THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 40, Issue of October 4, pp. 37064–37069, 2002

Printed in U.S.A.

Iron Detoxification Properties of *Escherichia coli* Bacterioferritin

ATTENUATION OF OXYRADICAL CHEMISTRY*

Received for publication, June 10, 2002
Published, JBC Papers in Press, July 17, 2002, DOI 10.1074/jbc.M205712200

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Bacterioferritin (EcBFR) of *Escherichia coli* is an iron-mineralizing hemoprotein composed of 24 identical subunits, each containing a dinuclear metal-binding site known as the "ferroxidase center." The chemistry of Fe(II) binding and oxidation and Fe(III) hydrolysis using H_2O_2 as oxidant was studied by electrode oximetry, pH-stat, UV-visible spectrophotometry, and electron paramagnetic resonance spin trapping experiments. Absorption spectroscopy data demonstrate the oxidation of two Fe(II) per H_2O_2 at the ferroxidase center, thus avoiding hydroxyl radical production via Fenton chemistry. The oxidation reaction with H_2O_2 corresponds to $[Fe(II)_2\text{-}P]^Z + H_2O_2 \rightarrow [Fe(III)_2O\text{-}P]^Z + H_2O$, where $[Fe(II)_2\text{-}P]^Z$ represents a diferrous ferroxidase center complex of the protein P with net charge Z and $[Fe(III)_2O\text{-}P]^Z$ a μ-oxo-bridged diferric ferroxidase complex. The mineralization reaction is given by $2Fe^{2+} + H_2O_2 + 2H_2O \rightarrow 2FeOOH_{(core)} + 4H^+$, where two Fe(II) are again oxidized by one H_2O_2. Hydrogen peroxide is shown to be an intermediate product of dioxygen reduction when O_2 is used as the oxidant in both the ferroxidation and mineralization reactions. Most of the H_2O_2 produced from O_2 is rapidly consumed in a subsequent ferroxidase reaction with Fe(II) to produce H_2O. EPR spin trapping experiments show that the presence of EcBFR greatly attenuates the production of hydroxyl radical during Fe(II) oxidation by H_2O_2, consistent with the ability of the bacterioferritin to facilitate the pairwise oxidation of Fe(II) by H_2O_2, thus avoiding odd electron reduction products of oxygen and therefore oxidative damage to the protein and cellular components through oxygen radical chemistry.

The ferritins are a family of iron storage proteins that reversibly sequester iron in a mineral form that can be mobilized for intracellular metabolism. Ferritins from *Escherichia coli* are of two types: the non-heme bacterial ferritins EcFtnA[1] and EcFtnB, and the heme-containing bacterioferritin EcBFR. Although EcFtnA is a true ferritin with an iron storage role, the function of EcBFR is less clear (1). Both types of ferritin are composed of 24 identical subunits each containing ferroxidase and nucleation sites (1–5). These sites are important for rapid oxidation of Fe(II) to Fe(III) and its subsequent hydrolysis to form a mineral core on the inner surface of the protein. In the *E. coli* bacterioferritin, up to 12 protoporphyrin IX heme groups of unknown function bind between 2-fold symmetry-related subunits and are ligated by methionines Met-52 and Met-52′. Although the hemes do not possess catalase activity, they may be involved in mediating iron-core reduction and iron release from the protein (1–3, 6).

The three-dimensional structures of ferritins from different origins are highly conserved despite homology as low as 15% in amino acid sequence in some cases. The typical 24-mer ferritins assemble in highly stable complexes with 4/3/2 symmetry, forming a large central cavity of about 80 Å diameter which can accommodate up to 4500 iron atoms as a ferric hydrous oxide mineral core. Whereas ferritins from various organisms share many common structural features, their chemistries of iron deposition differ significantly (1, 2, 6–11). Such differences are undoubtedly important for the physiological function of each protein in iron storage and detoxification. Most notably, hydrogen peroxide is the product of dioxygen reduction in the ferroxidase reaction of mammalian ferritins, whereas water is produced in EcBFR and in the 12-mer ferritin from *Listeria innocua* (LiFtn) (6, 9, 11).

To account for the complete reduction of O_2 to H_2O in EcBFR, two ferroxidation mechanisms have been proposed (6), one involving a high valent iron oxidation state (Fe^{4+}) similar to that of the di-iron enzymes ribonucleotide reductase and methane monooxygenase (12, 13), and the other involving the reduction of O_2 to H_2O_2 at one di-iron ferroxidase center followed by rapid reduction of H_2O_2 to H_2O at a second di-iron ferroxidase center. The previous observation that H_2O_2 is a better oxidant for Fe(II) in bacterioferritin than O_2 (6) is consistent with the latter mechanism and has implications for the role of the protein in avoiding Fenton chemistry inherent in the odd electron oxidation of iron(II) by H_2O_2 (Equation 1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\cdot} \quad \text{(Eq. 1)}$$

To date, there has been no study demonstrating the production of H_2O_2 in EcBFR when O_2 is the Fe(II) oxidant nor has the reaction between Fe(II) and H_2O_2 in EcBFR been examined in any detail. We describe here experiments that further explore the iron redox chemistry of EcBFR. The data reveal that H_2O_2 is a better oxidant than O_2 for Fe(II) at the dinuclear ferroxidase center and that Fe(II) is oxidized pairwise by H_2O_2, thus

* This work was supported by NIGMS Grant R37 GM20194 from the National Institutes of Health (to N. D. C.) and by the Biotechnology and Biology Science Research Council (to G. R. M and N. L. B). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: EcFtnA, *E. coli* bacterial ferritin type A; EcBFR, *E. coli* bacterioferritin; EcFtnB, *E. coli* bacterial ferritin type B; HuHF, recombinant human H-chain ferritin; Dps, DNA-binding protein from starved cells; Mops, 3-(*N*-morpholino)propanesulfonic acid; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

avoiding hydroxyl radical production through Fenton chemistry (Equation 1). A stoichiometry of one H_2O_2 per two Fe(II) oxidized is also obtained in the mineralization reaction of EcBFR. Ultraviolet spectroscopic titration data in conjunction with the observed hydrolysis chemistry suggest formation of a μ-oxo-bridged complex at the ferroxidase center of the protein when either H_2O_2 or O_2 are oxidants for Fe(II). A fluorescence assay demonstrates that H_2O_2 is an intermediate product of O_2 reduction during both the ferroxidase and mineralization reactions. Most of this H_2O_2 is then rapidly consumed in subsequent Fe(II) oxidation without generating radicals, an indication that the protein is capable of nullifying the toxic combination of Fe(II) and H_2O_2.

MATERIALS AND METHODS

The purification of EcBFR from cell extracts obtained from an overexpression system was achieved by methods described previously (14) but using an overexpression system different from the earlier one, *i.e.* a pET vector instead of the previous pALTER. Plasmid pGS758 (6), which contained the gene for *E. coli* bacterioferritin, was digested with *Nde*I/*Eco*RI, and the fragment containing the *bfr* gene was ligated into pET21a (Novagen) digested with *Nde*I/*Eco*RI. The resulting plasmid was used to transform *E. coli* strain BL21 (DE3). 250-ml cultures were grown in LB medium containing 100 μg/ml ampicillin to an $A_{650\ nm}$ of 0.8–0.9 and then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (final concentration). After induction for 3 h, cells were harvested, washed, and stored at −70 °C until use. The heme content of the purified EcBFR was ~2.5 heme per 24-mer.

Non-heme iron was removed from EcBFR by reduction with sodium dithionite and complexation with 2,2′-bipyridyl (15). The protein concentration was determined spectrophotometrically using an absorptivity of 33,000 cm^{-1} M^{-1} at 280 nm for the apoprotein (6) or by the Advanced Protein Assay (www.cytoskelton.com, patent pending). Freshly prepared stock solutions of H_2O_2 were assayed by the amount of O_2 produced upon addition of catalase as measured by Clark-type electrode oximetry or from its absorbance at 240 nm (ϵ = 43.6 cm^{-1} M^{-1}). The enzyme catalase (EC 1.11.1.6, 65,000 units/mg) was purchased from Roche Molecular Biochemicals, the Amplex Red hydrogen peroxide assay kit from Molecular Probes (Eugene, OR), and EMPO from Oxis Research (Portland, OR). All other compounds including Mes and Mops buffers, sodium chloride, and ferrous sulfate were of reagent grade quality.

The use and standardization reactions of the oxygen electrode/pH-stat apparatus are described in detail elsewhere (6, 9). For the anaerobic experiments, all solutions were thoroughly deoxygenated using high purity grade argon gas (99.995%, <5 ppm O_2). The evolution of O_2 after the addition of catalase to H_2O_2 containing EcBFR solutions or O_2 consumption during Fe(II) oxidation in EcBFR was followed either at room temperature (22 °C) or at 25 °C by a Clark-type oxygen microelectrode. Typical conditions for measurements were 1 μM protein in 100 mM NaCl and 0.3 mM Mes, pH 6.5 to 7.0 (controlled by the pH-stat), with increments of 24–48 Fe(II)/protein, added as freshly prepared ferrous sulfate solution at pH 3.5 (J. T. Baker Inc.). The addition of 0.3 mM Mes buffer to the protein solution improved the stability of the pH-stat control without significantly buffering the solution. Background corrections for the free acid in the ferrous sulfate solutions were made in all calculations. Conditions for each experiment are given in the figure legends.

The ultraviolet-visible difference spectrophotometric titrations of Fe(II) oxidation by H_2O_2 in EcBFR were performed on a Cary 50 spectrophotometer. For the anaerobic experiments, 1 ml of deoxygenated apo-EcBFR solution was added to an argon-purged quartz cuvette with a micro stir bar at the bottom. A constant positive atmosphere of pure argon was maintained through the rubber septum of the cuvette during the experiment to avoid oxygen diffusion into the cell. An apoprotein solution in the same buffer was used as a blank for absorbance measurements. The experiments were terminated when no further increase in the absorbance was seen following the addition of Fe(II) to the protein solution.

EPR measurements were carried out on a laboratory assembled EPR spectrophotometer described elsewhere (16). In the EMPO spin trapping experiments for hydroxyl radical (17), the spectra were recorded immediately after the addition of the last reagent. The conditions of the experiment and the parameters of the spectrometer are indicated in the figure legends.

In the Amplex fluorescence assay for H_2O_2, 50 mM Mops buffer, pH 7.4, was used in place of the 50 mM phosphate buffer, pH 7.4, supplied with the assay kit in order to avoid possible interference by the phosphate on the iron chemistry in ferritin. Fluorescence standard curves were constructed using duplicate samples and measured at the same time as the unknowns which were both incubated for 30 min at room temperature and protected from light. Fluorescence intensity was measured on a Varian Cary Eclipse fluorimeter using excitation and emission wavelengths of 560 and 590 nm, respectively. The detection limit of the Amplex H_2O_2 fluorescence assay and the standard deviation in the fluorescence intensity measurements are reported elsewhere (18).

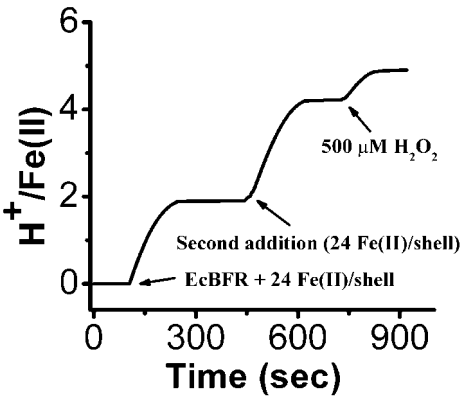

FIG. 1. **Proton production curve *versus* time for two sequential anaerobic additions of 24 Fe(II)/shell (24 μM Fe(II)) followed by 500 μM H_2O_2.** Conditions: 1.0 μM EcBFR in 0.3 mM Mes, 100 mM NaCl, pH 6.98, 25 °C. Stock solutions of Fe(II) (16 mM) and H_2O_2 (166 mM) were prepared in water pH 3.5.

RESULTS

Previous studies of iron uptake by EcBFR using O_2 as oxidant have shown that Fe(II) binding at the dinuclear centers of the protein followed by its rapid oxidation and core formation occur in three distinct phases (6, 19). We describe the stoichiometric equations and the kinetics of iron oxidation and hydrolysis in EcBFR using H_2O_2 as the oxidant. We demonstrate for the first time that H_2O_2 is an intermediate product of iron oxidation by O_2 in EcBFR and report that bacterioferritin minimizes the production of highly reactive hydroxyl radicals generated by the Fenton reaction (Equation 1) facilitating pairwise oxidation of Fe(II) by H_2O_2.

Fe(II) Binding and Oxidation in EcBFR by H_2O_2—The number of protons produced upon Fe(II) binding to the protein followed by oxidation with H_2O_2 was monitored by titration with standard NaOH (5 mM) to maintain the pH at 7.0 with the pH-stat apparatus. The anaerobic addition of 24 Fe(II) to the apoprotein in the absence of H_2O_2 resulted in the production of two protons per Fe(II) added (first and second injections, Fig. 1), consistent with previous results obtained with EcBFR at pH 6.5 (6).[2] The binding reaction for this first step is thus written as shown in Equation 2,

$$2Fe^{2+} + P^Z \rightarrow [Fe(II)_2{-}P]^Z + 4H^+ \qquad \text{(Eq. 2)}$$

where $[Fe(II)_2\text{-}P]^Z$ represents a diFe(II)-protein complex at the ferroxidase center.

To determine the number of protons produced during Fe(II) oxidation by H_2O_2 in EcBFR, excess H_2O_2 (10 H_2O_2/Fe(II)) was added to the protein solution containing 48 Fe(II)/shell, added in increments of 24 Fe(II)/shell as shown in Fig. 1. The amount of base delivered after the addition of H_2O_2 (third injection, Fig. 1) corresponds to that required to neutralize the free acid in the

[2] To accurately measure the number of protons released upon Fe^{2+} binding, a pH-stat proportional band setting of 1.0 was used, and a relatively slow response was seen. However, the rapid response reported in Ref. 6 was due to the smaller proportional band setting of 0.2 required to measure the initial rate of proton production.

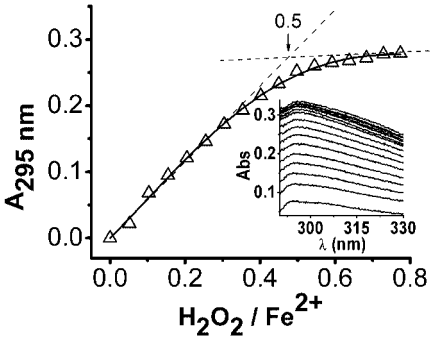

FIG. 2. **Spectrometric titration of apo-EcBFR containing 48 Fe(II)/shell (76.8 μM Fe(II)) with H_2O_2 under anaerobic conditions.** *Inset*, family of UV-visible difference spectra with EcBFR (1.6 μM) in 0.1 M Mes, 50 mM NaCl, pH 6.50. Each addition corresponds to 0.05 H_2O_2/Fe(II). The smooth curve through the data points is an empirical fit to a 4th order polynomial constrained to pass through the origin. The *straight lines* are computed tangents evaluated at the beginning and end of the fitted curve.

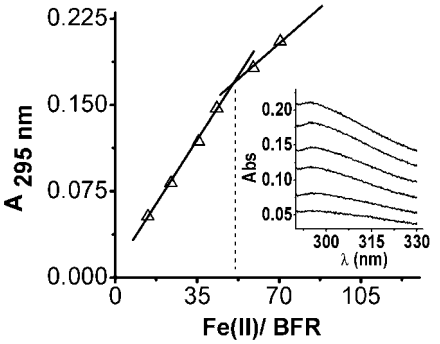

FIG. 3. **Spectrometric titration of apo-EcBFR with ferrous sulfate in the presence of H_2O_2.** Conditions: 1 μM EcBFR, 0.1 M Mes, 50 mM NaCl, pH 6.50. Each point represents a separate sample where various amounts of $FeSO_4$ (14, 24, 36, 43, 59, and 70 Fe(II)/shell, respectively) were added anaerobically to the apoprotein followed immediately by 120 μM H_2O_2.

hydrogen peroxide solution, which was prepared at pH 3.5 in deoxygenated water. In another experiment, either 24 or 48 Fe(II)/shell were added anaerobically to the protein solution followed immediately by the addition of H_2O_2. In both cases, no H^+ was produced.

The anaerobic addition of Fe(II) to EcBFR showed no UV absorbance, but upon the addition of H_2O_2 to the protein solution containing 48 Fe(II)/shell, a band with a maximum absorbance at 295 nm emerged (Fig. 2, *inset*). The spectrometric titration shown in Fig. 2 has a discontinuity in absorbance at 0.5 H_2O_2/Fe(II), indicating that one H_2O_2 oxidizes two Fe(II).[3] Because two Fe(II) are oxidized by one H_2O_2 and no H^+ is generated upon H_2O_2 addition, we write Equation 3 for the oxidation reaction as follows:

$$[Fe(II)_2{-}P^Z] + H_2O_2 \rightarrow [Fe(III)_2O\text{-}P]^z + H_2O \qquad \text{(Eq. 3)}$$

where $[Fe(III)_2O\text{-}P]^Z$ represents a μ-oxo-bridged species at the dinuclear center of the protein.

The absorptivity for the observed oxidation product $[Fe(III)_2O\text{-}P]^Z$ is 3460 M^{-1} cm^{-1} per iron at 295 nm, which is very similar to the value of 3380 M^{-1} cm^{-1} observed for EcBFR when O_2 was the oxidant (6). Similar spectrometric titrations have been reported for human H-chain and horse spleen ferritins with absorptivities of 2990 and 3540 M^{-1} cm^{-1}, respectively (9). Absorbances within this spectral region of ferritins and other dinuclear Fe(III)-proteins generally arise from μ-oxo-bridged Fe(III) dimer species (6, 9, 20–22). Therefore, we attribute the absorbing species seen in Fig. 2 to a μ-oxo-bridged dinuclear complex formed at the ferroxidase center of the protein. This assignment is also based on data from a preliminary Mössbauer study with EcBFR (23) and spectrometric titration data for EcBFR using O_2 as the Fe(II) oxidant (6).

In order to determine the Fe(II)/protein stoichiometry for Fe(II) oxidation, a careful anaerobic spectrometric titration of EcBFR with Fe(II) followed by addition of H_2O_2 was carried out. Excess H_2O_2 was added to different samples of apo-EcBFR previously loaded with various amounts of Fe(II) (see Fig. 3 legend). A discontinuity in absorbance at 48 Fe(II)/protein (Fig. 3) is evident, consistent with the binding and oxidation of two iron at each of the 24 ferroxidase centers of the protein. Similar UV titrations have been reported (6, 9) for the ferroxidation reactions of EcBFR and HuHF with O_2 as the oxidant. The result in Fig. 3 implies that the 24 ferroxidase centers of EcBFR are involved in the rapid pairwise oxidation of Fe(II) by H_2O_2 and that Equation 3 corresponds to the ferroxidation reaction.

Fig. 4 shows the effect of catalase on the amount of unreacted H_2O_2 when 48 Fe(II)/protein shell are added anaerobically to 1 μM EcBFR followed by 48 μM H_2O_2. For the addition sequence EcBFR + 48 Fe(II) + 48 H_2O_2 + catalase (added 150 s later), about 12 μM O_2 is produced, implying that 24 μM H_2O_2 had reacted with Fe(II) to give an Fe(II)/H_2O_2 stoichiometry of 2:1 (Fig. 4), a result in accord with the absorbance measurements of Fig. 2. In the control experiment, evolution of 24 μM O_2 was observed when 48 μM H_2O_2 were added to buffer alone followed by catalase as expected from the disproportionation reaction of H_2O_2 (Equation 4),

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2}\, O_2 \qquad \text{(Eq. 4)}$$

EcBFR has a specific activity of ~2 O_2/subunit/min at pH 7.0 (two Fe(II)/subunit) when O_2 is the oxidant for Fe(II) (6). To determine the relative reactivities of H_2O_2 *versus* O_2 toward Fe(II) oxidation in EcBFR, 48 Fe(II)/shell were added to a protein sample containing both 48 μM H_2O_2 (48 H_2O_2/shell) and 260 μM O_2 (260 O_2/shell).[4] In a series of three experiments, an average of 30 ± 5% of the oxygen was consumed relative to that in the control experiment where H_2O_2 was absent. In another series of experiments, 160 Fe(II)/shell were added to the apoprotein in the presence of 260 μM O_2 (2600 O_2/shell) and 270 μM H_2O_2 (2700 H_2O_2/shell). In this case only 8.5% of the O_2 was consumed compared with the control experiment in the absence of H_2O_2. In these experiments the amount of each oxidant was sufficient to oxidize all the iron present in the solution. Both types of experiments indicate that in EcBFR H_2O_2 is a 10-fold faster oxidant of Fe(II) than O_2 and thus is a superior substrate for the ferroxidation reaction.

Fe(II) Mineralization in EcBFR—The stoichiometry of Fe(II) oxidation by H_2O_2 was also determined when Fe(II) was added beyond the 48 Fe(II)/shell required for saturation of the ferroxidase sites. The anaerobic addition of 500 Fe(II)/protein followed by H_2O_2 titration resulted in an increase in the absorbance intensity (Fig. 5, *inset*), giving a ratio of two Fe(II)/H_2O_2 (Fig. 5). The absorptivity of the mineralized iron was determined to

[3] The increase in absorbance beyond the 48 Fe(II)/protein ratio seen in Figs. 2 and 5 was due to the slight excess of H_2O_2 needed to fully oxidize all the iron added and does not indicate a two-step oxidation process. The ratio of H_2O_2/Fe^{2+} was determined from the intercept of the two computed tangents.

[4] A small amount of Fe(II) added to the apoprotein undergoes Fenton chemistry in the presence of H_2O_2 (see EPR results). Therefore, the greater reactivity of H_2O_2 *versus* O_2 has contributions from the pairwise oxidation of Fe(II) by H_2O_2 at the ferroxidase center of the protein and from Fe(II) reaction with H_2O_2 via Fenton reaction.

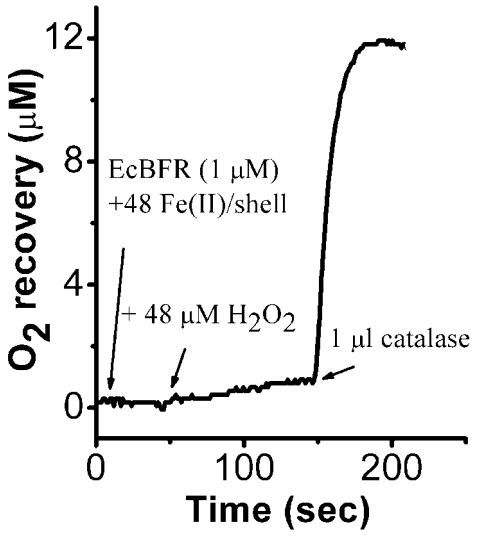

FIG. 4. **Reaction of H_2O_2 with the iron containing EcBFR (1 μM, 48 Fe(II)/shell, 0.3 mM Mops, 50 mM NaCl, pH 7.0).** H_2O_2 (48 μM) was added to the protein solution 50 s after the anaerobic addition of iron followed by 1 μl of catalase (1300 units) 150 s later. The amount of O_2 recovered corresponds to the amount of H_2O_2 remaining after 150 s of the reaction.

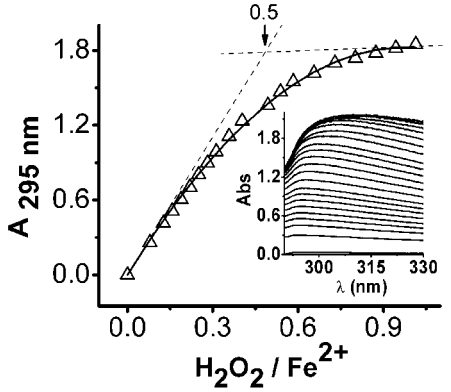

FIG. 5. **Spectrometric titration of apo-EcBFR containing 500 Fe(II)/shell (1 mM Fe(II)) with H_2O_2 under anaerobic conditions.** *Inset*, family of UV-visible difference spectra with EcBFR (2 μM) in 0.1 M Mes, 50 mM NaCl, pH 6.50. All the solutions were thoroughly degassed with pure argon gas before the experiment. The smooth curve through the data points is an empirical fit to a 3rd order polynomial constrained to pass through the origin. The *straight lines* are computed tangents evaluated at the beginning and end of the fitted curve.

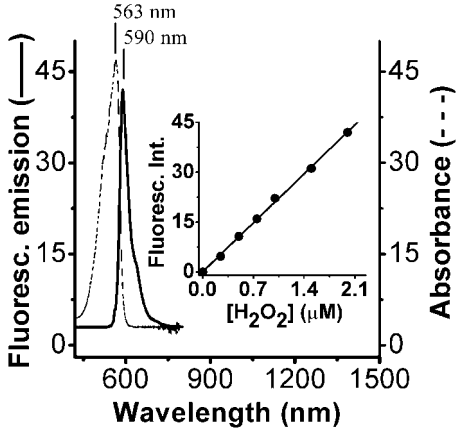

FIG. 6. **Absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reaction.** *Inset*, standard curve for the fluorescence assay of H_2O_2 including the blank correction. All the solutions (standards, controls, and samples) contained 200 μM Amplex Red reagent and 1 unit/ml horseradish peroxidase in 50 mM Mops, pH 7.40. The concentrations of the protein and iron stock solutions were 0.15 μM and 3.6 mM, respectively. The fluorescence was measured at 590 nm using an excitation wavelength of 560 nm. The fluorescence of a blank containing the apoprotein, Amplex Red reagent, and horseradish peroxidase was subtracted from the fluorescence of all the iron protein solutions.

be 2310 M^{-1} cm^{-1} at 295 nm and is similar to the value of 2030 M^{-1} cm^{-1} obtained for EcBFR when O_2 was the oxidant (6). Similar values have been observed for μ-oxo-bridged Fe(III) clusters in other proteins and model complexes (9, 20, 22).

To determine the number of protons produced during the mineralization reaction with H_2O_2 as the oxidant, 48 Fe(II)/shell were oxidized anaerobically by 24 μM H_2O_2 (24 H_2O_2/shell, 2 Fe(II)/H_2O_2) followed by the anaerobic addition of an additional 48 Fe(II)/shell 5 min later and then 48 μM H_2O_2 (48 H_2O_2/shell). A value of 2.0 ± 0.1 H^+/Fe(II) was obtained. In another experiment, a stoichiometry of H^+/Fe(II) = 2.3 ± 0.2 was obtained after the anaerobic addition of 220 Fe(II)/shell to 0.2 μM apo-EcBFR followed by 44 μM H_2O_2 (220 H_2O_2/shell). In both samples, enough H_2O_2 was added to oxidize all of the iron present. Accordingly, we write the net mineralization reaction for EcBFR as Equation 5,

$$2Fe^{2+} + H_2O_2 + 2H_2O \rightarrow 2FeOOH_{(core)} + 4H^+ \qquad \text{(Eq. 5)}$$

where $FeOOH_{(core)}$ is a mineral core with UV absorption properties similar to that observed previously (6, 9) for bacterioferritins and mammalian ferritins when O_2 was used as the oxidant.

Fluorescence Assay for H_2O_2 Production—Experiments were also performed to determine whether H_2O_2 is produced as an intermediate during the ferroxidation reaction of Fe(II) with O_2 as the oxidant. Fig. 6 illustrates the fluorescence standard curve for H_2O_2 measurements when the Amplex Red reagent/horseradish peroxidase mixture was added to the protein solution before the addition of 48 Fe(II)/shell. After subtracting the blank correction (apoprotein plus the fluorescence reagents), an average of one H_2O_2 per 9.1 ± 0.1 Fe(II) oxidized was detected (n = 2). Because there are competing reactions for the H_2O_2 produced (disproportionation, reaction with Fe(II), etc.), the value from the fluorescence assay is a lower limit to the true amount of H_2O_2 produced in EcBFR.

H_2O_2 production was also observed during the mineralization reaction of EcBFR in air. For the addition sequence, apo-EcBFR + 48 or 96 Fe(II)/shell + fluorescence reagents (5 min later) + another 48 or 96 Fe(II)/shell, or the sequence, apo-EcBFR + fluorescence reagents + 96 Fe(II)/shell, an average of 1 H_2O_2 per 18 Fe(II) oxidized was detected. The control reaction (7.2 or 14.4 μM Fe(II) in oxygenated buffer in the presence of the fluorescence reagents) did not show the production of H_2O_2. The conditions for these experiments are indicated in the legend of Fig. 6. These results taken together indicate that H_2O_2 is an intermediate product during Fe(II) oxidation in both the ferroxidation and mineralization phases of EcBFR.

Hydroxyl Radical Spin Trapping—The oxidation stoichiometry of two Fe(II)/H_2O_2 (Fig. 2) implies that Fenton chemistry should minimally occur in EcBFR. EPR spin trapping experiments were therefore undertaken to determine whether hydroxyl radical production is attenuated by the presence of the protein. All solutions were thoroughly deoxygenated with argon before each experiment. A control reaction corresponding to the addition sequence, DTPA + EMPO + H_2O_2 + Fe(II) in Mops buffer, pH 7.4, was recorded (Fig. 7, *spectrum A*). The strong EPR signal seen in spectrum A reflects the efficiency of the 1 electron oxidation of the Fe(II)-DTPA complex in generating a hydroxyl radical through the Fenton reaction (17).

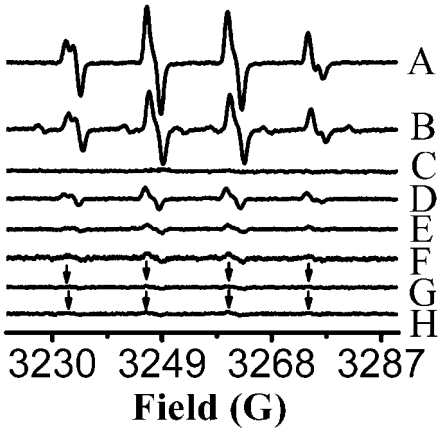

FIG. 7. **X-band EPR signal of EMPO-OH adduct.** *Spectrum A*, anaerobic addition sequence DTPA + EMPO + H_2O_2 + Fe(II); *spectrum B*, anaerobic addition sequence EMPO + H_2O_2 + Fe(II); *spectrum C*, anaerobic addition sequence apo-EcBFR + EMPO + H_2O_2; *spectrum D*, anaerobic addition sequence apo-EcBFR + EMPO + H_2O_2 + Fe(II); *spectrum E*, anaerobic addition sequence apo-EcBFR + EMPO + Fe(II) + H_2O_2; *spectrum F*, aerobic addition sequence apo-EcBFR + EMPO + Fe(II); *spectrum G*, aerobic addition sequence EcBFR containing 200 Fe(III)/shell + EMPO + H_2O_2; *spectrum H*, aerobic addition sequence EcBFR containing 500 Fe(III)/shell + EMPO + H_2O_2. The *arrows* denote weak signals resulting from small percentage of hydroxyl radical production. Conditions: 2.08 μM EcBFR or 100 μM DTPA, 25 mM EMPO, 100 μM $FeSO_4$ for *spectra A*, *B*, *D*, *E*, and *F*, 416 μM $FeSO_4$ for *spectrum G* and 1.04 mM $FeSO_4$ for *spectrum H*, 500 μM H_2O_2, 50 mM Mops, pH 7.0, room temperature.

Spectrum B was obtained in another control experiment carried out in the absence of DTPA for the addition sequence, EMPO + H_2O_2 + Fe(II), and has an intensity of 68% of that when DTPA is present. A third control experiment showed no EPR signal when EMPO and H_2O_2 were added to the apoprotein alone (spectrum C). For the addition sequence, EcBFR + EMPO + H_2O_2 + Fe(II), a signal corresponding to 21% of that of spectrum A was produced (spectrum D). A much weaker EPR signal (8% of spectrum A) was obtained when EcBFR was first loaded with 48 Fe(II)/shell in the presence of EMPO, followed by the addition of H_2O_2 (spectrum E). Thus, detectable hydroxyl radical production during Fe(II) oxidation by H_2O_2 is significantly attenuated by the protein.

To test the possibility that hydroxyl radicals are produced during the aerobic addition of Fe(II) to apo-EcBFR, the addition sequence EcBFR + EMPO + 48 Fe(II)/shell was carried out. An EPR signal identical to that of spectrum E (*spectrum F*, Fig. 7) was again obtained corresponding to 8% of spectrum A. Therefore, when O_2 is used as the Fe(II) oxidant, a small amount of the H_2O_2 produced at the ferroxidase center subsequently reacts with iron via the Fenton reaction.

Two other samples were prepared, 200 Fe(II)/shell (*spectrum G*, Fig. 7) and 500 Fe(II)/shell (*spectrum H*, Fig. 7) in order to determine whether H_2O_2 addition to a preformed Fe(III) mineral core would generate hydroxyl radicals. In these samples, Fe(II) was added to the apoprotein in the presence of O_2 and continuously stirred for 6–8 h to ensure complete oxidation and followed by the addition sequence EMPO + H_2O_2. A very weak EPR signal (2.8% of spectrum A) was obtained in both cases and indicates that a small amount of hydroxyl radical of unknown origin is produced.

DISCUSSION

Ferritins have developed a variety of mechanisms to counter the problem that iron imposes as a facilitator of oxygen radical chemistry. The present work shows that EcBFR utilizes H_2O_2 to oxidize iron pairwise, thus preventing the formation of hydroxyl radical through Fenton chemistry. A stoichiometric ratio of 0.5 H_2O_2 per Fe(II) is obtained from spectrophotometric data for both ferroxidation (Fig. 2) and mineralization (Fig. 5) reactions, indicating that EcBFR is capable of nullifying the harmful combination of Fe(II) and H_2O_2. A similar stoichiometry was obtained using the oximetry apparatus, where the effect of the enzyme catalase on the remaining H_2O_2 in solution, after its reaction with Fe(II), indicates pairwise oxidation of Fe(II) by H_2O_2 (Fig. 4). These data are consistent with the fact that traditional ferritins have an iron housekeeping role, making iron less likely to participate in free radical-generating reactions (1, 10).

The EPR spin trapping experiments showing little $HO^{\cdot}$ radical production are in agreement with the two Fe(II)/H_2O_2 stoichiometry and indicate a dependence of the amount of hydroxyl radical trapped on the order of addition of reagents (Fig. 7). Namely, when Fe(II) is first bound to the protein followed by the addition of H_2O_2 (*spectrum E*, Fig. 7), only 8% of the hydroxyl radical is generated compared with the control experiment (*spectrum A*, Fig. 7). A stronger EPR signal is obtained (21% of control) when H_2O_2 is first added to the apoprotein followed by Fe(II) (*spectrum D*, Fig. 7), presumably because of competition between Fe(II) binding at the ferroxidase center and the Fenton reaction. Nevertheless, it is clear that Fenton chemistry is significantly reduced in EcBFR regardless of the order of addition of H_2O_2 and Fe(II), but reduction of $HO^{\cdot}$ radical formation is most pronounced if Fe(II) is pre-bound to the protein prior to introduction of H_2O_2.

The fluorescence assay experiments reported here indicate that H_2O_2 is an intermediate product of dioxygen reduction when O_2 is used as the oxidant in both the ferroxidation and mineralization reactions. The present results support the two-step ferroxidation mechanism where H_2O_2 is produced at one ferroxidase center and is rapidly consumed at the other to produce H_2O (see Introduction). The 10-fold more rapid reaction of H_2O_2 with the di-Fe(II) ferroxidase site compared with O_2 (see "Results") is consistent with this mechanism. Because the two protons generated per Fe(II) are from ferrous binding to the apoprotein, we write the mechanism of iron oxidation by O_2 as Equations 6–12.

Diferrous binding,

$$2Fe^{2+} + P^z \rightarrow [Fe(II)_2{-}P]^z + 4H^+ \quad \text{(Eq. 6)}$$

Ferroxidation,

$$\textit{Step one}, [Fe(II)_2{-}P]^z + O_2 + H_2O \rightarrow [Fe(III)_2O{-}P]^z + H_2O_2 \quad \text{(Eq. 7)}$$

$$\textit{Step two}, [Fe(II)_2{-}P]^z + H_2O_2 \rightarrow [Fe(III)_2O{-}P]^z + H_2O \quad \text{(Eq. 8)}$$

$$\textit{Net reaction}; 2[Fe(II)_2{-}P]^z + O_2 \rightarrow 2[Fe(III)_2O{-}P]^z \quad \text{(Eq. 9)}$$

Mineralization,

$$\textit{Step one}, 2Fe^{2+} + O_2 + 4H_2O \rightarrow 2FeOOH_{(core)} + H_2O_2 + 4H^+ \quad \text{(Eq. 10)}$$

$$\textit{Step two}, 2Fe^{2+} + H_2O_2 + 2H_2O \rightarrow 2FeOOH_{(core)} + 4H^+ \quad \text{(Eq. 11)}$$

$$\textit{Net reaction}; 4Fe^{2+} + O_2 + 6H_2O \rightarrow 4FeOOH_{(core)} + 8H^+ \quad \text{(Eq. 12)}$$

H_2O_2 is depicted as an intermediate in both the ferroxidase and mineralization reactions; however, previous experiments using the enzyme catalase have failed to detect any measurable amounts of free H_2O_2 in solution during Fe(II) oxidation by O_2 in EcBFR (6). In order for catalase to react with H_2O_2, H_2O_2 must be released into the bulk solution where it can diffuse out of the protein cavity and react with the enzyme. In order to see O_2 production in solution as per Equation 4, the reaction of H_2O_2 with catalase must also occur more rapidly than its reaction with Fe(II). Although not observed with EcBFR, this

behavior was seen with mammalian ferritins where H_2O_2 produced at the ferroxidase center of these proteins accumulates to measurable levels in the bulk solution and can be detected by the addition of catalase (9, 24–26). In accord with these observations, experiments with HuHF (not shown) indicate that O_2 is the preferred oxidant for Fe(II) over H_2O_2, the opposite of what is found for EcBFR. Because small amounts of H_2O_2 were detected by the fluorescence assay in EcBFR (see "Results"), and previous catalase experiments had no effect on the iron oxidation rate and on the Fe(II)/O_2 stoichiometry (6), H_2O_2 must become quickly consumed by other Fe(II) bound to the ferroxidase centers as shown in the reaction mechanism. It is evident, however, that a small amount of Fe(II) reacts via Fenton chemistry (Fig. 7).

One might anticipate the formation of a peroxo-diFe(III) intermediate during Equation 7 as found for other ferritins when O_2 is the oxidant (27–29). To date, there has been no indication of a peroxodiferric intermediate formation in EcBFR; more studies of the iron-complex intermediates formed in the early and late stages of its ferroxidase reaction are needed. The fact that no peroxo intermediate has been observed with bullfrog H-chain protein, although such an intermediate is seen in other ferritins (27–29), does not exclude its formation as it may be formed in low amounts, being rapidly consumed in a subsequent reaction as suggested by Pereira *et al.* (30). Initially, the peroxo complex was difficult to see in EcFtnA because of its low absorbance but is more evident in site-directed variants of this protein (21, 27).

That the chemistry examined in this work takes place at the 24 ferroxidase centers of EcBFR is implied by the stoichiometry of 48 Fe(II)/shell when H_2O_2 is used as oxidant (Fig. 3). The fact that the molar absorptivities and absorption maxima of the ferroxidase center iron and the mineral core in EcBFR when H_2O_2 is used as the oxidant are essentially the same as those when O_2 is used as the oxidant (6) suggests the formation of the same μ-oxo or μ-hydroxyl species within the protein independent of the oxidant employed.

Bacterioferritin (EcBFR) and bacterial ferritins (EcFtnA and EcFtnB) are only distantly related in evolution (1, 7) and appear to have different primary functions. Whereas EcFtnA is primarily expressed during the growth phase, in keeping with a general housekeeping role in iron metabolism, EcBFR is a stationary phase protein consistent with an involvement in stress relief (1). Further evidence for a role for bacterioferritin in the response to oxidative stress comes from the work of Ma *et al.* (32) who showed that *Pseudomonas aeruginosa* bacterioferritin A is required for protection against H_2O_2, and Chen and Morse (33) who demonstrated that *Neisseria gonorrhoeae* bacterioferritin protected the organism from iron-mediated oxidative stress. The demonstration that the bacterioferritin comigratory protein (34), which is induced by oxidative stress, is a thiol-peroxidase (35) indicates that the cell has multiple means of removing H_2O_2 when it is oxidatively stressed under iron-rich conditions. As noted earlier, both the ferroxidase center and the mineralized core of EcBFR consume H_2O_2 (Figs. 2, 3, and 5), consistent with this ferritin being important for relief from free radical toxicity within the cell. In this context the H_2O_2 reaction might be as significant as an iron storage function of bacterioferritins, with the Fe(II)-protein complex acting as a peroxidative co-factor in a similar manner to the thiol and heme groups of other peroxidases. In this connection, a recent investigation of *E. coli* Dps, a DNA-binding protein, showed it to have a ferritin-like capacity to bind and store iron using H_2O_2 as oxidant, thus preventing the production of reactive oxygen species and oxidative damage to DNA (31). EcBFR may have a similar role in protecting cellular components against radical assault.

In conclusion, the ability to utilize and sequester iron safely is a fundamental requirement of nearly all living organisms, and although the precise physiological function of bacterioferritin remains to be established, a role in preventing iron-induced radical toxicity is suggested by the present study. The fate of the H_2O_2 produced during iron deposition in ferritins is of considerable importance. EcBFR may provide a protective effect within the cytosol by oxidizing iron pairwise with H_2O_2, similarly to the Dps protein (31), thus avoiding Fenton chemistry.

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