the encapsulated mineral is rather more like a ferric phosphate than a ferric oxide.[24,25] Mammalian ferritins too can be remineralized to form a phosphate-containing mineral core. The pathway for the uptake of phosphate has not been established, although it has been suggested that gradients in the electrostatic potential of the protein could direct anion uptake through the 4-fold channel.[8] The second potential mechanism does not require direct contact with the mineral core but rather electron transfer through the protein shell. Photoinduced electron transfer has previously been shown to occur through the protein shell of ferritin when electron acceptors were used that were too large to enter the protein shell (such as an electrode surface or cytochrome *c*).[26,27]

We investigated the possibility that the Cr(III) product observed within the protein cage was not formed there, but instead diffused into the protein cage after its formation on the outside of the protein. In this scenario, the Cr(III) product might be expected to enter the protein cage through the 3-fold channel, which is electrostatically poised for the uptake of metal cations.[8] Experiments were conducted that exposed the ferritin to Cr(III) solutions with and without light for the same length of time as in the photoreduction experiments. In both cases, XPS analysis showed no evidence of Cr on the inside or adsorbed onto the outside of the protein. This result suggested that the Cr(III) experimentally observed during the irradiation of Cr(VI)/ferritin solutions was not due to the diffusion of Cr(III) (formed on the exterior of the protein) and subsequent migration into the protein cavity. Instead, these results indicate that at least a fraction of the Cr(VI) that was reduced to Cr(III) occurred within the protein shell, presumably on the iron oxide core. Cr(III) complexes are substitutionally inert and ligand-exchange reactions at these metal centers are extremely slow.[28] For Cr^{3+}(aq) to traverse the protein shell through the 3-fold channel, multiple exchange reactions would be required between the metal ion and amino acid side chains of the protein. These reactions are expected to be slow as borne out by our control Cr(III) uptake experiments. However, it has

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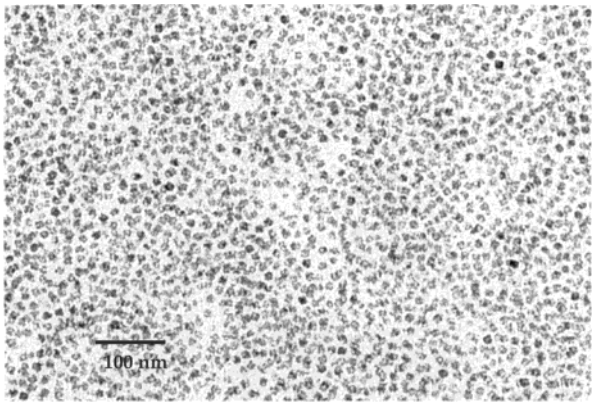

Figure 6. TEM of native ferritin showing the multiple Fe(O)OH crystals that make up its inorganic core. We believe that the electron-transfer reactions occur where the mineral contacts the interior of the protein. Electron transfer through the protein leads to the Cr(VI) reduction.

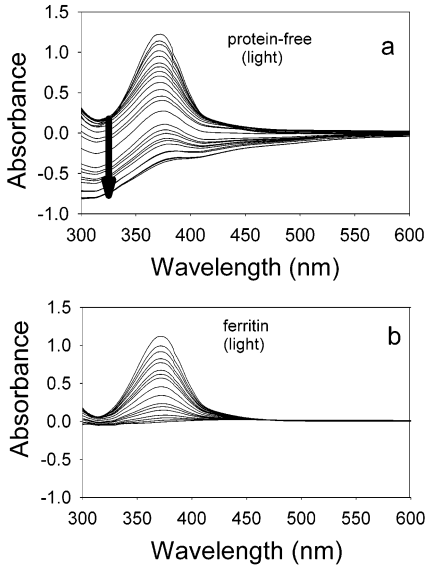

Figure 7. Absorption data showing the reduction of Cr(VI) (372-nm absorbance) by (a) protein-free Fe(O)OH particles and (b) ferritin (1000 Fe loading) in the presence of light at pH 7. The initial Cr:Fe concentration ratio was 1:10. The tartrate concentrate was 0.1 M at a pH of 7. Each scan is separated by a time interval of 5 min (from top to bottom in each data set). The protein-free Fe(O)OH initially facilitates Cr(VI) reduction, but it precipitates from solution during the Cr(VI) reduction process. This precipitation leads to a large decrease in the overall absorption of the solution compared to the reference solution (no Cr), leading to a "negative" absorption (indicated by the dark solid arrow). Ferritin shows no loss of solubility during Cr(VI) reduction, suggesting that the protein maintains the stability of the photocatalytic particles in solution.

also been demonstrated that cations inside the protein cage exchange slowly with the bulk media,[29] suggesting that any Cr(III) trapped inside the protein cage was most likely formed there.

We observed no significant difference in the Cr(VI) reduction rate between ferritins with 3000 and 1000 Fe loading, even though a significant difference in particle surface area is expected. Assuming a spherical morphology for the Fe(O)OH core (bulk density of 3.96 g cm^3), the particle diameter can be estimated to be 7.0 and 5.5 nm for the 3000 and 1000 Fe particle loadings, resulting in a factor of 1.6 difference in surface area between the two particles. This insensitivity of the Cr(VI) reduction rate with particle size might be taken to suggest that the reduction rate is rate-limited by the diffusion of Cr(VI) into the ferritin and/or the diffusion of Cr(III) back into solution.

It must be emphasized, however, that whether the majority of the Cr(VI) reduction occurred inside or outside of ferritin cannot be unambiguously determined from our experiments. On the basis of prior studies of ferritin, it would appear that the reduction of Cr(VI) to Cr(III) need not occur via intimate contact with the Fe(O)OH photocatalyst. Photoinduced electron transfer, for example, from the mineral particle of ferritin, through the protein shell, to an acceptor on the outside of the protein has previously been implicated in prior studies.[30] (Prior experiment and theory[31] suggest that electron transfer can take place in protein systems over the distances that would be required in ferritin. about 2 nm). Transmission electron microscopy revealed that the mineral core of the native ferritin used in these experiments was polycrystalline, composed of many small iron oxide particles adhering to the interior of the protein shell (Figure 6). Prior studies of ferritin show that nucleation of the mineral particle inside the protein cage of ferritin occurs at symmetry-related clusters of glutamic acid residues, which create a protein surface of high charge density. In the absence of the ferroxidase site, which enzymatically oxidizes Fe(II) to Fe(III), this highly charged interface is sufficient for inducing oxidative hydrolysis and mineral formation within the confines of the protein cage.[10] The contact points between the mineral and protein cage may be where electron transfer through the protein shell occurs. Hence, even in the lowest Fe-loading scenario (100−500 Fe atoms/ferritin) there may be numerous Fe(O)OH−protein contact sites that can act as the photoactive medium. Hence, ferritin with Fe loadings of 3000 and 1000 might be expected to show similar Cr(VI) reduction rates since both systems have similar contact areas between the mineral particle and protein. In essence, the part of the surface of the Fe(O)OH particle, at higher loadings, that does not contact the protein may not take part in the redox chemistry.

While the location of the Cr(VI) reduction is ambiguous, we can assert that the majority of the Cr(III) product remained in solution as soluble molecular species. The fraction of Cr(III) experimentally observed within the protein is relatively small. It is pointed out that this Cr(III) fraction could conceivably poison the iron oxide core and be the cause for the slow degradation of the ferritin catalytic activity over time (Figure 2). Under our experimental conditions, however, the catalytic particle, for the most part, was not significantly altered (through formation of a passive layer) by the Cr(III) product. This result was in stark contrast to additional results obtained in our laboratory for protein-free Fe(O)OH particles. These comparative experiments were carried out by treating ferritin with 1 M NaOH, which was shown in prior studies to remove the protein

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shell.[32] Figure 7a shows absorption data for protein-free particles in a Cr(VI)-bearing solution [Cr(VI) absorption at 372 nm] over a period of 2 h. Irradiation of the solution led to the reduction of Cr(VI), but also to the rapid growth and precipitation of the protein-free particles. It is pointed out that in these particular experiments Cr(VI) was not completely reduced due to the aggregation resulting from the lack of stabilizing protein. Figure 7b exhibits data for the ferritin case and emphasizes the stability of the catalytic particles, and the ability of the catalyst to completely reduce Cr(VI). In the case of the protein-free particles, it is probable that Cr(III) produced during the reaction complexed with the Fe(O)OH particle, which would carry a negative charge (since the pH of reaction conditions is higher than the P_{zc} of the Fe_2O_3), causing cross-linking and the observed aggregation. Furthermore, it is well-known that ferric oxides such as Fe(O)OH undergo photocorrosion reactions in solution in the presence of a reductant [conversion of Fe(III) to soluble Fe(II)].[21,33] A similar phenomenon is expected to occur in the protein-free particle case, and the reoxidation of Fe(II) to Fe(III) presumably contributed to the particle aggregation. We believe that ferritin inhibited such a process since any Fe(II) produced within the protein would be rapidly converted back to Fe(III), leading to a stable catalytic particle, a composite of protein and iron oxide.

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Summary

We have demonstrated that the iron oxide nanoparticle encapsulated within the protein cage of ferritin acts as a semiconductor photocatalyst for the reduction of Cr(VI) to Cr(III). It is likely that this reduction occurs out of a photoinduced electron−hole pair in the iron oxide semiconductor. We speculate that reducing equivalents from the mineral to the CrO_4^{2-} (or $Cr_2O_7^{2-}$ depending on the pH) proceed via electron transfer through the protein shell and through direct mineral−CrO_4^{2-} contact. Cr(III) found inside the protein cage after the reaction was likely formed within the protein cavity. No detectable Fe(II) was generated outside the protein cage, perhaps due to efficient reoxidation and mineralization of Fe(O)OH within the protein under the experimental conditions. Thus, there is only a small degradation of the catalyst, even after many catalytic turnovers. The protein stabilization of the semiconductor catalyst in the ferritin system may represent a significant advantage for future photocatalytic applications, such as in environmental remediation chemistry.

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