Is Hydrogen Peroxide Produced during Iron(II) Oxidation in Mammalian Apoferritins?†

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ABSTRACT: The ferritins are a class of iron storage and detoxification proteins that play a central role in the biological management of iron. These proteins have a catalytic site, "the ferroxidase site", located on the H-type subunit that facilitates the oxidation of Fe(II) to Fe(III) by O_2. Measurements during the past 10 years on a number of vertebrate ferritins have provided evidence that H_2O_2 is produced at this diiron ferroxidase site. Recently reported experiments using three different analytical methods with horse spleen ferritin (HoSF) have failed to detect H_2O_2 production in this protein [Lindsay, S., Brosnahan, D., and Watt, G. D. (2001) *Biochemistry 40*, 3340−3347]. These findings contrast with earlier results reporting H_2O_2 production in HoSF [Xu, B., and Chasteen, N. D. (1991) *J. Biol. Chem. 266*, 19965−19970]. Here a sensitive fluorescence assay and an assay based on O_2 evolution in the presence of catalase were used to demonstrate that H_2O_2 is produced in HoSF as previously reported. However, because of the relatively few H-chain ferroxidase sites in HoSF and the reaction of H_2O_2 with the protein, H_2O_2 is more difficult to detect in this ferritin than in recombinant human H-chain ferritin (HuHF). The proper sequence of addition of reagents is important for measurement of the total amount of H_2O_2 produced during the ferroxidation reaction.

The ferritins are a class of iron oxidation and hydrolysis proteins that function as intracellular reservoirs of iron(III) (*1*). These proteins facilitate both iron(II) oxidation and iron(III) mineralization and form hydrous ferric oxide mineral cores, "$FeOOH_{(s)}$" within the interior of their shell-like structures (*1*−*3*). Mammalian ferritins are composed of 24 subunits of two types, H and L, with similar amino acid sequences but distinctly different functions (*3*). The H-subunit contains a dinuclear ferroxidase site and appears to be largely involved in promoting Fe(II) oxidation (*4*) while the more acidic L-subunit is thought to provide an environment for mineralization within the protein cavity (*5*).

Achieving an understanding of the mechanism by which ferritin acquires its complement of iron requires a knowledge of the products of iron oxidation and hydrolysis and the stoichiometric equations for the reactions involved. Previous studies of iron deposition in horse spleen apoferritin (apoHoSF)[1] employing mass spectrometric measurements of O_2 consumption and base titration obtained reaction stoichiometries of $Fe(II)/O_2 = 2/1$ and $H^+/Fe(II) = 2/1$ when small amounts of iron were added to the protein [≤24 Fe(II)/shell] (*6*), stoichiometries in accord with earlier work (*7*). These stoichiometries implied that the net iron mineralization reaction via the ferroxidase site is given by eq 1 where

$$2Fe^{2+} + O_2 + 4H_2O = 2FeOOH_{(s)} + H_2O_2 + 4H^+ \quad (1)$$

hydrogen peroxide is the product of dioxygen reduction. The formation of H_2O_2 in eq 1 was probed *directly* by the addition of catalase, an enzyme that specifically catalyzes the disproportionation of hydrogen peroxide to water and dioxygen according to eq 2 (*8*). In the presence of catalase, the

$$2H_2O_2 = 2H_2O + O_2 \quad (2)$$

measured $Fe(II)/O_2$ stoichiometry increased to 4/1 from the value of 2/1 in its absence whereas the $H^+/Fe(II)$ stoichiometry remained at 2/1 (*6*). This result indicated that, in the presence of catalase, the net reaction is given by eq 3.

$$4Fe^{2+} + O_2 + 6H_2O = 4FeOOH_{(s)} + 8H^+ \quad (3)$$

Equation 3 reflects the fact that catalase recycles the H_2O_2 produced in eq 1 to O_2 and H_2O according to eq 2. Note that eq 3 is twice eq 1 plus eq 2. The complete oxidation of the iron(II) in these experiments was verified by Mössbauer spectroscopy (*6*).

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[1] Abbreviations: BfHF, bullfrog H-chain ferritin; BfMH, bullfrog M-chain ferritin; DPP, differential pulsed polarography; EcBFR, *Escherichia coli* bacterioferritin; EcFtnA, *Escherichia coli* bacterial ferritin; EXAFS, X-ray absorption fine structure; HoSF, horse spleen ferritin; HuHF, human H-chain ferritin; ITC, isothermal titration calorimetry; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; *N*, the number of samples; SDS−CGE, sodium dodecyl sulfate−capillary gel electrophoresis; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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Subsequent measurements on HoSF in two laboratories using electrode oximetry (*9*−*13*) confirmed the 2/1 Fe(II)/O_2 stoichiometry obtained from mass spectrometry (*6*). Other experiments showed that once sufficient mineral is deposited in ferritin [≥200 Fe(II)/shell], the protein ferroxidase reaction 1 no longer occurs to a major extent (*6*, *9*, *11*). Iron oxidation and hydrolysis appear to take place directly on the mineral surface with reduction of dioxygen completely to water (eq 3). The same reaction (eq 3) occurs in the normal autoxidation of Fe(II) in the absence of protein (*11*, *12*).

A ferroxidation reaction having an Fe(II)/O_2 stoichiometry of 2/1 has also been measured with recombinant human H-chain ferritin (HuHF), a homopolymer of 24 H-subunits (*11*, *14*, *15*), and with bullfrog H-ferritin (BfHF) (*13*) using electrode oximetry. The production of H_2O_2 in both proteins was confirmed with catalase (*11*, *13*, *14*). More recently, a colored peroxodiFe(III) complex has been observed in freeze−quench experiments with bullfrog M ferritin (BfMF) and proposed to be the source of H_2O_2 in this protein (*16*, *17*). The structure and spectral properties of this colored intermediate have been thoroughly characterized by optical data (*16*), Mössbauer (*17*) and resonance Raman spectroscopy (*18*), and EXAFS (*19*). Similar optical spectra of blue intermediates in HuHF and EcFtnA have been attributed to formation of peroxodiFe(III) complexes in these proteins (*20*, *21*).

Hydrogen peroxide is not the end product of O_2 reduction in all ferritins, however. In *Escherichia coli* bacterioferritin (*22*) and *Listeria innocua* ferritin (*23*), ferroxidation reactions having Fe(II)/O_2 stoichiometries of 4/1 have been observed, indicating complete reduction of O_2 to H_2O in these proteins. In these microbial ferritins, H_2O_2 may be produced as an intermediate active oxygen species but is then rapidly consumed in subsequent iron reactions, being ultimately reduced to water (*22*, *23*). However, other explanations are possible (*22*).

Recently, Watt and co-workers (*24*) reported unsuccessful attempts to detect H_2O_2 production in commercial horse spleen apoferritin from Sigma Chemical Co. They employed three analytical methodologies for measuring hydrogen peroxide not previously used in ferritin research. One is based on the reaction of H_2O_2 with Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase (HRP) to form fluorescent resorufin (the fluorescence method) (*25*), the second is based on the reaction of H_2O_2 with iodide to produce I_3^- which absorbs at 340 nm (the UV method), and the third method employed differential pulsed polarography to detect the reduction of H_2O_2, O_2, and Fe^{2+} at their respective potentials (the polarographic method). Of the three, the fluorescence method is the most sensitive (*24*). After extensive experimentation and repeated attempts with all three analytical methods, they were unable to detect the presence of hydrogen peroxide following aerobic addition of Fe(II) to apoHoSF, nor did they observe an Fe(II)/O_2 stoichiometry of 2/1. Rather, polarography measurements gave a value of 3.6 Fe(II)/O_2. From these results they concluded that water, not hydrogen peroxide, is the final product of dioxygen reduction during Fe(II) oxidation in apoHoSF (eq 3) even at low Fe loading of the protein [5 Fe(II)/shell].

These recent findings have cast doubt on the conclusions of a large body of work indicating that H_2O_2 is produced during the protein-catalyzed oxidation of iron in horse spleen ferritin and other vertebrate ferritins (*6*, *7*, *9*−*15*). In the present paper we address this important issue by presenting previously unreported data on apoHoSF and apoHuHF and include results of recent experiments employing the catalase assay, the fluorescent assay, and isothermal titration calorimetry. The present data conclusively demonstrate H_2O_2 production during Fe(II) oxidation in both HoSF and HuHF. The inability to measure H_2O_2 in the recently reported experiments (*24*) stems in part from the protein and protocols used. HoSF contains a small number of H-chains (typically 2−4 H-chains per 24-mer) resulting in the production of relatively little H_2O_2 compared to the amount of protein present. HuHF, having 24 ferroxidase sites, produces considerably more H_2O_2 than does HoSF and is better suited for such studies. The small amount of H_2O_2 that is produced in HoSF is rapidly consumed by reactions with the protein and/or its complement of iron. Its production in HoSF can be directly measured if catalase or fluorescence reagents are present during iron(II) addition but is more difficult to observe if catalase or fluorescence reagents are added after the iron, at which point most of the H_2O_2 has disappeared. The reaction of H_2O_2 with ferritin was confirmed by the catalase and fluorescence assays and by isothermal titration calorimetry.

MATERIALS AND METHODS

Cadmium-free horse spleen holoferritin and horse spleen apoferritin were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human H-chain ferritin was prepared as previously described (*26*). The Sigma holoHoSF and HuHF were rendered iron free by anaerobic ultrafiltration with dithionite and bipyridyl (*27*). The Sigma apoHoSF, which is commercially prepared from holoferritin using thioglycolate, was further treated with dithionite and bipyridyl to remove possible traces of iron (*24*) and analyzed by SDS−CGE and SDS−PAGE.[2] The concentration of apoHoSF was determined by its absorbance at 280 nm ($\epsilon = 19\,500$ cm^{-1} M^{-1} per subunit) (*28*). The concentration of apoHuHF was measured by the advanced protein assay (http://cytoskeleton.com, patent pending) using bovine serum albumin as a standard from which a protein absorbance of $\epsilon = 23\,000 \pm 1000$ cm^{-1} M^{-1} ($N = 3$) at 280 nm was determined spectrophotometrically. The same procedure with HoSF gives $\epsilon = 19\,650 \pm 600$ cm^{-1} M^{-1} per subunit ($N = 3$), in good agreement with the literature value (*28*). The amount of Fe in Sigma holoHoSF was determined by the ferrozine assay (*29*) to be ∼2600 Fe(III)/protein shell. The concentration of H_2O_2 in stock solutions was determined from the amount of O_2 evolved upon addition of catalase or from its absorbance at 240 nm ($\epsilon = 43.6$ cm^{-1} M^{-1}) (*30*). Beef liver catalase (EC 1.11.1.6), 65 000 units/mg, was purchased from Boehinger-Mannheim GmbH (Germany) and the Amplex

[2] Attempts to measure the H-chain content of Sigma apoHoSF were unsuccessful. Experiments employing SDS−PAGE and SDS−CGE by Mr. John Grady in this laboratory failed to detect the presence of intact H-chains in this commercial apoferritin preparation. Only L-chains and smaller fragments were detected. However, the observed production of H_2O_2 in apoHoSF indicates that some functional H-chains are present. These H-chains perhaps have proteolytic nicks in their sequence and dissociate into smaller peptide fragments upon denaturation of the protein with SDS.

Red hydrogen peroxide assay kit from Molecular Probes (Eugene, OR). All other compounds were of reagent grade quality.

The electrode oximetry/pH-stat apparatus and standardization reactions for its use have been described in detail elsewhere (*11*). In the present experiments, the consumption of O_2 during iron(II) oxidation or its evolution during the catalase-promoted disproportionation of H_2O_2 (eq 2) was followed at 20 °C by a Clark-type oxygen microelectrode while maintaining the pH at values between 6.0 and 8.0 using 100 mM Mes, 50 mM Mops, or a 1:1 mixture of 50 mM Mes and 50 mM Mops with 50 mM NaCl (see figure captions). Experiments were performed by adding 1 μL of catalase (1300 units) to 0.45 mL of 1.0−10.0 μM protein either before or after the addition of Fe(II) as 20 mM $FeSO_4 \cdot 7H_2O$, pH 2.0, to give Fe/protein shell ratios of 20/1 or 48/1 for HuHF or 6/1, 8/1, or 24/1 for HoSF, respectively. The detection limit of the oximetry apparatus for O_2 evolution due to the disproportionation of H_2O_2 is 0.3 μM O_2 (three times the standard deviation of the electrode reading), corresponding to a detection limit of 0.6 μM for H_2O_2 (see eq 2). The reported detection limit for the Amplex H_2O_2 fluorescence assay is 50 nM hydrogen peroxide (*25*); however, in our hands the measured detection limit was 0.180 μM using the 99% confidence interval lines for the standard curve (*31*). This value compares favorably with the reported detection limit of 0.250 μM calculated from the standard deviation in the fluorescence intensity measurement (*24*). Corrections for background fluorescence employed the assay cocktail plus apoferritin without added iron and was typically 10% of the fluorescence reading for a solution of 3 μM H_2O_2. For experiments in which Fe(II) was added to apoHoSF in the presence of the fluorescence assay reagents, a buffer of 50 mM Mops, pH 7.4, was used in place of the 50 mM phosphate, pH 7.4, buffer supplied with the kit. In this way possible interference by the phosphate on the iron chemistry was avoided. Fluorescence standard curves were constructed using triplicate samples and measured at the same time as the unknowns, which were also prepared in duplicate or triplicate. The standard curve is time dependent, requiring that samples and unknowns be handled together and in exactly the same way, and nonlinear with H_2O_2 concentration above approximately 3 μM H_2O_2. Fluorescence intensity was measured on an Aminco Bowman Series 2 luminescence spectrometer. Isothermal titration calorimetry (ITC) measurements were performed on a Calorimetry Sciences Corp. Model CSC 4200 calorimeter, and the analysis of the binding isotherm was done with the BindWorks 3.0 software program supplied by CSC. Absorbance measurements for the protein assays were made on a Cary 5 spectrophotometer.

RESULTS

Previous measurements of H_2O_2 production in HoSF and HuHF have been based on the change in apparent stoichiometry when iron(II) is added to the apoprotein in the presence or absence of catalase (*6, 9, 14*). The experiments outlined here show that the order of addition of Fe(II) and catalase to the apoprotein solution at pH 6.5 is critical to whether all of the hydrogen peroxide produced by the ferroxidase reaction (eq 1) can be detected. Figure 1A shows the effect of catalase on the amount of oxygen consumed when 20 Fe(II)/protein shell are added to 3 μM HuHF. For the addition sequence HuHF + catalase + Fe(II) [catalase added before Fe(II) added], an Fe(II)/O_2 stoichiometry of 4/1 is obtained (Figure 1A, upper curve). When catalase is absent during Fe(II) addition, a stoichiometry of 2 Fe(II)/O_2 is observed (Figure 1A, lower curve). The subsequent addition of catalase to the latter sample results in evolution of O_2, a clear indication of the presence of H_2O_2 (Figure 1A). However, the amount of O_2 generated is only 49 ± 3% ($N = 2$) of the 15 μM expected from the catalase-facilitated disproportionation of the H_2O_2 produced from the ferroxidation reaction, a result indicating that 5.1 ± 0.3 H_2O_2 per protein have been consumed by a reaction.

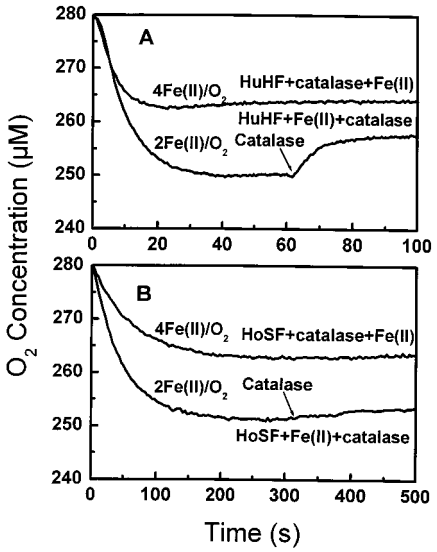

FIGURE 1: Dependence of Fe(II)/O_2 stoichiometry and H_2O_2 measurement on the order of addition of components. (A) HuHF (3 μM) and (B) HoSF (10 μM) in 0.1 M Mes and 50 mM NaCl, pH 6.5. In both (A) and (B), the addition of Fe(II) initiates O_2 consumption. When catalase is absent during Fe(II) addition, an Fe(II)/O_2 stoichiometry of 2/1 is observed (lower curve). Subsequent addition of catalase to HuHF results in O_2 evolution, indicative of the presence of H_2O_2 (A, lower curve). Much less O_2 is evolved upon catalase addition to HoSF (B, lower curve). When catalase is present during Fe(II) addition, less net O_2 is consumed and an apparent stoichiometry of 4/1 is obtained in both HuHF and HoSF (see introduction).

We also measured H_2O_2 production in HuHF by the fluorescence method (Materials and Methods). Figure 2 illustrates a typical standard curve for the fluorescence assay with error bars in individual points indicated and the 99% confidence interval and the detection limit shown. The Amplex Red reagent/horseradish peroxidase cocktail was added to the protein solution 60 s after the addition of 48 Fe(II)/shell to 0.5 μM apoHuHF. An average of 11.3 ± 0.5 H_2O_2 per protein ($N = 3$) was detected or 47% of the expected 24 H_2O_2, in accord with the results from the catalase assay. In this instance 12.7 ± 0.5 H_2O_2 react per protein at an iron loading of 48 Fe/HuHF.

A similar set of experiments was performed on HoSF at pH 6.5. Since HoSF typically has only 2−4 ferroxidase sites per protein, an Fe/shell ratio of 6/1 and a higher protein concentration of 10 μM were employed (Figure 1B).[2] As with HuHF, the addition of catalase before Fe(II) addition resulted in an apparent Fe(II)/O_2 stoichiometry of 4/1 whereas in the absence of catalase during Fe(II) addition a stoichiometry of 2/1 was obtained, consistent with the production of H_2O_2 (Figure 1B). However, in contrast to HuHF, the

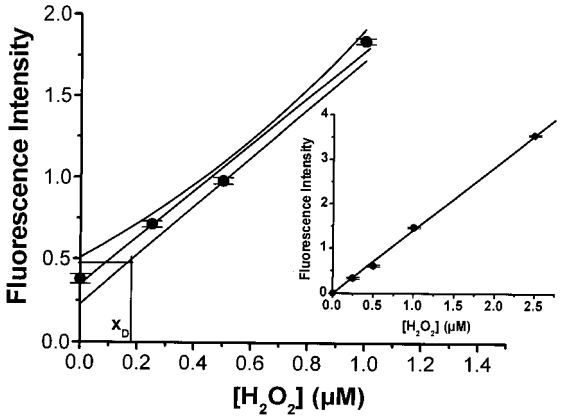

FIGURE 2: Standard curve for the fluorescence assay of hydrogen peroxide. Error bars are indicated for triplicate determinations. The 99% confidence interval is shown for the regression line of the lowest four data points (excluding the blank subtraction) from which a detection limit (x_D) of 0.180 μM for H_2O_2 is obtained. Inset: Complete standard curve including the blank correction. Solutions containing 200 μM Amplex Red reagent and 1 unit/mL horseradish peroxidase (HRP) plus the indicated concentration of H_2O_2 in 50 mM Mops, pH 7.4, were incubated for 30 min at room temperature before fluorescence measurement at 590 nm with excitation at 560 nm. In H_2O_2 measurements on the protein, the fluorescence of a blank containing the Amplex Red reagent, HRP, and apoferritin was subtracted from the fluorescence of the solution of the iron protein.

addition of catalase to Sigma apoHoSF after Fe(II) addition resulted in generation of a very small amount of O_2, corresponding to only 14 ± 3% of the 15 μM O_2 expected (Figure 1B, bottom curve). In another experiment under the same conditions but using apoprotein prepared from Sigma holoprotein (Materials and Methods), no H_2O_2 was detected upon addition of catalase following Fe(II) addition to the apoprotein. Thus H_2O_2 is generally difficult to detect in HoSF when catalase is added after Fe(II).

To confirm that the order of addition is critical to whether H_2O_2 can be detected in HoSF, we also employed the fluorescence assay. For 8 Fe(II)/shell added to apoHoSF (1.5 μM protein, 50 mM Mops, pH 7.4), one predicts from eq 1 that 6 μM H_2O_2 would be produced. When the reagents are added 30 s following Fe(II) addition to the protein and fluorescence measured 30 min later, we obtain a value of 0.97 ± 0.05 μM H_2O_2 ($N = 2$). This result compares with the higher value of 2.0 ± 0.1 μM H_2O_2 ($N = 2$) when the reagents are present during Fe(II) addition. Both of these values are well above the detection limit of 0.180 μM for the fluorescence method (Materials and Methods, Figure 2) but less than the theoretical amount, namely, 16% and 33% of the maximum 6 μM, respectively.[3] Thus both the catalase and fluorescence assays demonstrate H_2O_2 production in HoSF but indicate that much of the H_2O_2 produced is rapidly consumed after its formation. The full amount of H_2O_2 produced at the ferroxidase site can only be quantitatively measured when catalase is present during the addition of Fe(II), in which case an apparent Fe(II)/O_2 stoichiometry of 4/1 is observed for both HuHF and HoSF (Figure 1, upper curves).

[3] The presence of Fe(II) is known to enhance fluorescence in this assay, but its effect can be eliminated by the addition of EDTA (*24*). We therefore added 5 μL of 12 mM EDTA after Fe(II) addition to the 1.0 mL samples; however, it had no effect on the observed fluorescence intensity.

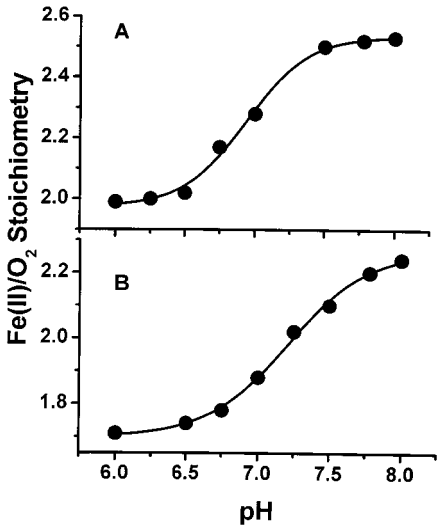

FIGURE 3: Dependence of the Fe(II)/O_2 stoichiometry on pH for (A) HuHF [1 μM, 48 Fe(II)/shell] and (B) HoSF [6 μM, 8Fe(II)/shell] in 50 mM Mes/50 mM Mops/50 mM NaCl, pH 6−8.

We also determined the pH dependence of the Fe(II)/O_2 stoichiometry for HuHF and HoSF since pH may have an effect on H_2O_2 production. For both proteins, the Fe(II)/O_2 stoichiometry increases with increasing pH and follows similar profiles (Figure 3). The stoichiometry with HuHF changes from 1.98/1 at pH 6.0 to 2.53/1 at pH 8.0 whereas with HoSF it changes from 1.72/1 to 2.25/1 over the same pH range. These data reflect the increased fraction of iron that undergoes autoxidation at the higher pH, a process that occurs with a stoichiometry of 4/1 (eq 3) (*6*, *11*, *13*). A general upward trend in Fe(II)/O_2 stoichiometry with pH has been noted previously with HoSF (*7*). Over the entire pH range, the observed stoichiometries are still near 2/1 and imply that significant H_2O_2 production occurs even at the highest pH. Nevertheless, most studies of the ferroxidation reaction in ferritin have been carried out at or below pH 7 to avoid complications from the Fe(II) autoxidation reaction. For HoSF the somewhat low Fe(II)/O_2 stoichiometry of 1.72 at pH 6.0 perhaps reflects a small amount of superoxide production at this low pH.

Experiments were also performed in an attempt to determine the fate of the H_2O_2 generated during iron(II) oxidation. In our initial experiments, we examined whether added H_2O_2 reacts directly with either apoHoSF or apoHuHF. When 28 μM H_2O_2 was added to 10 μM Sigma apoHoSF, which is prepared using thioglycolate, 10.0 μM O_2 evolved upon addition of catalase, corresponding to the disproportionation of 20.0 μM H_2O_2 (Figure 4A). This finding indicates that 8.0 μM H_2O_2 reacts with 10 μM apoHoSF or only 0.8 H_2O_2 per shell. For apoHoSF prepared from Sigma holoprotein using dithionite (Materials and Methods), no reaction of H_2O_2 with the protein was observed (data not shown). Figure 4B illustrates a control experiment where 28 μM H_2O_2 is added to buffer alone followed by catalase 150 s later. Evolution of 13.7 μM O_2 occurs, corresponding to the disproportionation of 27.4 μM H_2O_2 or 98% of the amount of H_2O_2 added. Thus, measurement of H_2O_2 added to buffer is essentially quantitative.

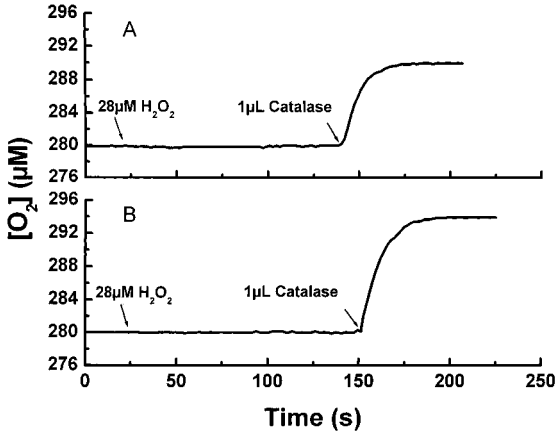

FIGURE 4: Reaction of H_2O_2 with (A) apoHoSF (10 μM) and (B) blank (buffer alone). H_2O_2 (28 μM) was added to the protein solution (A) or to buffer (B) followed by 1300 units of catalase 150 s later. The amount of O_2 evolved (eq 2) is a measure of the H_2O_2 remaining after 150 s. Only 0.8 H_2O_2 per apoHoSF was not measured. The buffer was 0.1 M Mes and 50 mM NaCl, pH 6.5.

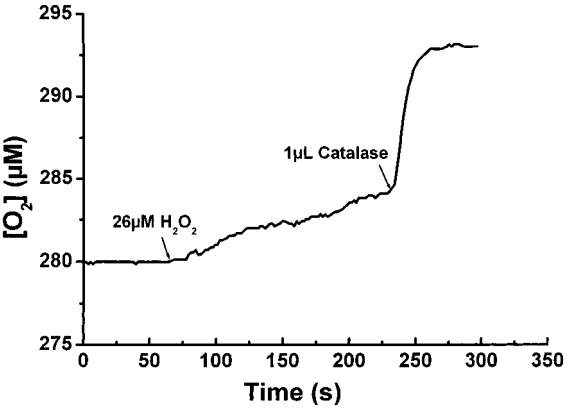

FIGURE 5: Reaction of H_2O_2 with apoHuHF (1 μM). H_2O_2 (26 μM) was added to the protein solution followed by 1300 units of catalase 165 s later. The amount of O_2 evolved (eq 2) is a measure of the H_2O_2 remaining after 165 s. All of the added H_2O_2 was measured. The buffer was 0.1 M Mes and 50 mM NaCl, pH 6.5.

Experiments with apoHuHF yielded similar results (Figure 5). Addition of 26 μM H_2O_2 to 1 μM apoHuHF resulted in the slow evolution of O_2, an indication that the apoHuHF solution has weak catalase activity itself (Figure 5). Subsequent addition of catalase causes a pronounced evolution of O_2. A total of 13.0 μM O_2 evolved during the entire experiment, indicating measurement of all the 26 μM H_2O_2 added.

Figure 6 illustrates an experiment to determine whether the presence of iron(III) in the HuHF prior to the addition of H_2O_2 facilitates reaction of H_2O_2 with the protein. Fe(II) was added to 2 μM apoHuHF to give an Fe(II)/shell ratio of 48/1 and allowed to completely oxidize overnight. Then 56 μM H_2O_2 was added, followed by catalase 120 s later (Figure 6). Again the protein exhibits some catalase activity as evidenced by the slow evolution of O_2 following H_2O_2 addition. Subsequent addition of the enzyme catalase further augments O_2 evolution (Figure 6). A total of 21 μM O_2 was generated, corresponding to the disproportionation of 42 μM H_2O_2. Thus 14 μM H_2O_2 has reacted, or 7.0 H_2O_2 per HuHF molecule. The average H_2O_2 reaction stoichiometry for three protein preparations was 7.8 $\pm$ 0.8 H_2O_2 per HuHF. A similar experiment with HoSF containing 8 Fe(III)/shell gave a

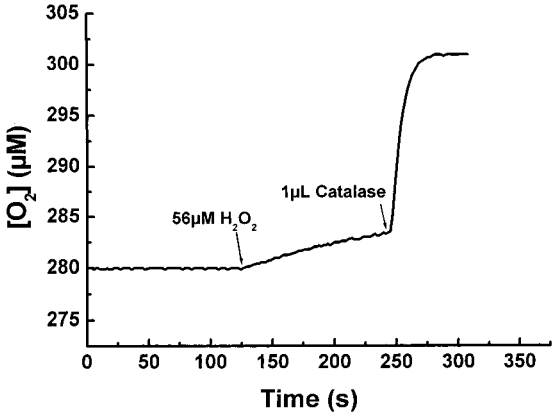

FIGURE 6: Reaction of H_2O_2 with HuHF (2 μM, 48 Fe/shell). H_2O_2 (56 μM) was added to the protein solution followed by 1300 units of catalase 2 min later. The amount of O_2 evolved (eq 2) is a measure of the H_2O_2 remaining after 2 min. 7.8 $\pm$ 0.8 (N = 3) H_2O_2 per HuHF were not recovered. The buffer was 0.1 M Mes and 50 mM NaCl, pH 6.5.

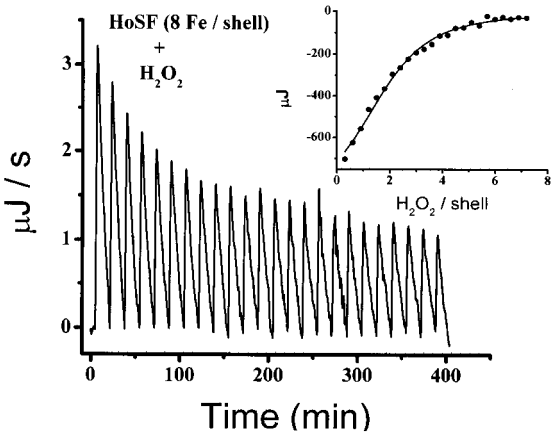

FIGURE 7: Measurement of the reaction of H_2O_2 with horse spleen ferritin by isothermal titration calorimetry. 1.3 mL of 5 μM HoSF containing 8 Fe(III)/shell was titrated with 10 μL injections of 0.195 mM H_2O_2 in buffer at 25 °C. The buffer was 0.1 M Mes and 50 mM NaCl, pH 6.5. Inset: Integrated heat as a function of the H_2O_2 added. The data were fitted to a binding isotherm with n = 1.92 $\pm$ 0.22, $\Delta H°$ = $-$482 $\pm$ 76 kJ/mol, and K = 2.8 ($\pm$0.9) $\times$ 10^5 M^{-1}.

stoichiometry of 2.7 $\pm$ 0.4 (N = 3) H_2O_2 per protein molecule. A higher H_2O_2 reaction stoichiometry of 3.4 $\pm$ 0.3 (N = 3) per molecule was obtained for apoHoSF previously loaded with 24 Fe(III). Sigma holoHoSF containing ~2600 Fe(III) gave a stoichiometry of 36.0 $\pm$ 1.0 H_2O_2/shell (N = 8). Thus the amount of H_2O_2 reacting with HoSF increases with increasing iron content of the protein.

Isothermal titration calorimetry (ITC) experiments were also carried out to confirm the reaction stoichiometries of H_2O_2 with HoSF. In the ITC experiment, the heat of reaction is measured as the protein is titrated with H_2O_2. Figure 7 shows the titration of 5 μM HoSF previously loaded with 8 Fe(II)/shell and allowed to completely oxidize overnight. The area under the peak upon each addition of H_2O_2 corresponds to the enthalpy. The inset of the graph shows the reaction isotherm from the integrated heat and curve fit, from which a stoichiometry of 1.9 $\pm$ 0.2 H_2O_2/shell at 25 °C is obtained.[4] The stoichiometry obtained from the ITC experiment is somewhat lower than the value of 2.7 $\pm$ 0.4 derived from the catalase experiment. ITC measurements with Sigma holoHoSF containing ~2600 Fe(III) were also carried out.

In this instance the thermochemistry of the reaction of the H_2O_2 with the protein was superimposed on that from significant disproportionation of the H_2O_2, precluding a detailed analysis of the data. However, the protein reaction was seen to be complete after 30−40 H_2O_2/shell had been added (data not shown), a result consistent with the H_2O_2/shell stoichiometry of 36/1 from the above catalase experiment. An ITC experiment with HuHF containing 48 Fe(III) gave an H_2O_2 reaction stoichiometry of 11 ± 2 H_2O_2/shell as found in the catalase experiment.

ITC measurements were also carried out with the apoprotein. Isothermal titration of apoHuHF showed only disproportionation of H_2O_2. The measured enthalpy $\Delta H° = -103 \pm 6$ kJ/mol with apoHuHF compares with the value of -110 ± 2 kJ/mol when H_2O_2 was added to a solution of catalase alone at 25 °C and with the literature value of −98.4 ± 0.2 kJ/mol at 20 °C (*32*). Titration of apoHoSF revealed very weak catalase activity as evidenced by the slow production of heat, $\Delta H° = -107 \pm 17$ kJ/mol, and no end point corresponding to reaction with the protein.[5] Thus, the ITC measurements fully support the findings of the experiments with catalase, namely, that iron has to be present for any reaction with the protein to occur. Also, similar H_2O_2 reaction stoichiometries are obtained with oximetry and ITC measurements.

DISCUSSION

We have demonstrated that H_2O_2 is produced during the oxidative deposition of iron in both HoSF and HuHF (Results). The evolution of O_2 upon addition of catalase to HuHF following Fe(II) addition is a clear indication of hydrogen peroxide production (Figure 1A), a finding also confirmed by the fluorescence method. Approximately 50% of the theoretical amount of H_2O_2 was measured with the catalase assay, in accord with previous work with BfHF showing generation of 30−50% of the expected amount of O_2 upon addition of catalase following Fe(II) oxidation in this protein (*13*). In contrast, the amount of H_2O_2 produced in apoHoSF is decidedly less than in HuHF but still can be readily observed if the fluorescence assay reagents or catalase are present during iron addition (Figure 1, Results). H_2O_2 can also be detected in HoSF when catalase or fluorescence reagents are added after the Fe(II) although the amount measured is considerably reduced, ∼15% of the theoretical amounts by the two methods, respectively (Figure 1, Results). These levels are well above the detection limits of the two analytical methodologies (Figure 2, Materials and Methods).

That some of the H_2O_2 produced subsequently reacts with the protein and/or iron is illustrated by the dependence of the results on the order of addition of catalase (Figure 1). The 4/1 Fe(II)/O_2 stoichiometry observed when catalase is present prior to Fe(II) addition indicates that H_2O_2 is quantitatively produced at the ferroxidase site. The H_2O_2 so produced must be released into the bulk solution where it can diffuse out of the protein cavity and react with the catalase (eq 2). Otherwise, no change in the Fe(II)/O_2 reaction stoichiometry from 2/1 to 4/1 would have been observed when the reaction is run with catalase present. Furthermore, the reaction with catalase must occur more rapidly than the reaction of H_2O_2 with the iron-containing protein; otherwise, a change in stoichiometry from 2/1 to 4/1 in both HuHF and HoSF would not have been observed. The fluorescence assay is less efficient than the catalase assay at detecting all of the H_2O_2 produced in HoSF since a value of 2.0 μM is measured compared to the 6 μM predicted from eq 1 (Results). The fluorescence assay for H_2O_2 appears to be kinetically controlled since H_2O_2 reacts more readily with the ferritin than with the Amplex Red reagent in the presence of horseradish peroxidase.

The small amount of detectable H_2O_2 in HoSF by catalase addition or by addition of the fluorescence reagents after Fe(II) addition most likely accounts for the failure of Watt and co-workers (*24*) to observe hydrogen peroxide production in HoSF. Their reported H_2O_2 measurements appear to have been always made by adding reagents after iron(II) (*24*). If either catalase or the fluorescent assay reagents are present when Fe(II) is added to apoHoSF, the presence of H_2O_2 is quite apparent (Figures 1 and 2, Results). If the experiments had employed HuHF where H_2O_2 is generated in much greater amounts relative to the amount of protein present, H_2O_2 would almost certainly have been detected using one or all three of the analytical methods independent of the order of addition of iron.

Given that H_2O_2 is produced in vertebrate ferritins, is it unclear why an Fe(II)/O_2 stoichiometry of 3.6 for HoSF was obtained from differential pulsed polarography measurements (*24*), a stoichiometry implying nearly complete reduction of O_2 to water (eq 3). Previous stoichiometric measurements on HoSF employing mass spectrometry or electrode oximetry have obtained a value of 2/1 at pH 7.0, consistent with reduction of O_2 to H_2O_2 (eq 1) (*6*, *9*−*11*, *13*, *14*), a stoichiometry in accord with early measurements of Harrison and co-workers on HoSF (*7*). Thus the reported stoichiometry of ∼4/1 for HoSF from polarography measurements (*24*) is at variance with previous findings with horse, human, and bullfrog ferritins (see introduction) and remains unexplained.

The present data further show that, under the conditions of these experiments, H_2O_2 does not react significantly with apoHuHF or with apoHoSF prepared from the holoproteins using dithionite (Figures 4 and 5). The small amount of H_2O_2 reacting with Sigma apoHoSF preparations, ∼0.8 H_2O_2 per protein (Results), is probably due to trace amounts of thioglycolate strongly associated with the commercial apoprotein that is difficult to remove by exhaustive dialysis. The commercial preparation of apoferritin has a strong odor of thioglycolate. Sigma apoHoSF is the only protein used in the present work that had been exposed to thioglycolate.

The presence of Fe(III) in the human and horse proteins is required for the reaction of H_2O_2 with them (Figures 6 and 7, Results). In HoSF there is a general increase in reaction stoichiometry from ∼2 to 36 H_2O_2 per protein molecule as the iron content increases from 8 to ∼2600 Fe(III)/shell. The H_2O_2 chemistry observed here may be partly responsible for the reported radical production in ferritin (*33*, *34*) and alteration in the protein upon repeated

[4] In Figure 7, the observed heats at times greater than 250 min correspond to the slow disproportionation of H_2O_2. Because this heat was assumed to be the same throughout the reaction and subtracted from earlier values prior to constructing the isotherm, the parameters $\Delta H°$ and K (Figure 7, caption) from curve fitting may have a systematic error. The measured stoichiometry, i.e., end point, is reliable, however.

[5] Injections were spaced 20 min apart because of the slow disproportionation reaction observed with the ferritins. Due to the slow return to the baseline following each injection, the measured enthalpies for H_2O_2 disproportionation may be underestimated by as much as 10%.

additions of iron (*35*, *36*) and for the increased susceptibility of the holoprotein toward proteolysis upon exposure to H_2O_2 (*37*). Such processes may contribute to the degradation of ferritin to hemosiderin (*38*). A more detailed investigation of the stoichiometry of the H_2O_2 reaction with ferritin as well as spectroscopic and chemical characterization of the reaction products is currently in progress.

In summary, the present studies confirm and extend previous work on the oxidation chemistry of iron deposition in the ferritins. That hydrogen peroxide is produced in vertebrate ferritins has been conclusively shown and its reaction with the iron protein complex revealed. The observed reaction of H_2O_2 with ferritin (Figures 1 and 7) may help to protect the cell against the deleterious effects of the hydrogen peroxide produced during the initial stage of ferritin mineralization. After sufficient mineral has formed, the dominant iron oxidation and hydrolysis reactions proceed by eq 3 where active oxygen species are no longer generated.

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NOTE ADDED IN PROOF

The measurements of Watt and co-workers (*24*) were taken at a time interval where the H_2O_2 would have been consumed by a subsequent unknown reaction (G. D. Watt, personal communication).

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