Iron and Hydrogen Peroxide Detoxification Properties of DNA-binding Protein from Starved Cells

A FERRITIN-LIKE DNA-BINDING PROTEIN OF ESCHERICHIA COLI*

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The DNA-binding proteins from starved cells (Dps) are a family of proteins induced in microorganisms by oxidative or nutritional stress. *Escherichia coli* Dps, a structural analog of the 12-subunit *Listeria innocua* ferritin, binds and protects DNA against oxidative damage mediated by H₂O₂. Dps is shown to be a Fe-binding and storage protein where Fe(II) oxidation is most effectively accomplished by H₂O₂ rather than by O₂ as in ferritins. Two Fe²⁺ ions bind at each of the 12 putative dinuclear ferroxidase sites (P²) in the protein according to the equation, 2Fe²⁺ + P² → [(Fe(II)₂-P]P₂ + 2H⁺. The ferroxidase site (FS) bound iron is then oxidized according to the equation, [(Fe(II)₂-P]P₂ + H₂O₂ + H₂O → [Fe(III)₂O₃(OH)-P]P₂ + 3H⁺, where two Fe(II) are oxidized per H₂O₂ reduced, thus avoiding hydroxyl radical production through Fenton chemistry. Dps acquires a ferric core of ~500 Fe(III) according to the mineralization equation, 2Fe²⁺ + H₂O₂ + 2H₂O → 2Fe(III)O₃(OH)core + 4H⁺, again with a 2 Fe(II)/H₂O₂ stoichiometry. The protein forms a similar ferric core with O₂ as the oxidant, albeit at a slower rate. In the absence of H₂O₂ and O₂, Dps forms a ferrous core of ~400 Fe(II) by the reaction Fe²⁺ + H₂O₂ → Fe(II)O₃(OH)core + H⁺. The ferrous core also undergoes oxidation with a stoichiometry of 2 Fe(II)/H₂O₂. Spin trapping experiments demonstrate that Dps greatly attenuates hydroxyl radical production during Fe(II) oxidation by H₂O₂. These results and in vitro DNA damage assays indicate that the protective effect of Dps on DNA most likely is exerted through a dual action, the physical association with DNA and the ability to nullify the toxic combination of Fe(II) and H₂O₂. In the latter process a hydrous ferric oxide mineral core is produced within the protein, thus avoiding oxidative damage mediated by Fenton chemistry.

All aerobic organisms have evolved a variety of complex defense and repair mechanisms to protect their DNA from oxidative damage because of reactive oxygen species such as HO·, O₂⁻, and H₂O₂. These include highly regulated enzymatic systems that recognize and repair damaged DNA or that prevent damage by detoxifying the reactants that produce radicals (1–4). These inducible responses are associated with de novo protein synthesis, an energy requiring process. However, when bacteria are starved, their ability to cope with environmental assault by de novo protein synthesis becomes compromised by the lack of nutrients (1). A class of nonspecific DNA-binding Dps* (pexB) proteins is expressed in bacteria and accumulated to high levels prior to conditions of oxidative or nutritional stress, making the cell in stationary phase more resistant to H₂O₂ than actively growing cells (1, 5–9). Studies have shown that Dps plays a central role in protecting DNA from oxidative damage both in vitro and in vivo by directly binding to DNA (1, 6–9). It has been suggested that the protective activity of Dps may stem from its ability to sequester iron ions, but direct experimental evidence for this proposal has been lacking (1, 7, 10).

Dps has a shell-like structure of 3:2 tetrahedral symmetry assembled from 12 identical subunits with a central cavity measuring ~45 Å in diameter (7). Each Dps subunit is folded into a 4-helix bundle through hydrophobic interactions. The 3:2 symmetry of the assembled protein leads to two inequivalent environments along the 3-fold axes. One corresponds to the 3-fold interactions found in the 24-mer ferritins having 4:3:2 octahedral symmetry and involves the N-terminal end of the subunit, whereas the other 3-fold interaction takes place at the C-terminal end of Dps but is not typical of the ferritins (7).

*Escherichia coli* Dps and the relatively recently discovered 12-mer *Listeria innocua* ferritin assemble in essentially the same way but share only 19% identity in primary sequence (10, 11). Both proteins possess a negative electrostatic potential on the inner surfaces of their shells, providing an ideal microenvironment for iron mineralization (7, 10). Unlike typical 24-mer ferritins, the putative dinuclear ferroxidase sites of *L. innocua* ferritin are not located in the four-helix bundle of individual monomers but rather have ligands provided by two symmetry-related subunits (10). Most ligands comprising the putative ferroxidase center of *L. innocua* ferritin are conserved in *E. coli* Dps as well (7, 10). Pb²⁺ is bound at this location in the heavy atom derivative of the Dps crystal (7). The large "ferritin-like" 3-fold pores of 8 Å diameter are lined with Asp¹²¹, Asp¹²⁶, and Asp¹³⁰ from the symmetry-related subunits and are con-

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‡ The abbreviations used are: Dps, DNA-binding protein from starved cells; DTPA, diethylenetriamine pentaaacetic acid; EMPO, 5-ethoxy carbonyl-5-methyl-1-pyrroline-N-oxide; Mops, 3-(N-morpholino)propanesulfonic acid.
served between the two proteins (except that Asp121 is replaced by Lys in Dps). The negatively charged hydrophilic pores are likely pathways for iron entry into the protein cavity (7, 10). L. innocua ferritin has been shown to exhibit ferroxidase activity (12, 13) and can rapidly accumulate up to ~500 iron atoms within its central cavity when O$_2$ is the oxidant (11).

The striking similarity in overall structure between E. coli Dps and L. innocua ferritin and the protective effect of E. coli Dps on DNA against oxidative damage mediated by Fenton chemistry (1) suggest that E. coli Dps might also function as an iron-binding and storage protein. However, to date the iron binding, oxidation, and hydrolysis/mineralization reactions of Dps have not yet been investigated. In the present study, we describe experiments that demonstrate a ferritin-like function for Dps. Unexpectedly, O$_2$ was found to be a relatively poor oxidant for Fe(II) in Dps, the rate of O$_2$ consumption being only marginally faster than Fe(II) autoxidation in the absence of protein. In contrast to O$_2$, H$_2$O$_2$ causes rapid and complete oxidation of Fe(II). The initial rate of iron oxidation reaches a maximum at 24 Fe(II)/Dps, a result implying that there are 12 dinuclear ferroxidase sites in Dps, one per each of the 12 subunits. As in E. coli bacterioferritin and L. innocua ferritin (12, 14), the ferroxidase activity of Dps is not readily regenerated upon standing. A stoichiometry of 1 H$_2$O$_2$ per 2 Fe(II) oxidized is obtained in both ferroxidase and mineralization reactions of Dps, thus avoiding the production of the HO$^-$ radical through the Fenton reaction as follows.

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}_2^- \quad \text{(Eq. 1)}$$

By using spectrophotometry, pH-stat, sedimentation velocity measurements, and iron analysis, the protein was shown to load up to ~500 Fe(III) within its cavity. The results are discussed in terms of possible mechanisms of core formation in Dps and of the relationship between its ferritin-like function and DNA protection from oxidative damage.

**MATERIALS AND METHODS**

Preparation and purification of Dps were performed as described by Almiron et al. (6) with one modification. The Sephadex G-100 gel filtration step was replaced by dialysis against 20 mm Tris-HCl, pH 8.0. At this low ionic strength Dps precipitates and can be collected easily after centrifugation at 15,000 rpm for 10 min. Dps resuspended in 50 mm Tris-HCl, 2 mM NaCl, pH 8.0, was subjected to gel filtration on Sepharose 6B. ApoDps concentrations were determined spectrophotometrically using a molar absorptivity of 5.98 × 10$^4$ M$^{-1}$ cm$^{-1}$ at 280 nm on a 12-mer protein basis (13). The concentration of freshly prepared solutions of hydrogen peroxide was determined from the amount of O$_2$ evolved upon addition of catalase (Roche Molecular Biochemicals) as measured by Clark electrode orymetry or from its absorbance at 240 nm (ε = 43.6 M$^{-1}$ cm$^{-1}$) (15). EMPO was purchased from Oxis Research (Portland, OR). All chemicals were reagent grade or better, and were used without further purification.

The electrode oxymetry/pH-stat apparatus and standardization reaction for its use have been described in detail elsewhere (14, 16). In the present experiments, the consumption of O$_2$ during Fe(II) oxidation was followed at 25°C with a Clark-type oxygen microelectrode while maintaining the pH at 7.0 with a pH-stat. To accurately measure initial rates of proton production, a pH-stat proportional band setting of 0.1 was used; however, the rapid response of pH-stat at this setting resulted in an overshoot of the stoichiometric end point at the end of the reaction. Therefore, the stoichiometry was determined in a separate experiment using a proportional band setting of 1.2 or 1.5 where the response of pH-stat was slower and an accurate end point could be obtained. Typical conditions for the experiments were 0.1, 0.2, 1.2, or 4 μM Dps protein in 0.5 mM Mops and 150 or 200 mM NaCl, pH 7.0 (controlled by pH-stat), with increments of 6–24 or 100–1000 Fe(II) per protein added at 25°C as freshly prepared 10–20 mM FeSO$_4$ or Fe(NH)$_4$SO$_4$ (Baker Scientific) in pH 3.5 water. The use of 0.5 mM Mops buffer in the protein solution lends stability to the pH-stat control without significantly buffering the solution. Small corrections for free acid in the ferrous sulfate were made in all calculations.

The ultraviolet-visible difference spectrophotometric titration and kinetic experiments of Fe(II) oxidation by H$_2$O$_2$ were performed on a Cary 500 spectrophotometer. The instrument was zeroed using 0.1 or 0.5 mM Dps solution as the blank. Time-dependent absorbance kinetic traces at 25°C were collected using the Cary 500 Kinetic Software. The kinetic data were further analyzed with Origin 6.0 software (Microcal Inc.). The initial rates of Fe(II) oxidation measured by UV absorption spectroscopy were obtained from the linear A$t$ term of third-order polynomial curve to the experimental data, namely $Y = A_s + A_f + A_d + A_d^2$, where the derivative dY/dt = $A_s + 2A_f + 3A_d^2$ evaluated at t = 0 gives $A_s = (d\Delta A/dt)_0$. Here Y is either the change in absorbance $\Delta A$ at 305 nm or the amount of NaOH autotitrated into the same solution at time t in seconds (16).

EPR spectra were recorded on a laboratory assembled EPR spectrometer based on a Bruker ER 041 XK-H X-band microwave bridge operating at 9.24 GHz with 100 kHz field modulation. Samples in 1-mm inner diameter quartz capillaries were placed in a Varian TR-205 cavity for measurement at room temperature. Typical spectrometer parameters were: microwave power, 50 mW; modulation amplitude, 0.8 G; time constant, 0.3; scan rate, 7.14 G s$^{-1}$. In the EMPO spin trapping experiments for hydroxyl radical (17), all spectra were recorded ~1 min after addition of the last reagent. The concentrations of reagents are indicated in the figure captions.

To prepare the ferric core of Dps using H$_2$O$_2$ as the oxidant, 500 Fe(II) per Dps were added aerobically to 0.25 mM FeSO$_4$ in 50 mM Mops and 200 mM NaCl, pH 7.0, in 10 increments of 50 Fe(II)/Dps followed by 1 H$_2$O$_2$ (as a 12.5 mM solution) per 2 Fe(II) 2 min later with a 10-min interval between iron additions. The protein solution was stirred only during the additions of Fe(II) and H$_2$O$_2$. The holoprotein prepared in this way was dialyzed three times against buffer and then analyzed for protein content by the Advanced Protein Assay (cytoskeleton.com) and the iron content by the ferrozine method (18). To prepare the ferric core of Dps, Fe(II) was added in 8 increments of 50 Fe(II)/Dps at 10-min intervals under an argon atmosphere and in the presence of 1 mM dithionite, Na$_2$S$_2$O$_4$, in 50 mM Mops, 200 mM NaCl, pH 7.0, to give a total of 400 Fe(II)/protein. The iron(II) containing protein was ultracentrifuged 3 times under argon with a 1000-fold total volume change and analyzed for protein and iron contents.

Formation of a ferric core with O$_2$ as oxidant was assessed by adding two increments of 200 Fe(II)/Dps to Dps solutions (1 μM) equilibrated in air at 20°C. Ferrous ammonium sulfate solutions prepared in Thunberg tubes and kept under nitrogen were used. The iron incorporation reaction was followed at 310 nm; after reaching a constant absorbance (about 2 h) the samples were analyzed by sedimentation velocity.

Sedimentation velocity studies were carried out using the absorbance optics on a Beckman XLI analytical ultracentrifuge. Experiments were conducted at 30,000 rpm and 20°C at Dps concentrations between 0.25 and 1.0 μM. Radial absorbance scans were obtained at both 280 and 310 nm using the continuous scan mode to provide an effective radial resolution of 30 μm. The constant ratio of absorbance at 280 nm to that at 310 nm during the centrifugation run of the ferric core sample confirmed the homogeneity of the ferric core. Sedimentation coefficient distributions,$g(s^2)$, were obtained using the linear least-squares algorithm (lsqfit) incorporated in Sedfit (version 8.3) (19). Sedimentation coefficients were interpreted using standard methods (20).

Fluorescence spectra were measured on 4 μM Dps solutions in 50 mM Mops-NaOH, pH 7.0, containing 150 mM NaCl. The measurements were carried out at 25°C in gas-tight cells under a nitrogen atmosphere using a Fluoromax fluorimeter (Spex Industries). In the titration experiments, anaerobic 0.01 M FeSO$_4$ solutions were added in increments corresponding to 12, 24, 36, 48, and 100 Fe(II)/Dps.

DNA protection from oxidative damage in vitro was tested using pHUE21-2 plasmid DNA (3900 bp, 10 nm), purified by a Qiaprep spin plasmid miniprep kit (Qiagen, Chatsworth, CA). The total volume reaction was 10 μl in 20 mM Tris-HCl, pH 7.5. Dps (3 μM) was allowed to interact with plasmid DNA for 10 min prior to introduction of FeSO$_4$(50 μM) and H$_2$O$_2$(10 mM). The reaction mixtures were incubated for 15 or 30 min at room temperature; the reaction was stopped by incubation with 2% SDS at 85°C for 5 min. Dps was extracted with phenol and plasmid DNA was resolved by electrophoresis on 1% agarose gel. The gel was stained with ethidium bromide and imaged by ImageMaster VDS (Amersham Biosciences).

**RESULTS**

**Fe(II) Binding to Dps**—The anaerobic addition of Fe(II) to E. coli Dps produced no discernable change in the UV-visible spectrum, but resulted in significant quenching of the intrinsic
Iron Binding to E. coli Dps

Fig. 1. A, quenching of intrinsic fluorescence versus Fe(II)/Dps ratio for Fe(II) binding to Dps. The fluorescence spectra are shown in the inset. B, initial rate of H⁺ production versus Fe(II)/Dps ratio for Fe(II) binding to Dps (curve a, ●) and initial rate of H⁺ production versus Fe(II)/Dps ratio (curve b, ■). Conditions are as follows: A, 4.0 μM Dps, anaerobic 50 mM Mops, pH 7.0, 150 mM NaCl, 25 °C; B, curve a: 2.0 μM Dps, anaerobic 200 mM NaCl, pH-stat = 7.0 solution, Fe(II)/Dps = 6–42, 25 °C; curve b, 0.5 μM Dps, 3.0–21 μM FeSO₄, H₂O₂/Fe(II) = 2, 200 mM NaCl, 50 mM Mops, pH 7.0, 25 °C. Each point represents a separate sample.

Fe(II) oxidation—Initial experiments on Fe(II) oxidation in Dps were carried out using O₂ as the oxidant. Fig. 3 shows that the rate of O₂ consumption monitored by electrode oximetry was slow when Fe(II) was added aerobically to the apoprotein, being only marginally faster than Fe(II) autoxidation in buffer alone. Dps contrasts with all known ferritins investigated to date, including L. innocua ferritin, where O₂ is an efficient and rapid oxidant for Fe(II) (12, 14, 16, 21–24). For example, when compared on a subunit basis (2 Fe(II)/subunit) under the conditions in Fig. 3, Fe(II) oxidation in Dps consumes 1.2 × 10⁻² O₂/subunit/min compared with 10 O₂/subunit/min for human H-chain ferritin (16), an 830-fold difference between proteins. Furthermore, addition of 24 Fe(II)/Dps aerobically to a 0.5 μM protein sample caused a minimal absorbance change at 305 nm over a period of 7 min in contrast to ferritins where rapid Fe(II) oxidation by O₂ leads to formation of μ-oxo-bridged Fe(III) species that absorb at this wavelength (14, 16). A gradual increase in absorbance with Dps occurs over an extended time period, however.

Experiments were subsequently undertaken to determine whether H₂O₂ might be a more efficient oxidant of Fe(II) in Dps than O₂. Fig. 4 shows the addition of 12 μM Fe(II) to an aerobic solution of 0.50 μM Dps. In the presence of air there was little change in absorbance; however, upon the addition of H₂O₂ to the solution (2 H₂O₂/Fe(II)), the absorbance quickly increased to 0.35 units (Fig. 4). With H₂O₂ as the oxidant under the conditions in Fig. 4, the specific rate of Fe(II) oxidation was 100-fold larger than for O₂, 1.8 Fe/subunit/min versus 0.017 Fe/subunit/min, respectively. The molar absorptivity for the observed oxidation product was 29,200 M⁻¹ cm⁻¹ per iron at 305 nm, which is almost 10-fold higher than previously observed for the ferritins (~3000 M⁻¹ cm⁻¹ per Fe) (14, 16),⁳ perhaps reflecting the presence of more than one μ-oxo bridge in the diFe(III) complex that was formed (see “Discussion”).

Fig. 5 compares the difference spectrum (spectrum A) of

\[
2\text{Fe}^{2+} + \text{P}^2 - \{\text{Fe(II)}_{2} - \text{P}^{\text{Fe}}_{\text{Fe}}\}^{2+} + 2\text{H}^+ \quad (\text{Eq} \ 2)
\]

where \([\text{Fe(II)}_{2} - \text{P}^{\text{Fe}}_{\text{Fe}}]\) represents a diFe(II)-protein complex at each of the 12 putative ferroxidase sites.

The units of the ordinate in Fig. 6 of Ref. 16 should read micromolar/s.

³ The molar absorptivities for human H-chain ferritin and horse spleen ferritin were per iron not per dimer as previously reported (16).
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Dps + Fe(II) + H$_2$O$_2$ minus that of Dps + H$_2$O$_2$ with the difference spectrum (spectrum B) of Dps + H$_2$O$_2$ + Fe(II) minus that of Dps + H$_2$O$_2$. If Fe(II) was added to the protein prior to H$_2$O$_2$, the increase in absorbance was almost 2-fold larger, indicating more complete formation of the specific Fe(III) complex. Spectrum A was stable for ~1.5 h but ultimately decayed to 75% of its original absorbance by 24 h and to 48% in 4 days, suggesting slow clearance of iron from the original site of binding. After 24 h, spectrum B decayed to 93% of its original intensity. In subsequent experiments, Fe(II) was always added to the Dps solution prior to H$_2$O$_2$ to achieve the maximum amount of the specific complex.

To determine the stoichiometry of Fe(II) oxidation by H$_2$O$_2$, Dps containing 24 Fe(II)/protein was titrated with H$_2$O$_2$ (Fig. 6, curve a). The initial rate of Fe(II) oxidation reached a maximum at a ratio H$_2$O$_2$/Fe(II) = 0.5, indicating that one H$_2$O$_2$ oxidizes two Fe(II). In contrast, when Fe(II) was added to a solution of H$_2$O$_2$ in the absence of Dps, an oxidation stoichiometry of 1 H$_2$O$_2$/Fe(II) was observed (data not shown) as expected for the Fenton reaction (Equation 1). The number of protons generated during Fe(II) oxidation by H$_2$O$_2$ in Dps was 1.5 ± 0.1 H$^+$/Fe(II) at pH 7.0 (Fig. 2, 12 μM H$_2$O$_2$ addition).

Thus we write the Fe(II) oxidation (phase II) reaction as the following:

$$[\text{Fe(II)}_2 - \text{P}_{\text{Fe}}^{3+} + \text{H}_2\text{O}_2 + \text{H}_2\text{O} \rightarrow [\text{Fe(III)}_2\text{O}_2\text{OH}] - \text{P}_{\text{Fe}}^{3+} + 3\text{H}^+] \quad \text{(Eq. 3)}$$

The iron/protein stoichiometry for Fe(II) oxidation by H$_2$O$_2$ was determined by titrating the protein with Fe(II) followed by addition of excess H$_2$O$_2$ (Fig. 1B, curve b). The initial rate of Fe(II) oxidation (ΔA/min) reaches a maximum at 24 Fe(II)/Dps in accordance with the Fe(II) binding stoichiometry of 24 Fe(II)/Dps (Fig. 1A and 1B, curve a). These results imply that the 12 putative dinuclear ferroxidase sites are involved in the rapid pairwise oxidation of Fe(II) by H$_2$O$_2$.

Dps was also examined for catalase activity. The addition of 72 μM H$_2$O$_2$ to a solution of 1 μM Dps containing 24 Fe(III)/Dps in 50 mM Mops, 150 mM NaCl, pH 7.0, resulted in O$_2$ evolution according to the reaction H$_2$O$_2$ → H$_2$O + ½O$_2$ as measured by electrode oximetry. 1 μmol of the Fe(III)$_{24}$Dps complex disproportionates 0.12 μmol of H$_2$O$_2$/s. This level of catalase activity is about four times greater than that observed for human H-chain ferritin containing 48 Fe(III)/human H-chain ferritin (15).

ApDps itself exhibited no catalase activity, indicating that the bound iron(III) was responsible for the disproportionation of H$_2$O$_2$.

**Fe(III) Mineralization of Dps**—Samples of Dps previously loaded with 24 Fe(III)/Dps using H$_2$O$_2$ as the oxidant were loaded 12 h later with an additional 24 Fe(II) and various amounts of H$_2$O$_2$ were added. Fig. 6 (curve b) shows that the 305 nm absorbance, ΔA$_{305}$, reaches a maximum at 0.5 H$_2$O$_2$/Fe(II), corresponding to the oxidation of two Fe(II) per H$_2$O$_2$ when Fe(II) was added beyond the initial 24. The increase in absorbance for the second 24 Fe(II) added was considerably less than for the first 24 Fe(II), 0.08 versus 0.35 (c.f. Figs. 5A and 6, curve b), indicating that the Fe(III) species produced was different. Moreover, the oxidation of the second 24 Fe(II)/Dps was about 60% faster than the first 24 Fe(II)/Dps, 2.9 Fe/subunit/min versus 1.8 Fe/subunit/min, respectively, at 0.5 μM Dps, 12 μM Fe per addition and 2 H$_2$O$_2$/Fe(II).

**FIG. 4.** Spectrophotometric kinetic curve of Fe(III) oxidation by H$_2$O$_2$ in the presence of Dps. Conditions are as follows: 0.5 μM Dps, 12 μM FeSO$_4$, 24 μM H$_2$O$_2$, 150 mM NaCl, 50 mM Mops, pH 7.0, 25 °C. 12 μM Fe(II) was added to 0.5 μM apoprotein in the presence of air followed 1 min later by 24 μM H$_2$O$_2$. Significant Fe(II) oxidation only occurs upon addition of the H$_2$O$_2$.

**FIG. 5.** Dependence of the spectrum obtained from Fe(II) oxidation by H$_2$O$_2$ on the order of addition of H$_2$O$_2$ and Fe(II) to Dps. A, the spectrum of Dps + Fe(II) + H$_2$O$_2$ minus that of Dps + H$_2$O$_2$; B, the spectrum of Dps + H$_2$O$_2$ + Fe(II) minus that of Dps + H$_2$O$_2$. Conditions are as follows: 0.5 μM Dps, 12 μM FeSO$_4$, 24 μM H$_2$O$_2$, 150 mM NaCl, 50 mM Mops, pH 7.0, 25 °C.

**FIG. 3.** Oxygen consumption curves in the presence and absence of Dps upon addition of Fe(II) to the solution in the presence of air. Conditions are as follows: 4.0 μM Dps, 96 μM FeSO$_4$, 200 mM NaCl, 0.5 mM Mops, pH-stat = 7.0, 25 °C.
The 0.5 H₂O₂/Fe(II) stoichiometry was also confirmed in an experiment where 500 Fe(II) were added to apoDps followed by either 0.5 H₂O₂/Fe(II) or 2 H₂O₂/Fe(II). The same increase in absorbance at 305 nm was observed for either amount of H₂O₂ added, indicating that one H₂O₂ per two Fe(II) was sufficient for complete iron oxidation. Fig. 2 shows that 2.0 ± 0.2 H⁺ were produced during the oxidation of the second 24 Fe(II) by H₂O₂, consistent with the value of 1.9 ± 0.1 H⁺ per iron measured by pH-stat when 500 Fe(II) were oxidized (data not shown). Accordingly, we write the mineralization (phase III) reaction for Dps as follows.

\[
2\text{Fe}^{2+} + \text{H}_2\text{O}_2 + 2\text{H}_2\text{O} \rightarrow 2\text{Fe}^{3+}\text{OOH}_(\text{core}) + 4\text{H}^+ \quad \text{(Eq. 4)}
\]

To determine the maximum size of the ferric core in Dps, a spectrophotometric titration was carried out with Fe(II) using H₂O₂ as an oxidant with a 1 H₂O₂/2 Fe(II) ratio (Fig. 7, curve a). A discontinuity in absorbance at ~500 Fe(III)/Dps was observed, suggesting that Dps can accumulate up to ~500 Fe(III) with H₂O₂ as the oxidant as was also found for L. innocua ferritin with O₂ as the oxidant (11). Iron analysis following dialysis of the protein (“Materials and Methods”) gave 472 ± 11 Fe(III)/Dps (n = 3). The molar absorptivity at 305 nm for mineralized iron in Dps was 3120 m⁻¹ cm⁻¹ per iron compared with values in the range 2030–3540 m⁻¹ cm⁻¹ per iron for horse spleen ferritin, human H-chain ferritin, and E. coli bacteri ferritin (14, 16).

The formation of an iron core was confirmed by analytical ultracentrifugation of apoDps and Dps loaded with iron (Fig. 8A). ApoDps sediments as a single species with \(s_{20,w}^0 = 9.6\) S similar to the value for apo-L. innocua ferritin (11). When 500 Fe(III) were added to apoDps in 10 increments of 50 Fe(II)/Dps at intervals of 10 min followed by 25 H₂O₂/Dps each time, the apoDps peak at 9.6 S was replaced by components sedimenting at \(s_{20,w}^0 = 14.8, 21.9\) and 28.5 S. Because \(s_{20,w}^0\) is expected to increase by ~1 Svedberg per 100 Fe added to the core (25), we assign the 14.8 S species to Dps containing ~500 Fe(III). The minor component at 21.9 S is assigned to a cross-linked dimer of the 14.8 species, predicted to sediment at 20.9 S (= 1.414 × 14.8 S). The lesser component at 28.5 S may be a trimeric species. Although elucidation of the specific nature of the cross-link is beyond the scope of the present study, they probably arise from iron-induced radical chemistry (see below). Cross-linking is commonly observed for the ferritins (26–28).

To determine whether Dps is capable of forming a ferric core with O₂ as oxidant, 400 Fe(II)/Dps were added to 1 µM Dps solutions in two successive increments of 200 Fe(II) atoms. After about 2 h, a core was formed as indicated by the sedimentation velocity of the sample (Fig. 8B). The size of the core was similar to that obtained with H₂O₂ as oxidant (\(s_{20,w}^0 = 14.4\) S), but the molecular mass distribution was somewhat wider; the latter finding indicates that the reaction leading to core formation was less cooperative in the case of oxygen and leads to increased heterogeneity in the sample.

**Fe(II) Mineralization of Dps**—To determine whether Dps is capable of forming a ferric core, samples of Dps were placed in the pH-stat and various amounts of Fe(II) were added anaerobically. The production of H⁺ associated with Fe(II) incorporation into Dps was measured (Fig. 7, curve b). Fe(II) addition in the absence of Dps produces no H⁺ ions. A discontinuity in H⁺ production occurs at about 400 Fe(II)/Dps, suggesting that Dps can acquire up to 400 Fe(II). The H⁺/Fe(II) ratios for points beyond 400 Fe(II)/Dps represent averages of values for Fe(II) bound (1 H⁺/Fe(II)) and Fe(II) unbound (0 H⁺/Fe(II)), hence the linear decrease in stoichiometric ratio beyond the end point. We attempted to measure ferrous core formation spectrophotometrically but the ferrous core was UV transparent from 250 to 400 nm. However, the analytical ultracentrifugation data of Fig. 8B demonstrates that Fe(II) was incorporated into the protein. The iron containing protein sediments as a single somewhat heterogeneous component at 13.7 S consistent with an average core size of 400 Fe(II). Iron analysis of the sample following ultrafiltration gave 381 ± 13 Fe(II)/Dps (n = 3). Because 1 H⁺ was produced per Fe(II) incorporated in the core (Fig. 7B) and the anion chloride is required for electroneutrality, we write the ferrous core reaction as follows.

\[
\text{Fe}^{2+} + \text{H}_2\text{O} + \text{Cl}^- \rightarrow \text{Fe}^{3+}\text{OCl}_(\text{core}) + \text{H}^+ \quad \text{(Eq. 5)}
\]
DTPA by H2O2 is an efficient generator of hydroxyl radical (17) for the addition sequence Dps
Dps, a weaker EPR signal (25% of spectrum A) was produced corresponding to 65% of that when DTPA was present. With Fe(II). Again a significant amount of EMPO-OH was produced, aerobically followed by 1 H2O2 per 2 Fe(II) at 2 min for a total of 500 mM Fe(III)/Dps. Conditions are as follows: adding eight increments of 50 Fe(II)/Dps anaerobically in the presence 200 Fe(II)/Dps were added aerobically at 2 min intervals and the sam-

that of the EMPO-OH adduct in a control experiment using the chelator DTPA while adding reagents in the sequence DTPA + EMPO + H2O2 + Fe(II). The one-electron oxidation of Fe(II)-DTPA by H2O2 is an efficient generator of hydroxyl radical (17) and an intense spectrum from trapped HO• was observed (Spectrum A). Spectrum B corresponds to another control experiment where the oxidation of Fe(II) was carried out in the absence of DTPA for the addition sequence EMPO + H2O2 + Fe(II). Again a significant amount of EMPO-OH was produced, corresponding to 65% of that when DTPA was present. With Dps, a weaker EPR signal (25% of spectrum A) was produced for the addition sequence Dps + EMPO + H2O2 + Fe(II), Fe(II) being added immediately after H2O2. No EPR signal was observed (spectrum D) when Fe(II) was first allowed to bind to the protein before addition of H2O2 for the sequence Dps + EMPO + Fe(II) + H2O2. Relative to either control experiment, the presence of Dps substantially reduces the amount of hydroxyl radical trapped by EMPO (c.f. spectra A and B versus C and D).

To establish whether the ability of Dps to reduce hydroxyl radical production results in protection of DNA from cleavage because of Fe(II)-mediated Fenton reactions, an in vitro DNA damage assay was set up in which the effect of a combination of 50 µM Fe(II) plus 10 µM H2O2 on the integrity of plasmid pUHE21-2 (3900 bp) in the presence and absence of Dps was assessed. Prior to carrying out these assays, the amount of Dps necessary to saturate DNA by physical association was estab-

The present studies demonstrate that Dps possesses ferritin-like function and that iron incorporation is a multistep process involving Fe(II) binding, Fe(II) oxidation, nucleation, and growth of the mineral core as in classical ferrioxamines. However, Dps is unique in that C2, which is an efficient oxidant for all known ferrioxamines (12, 14, 16, 21–24), does not rapidly oxidize Fe(II) in Dps (“Results”). In Dps, rapid and complete oxidation of Fe(II) in both ferroxidase and mineralization reactions occurs with H2O2 at stoichiometry of 2 Fe(II) per H2O2 (Fig. 6, Equations 3 and 4). Pairwise oxidation of Fe(II) avoids hydroxyl radical production, a finding confirmed by the spin trapping experiments (Fig. 9). The stoichiometry of Fe(II) oxidation by H2O2 (Fig. 6), spin trapping measurements (Fig. 9), and the DNA protection experiments (Fig. 10) indicate that the protein is capable of nullifying the toxic combination of Fe(II) and H2O2.

FIG. 8. Sedimentation coefficient distribution g*(s) versus s^2 versus s^20,w (Svedberg) for Dps, apoDps (-----). For the ferric core, 10 increments of 50 Fe(II)/Dps were added aerobically followed by 1 H2O2 per 2 Fe(II) at 2 min for a total of 500 Fe(III)/Dps. Conditions are as follows: 0.25 mM Dps, 50 mM Mops, 200 mM NaCl, pH 7.0, 20 °C. B, ferric core Dps using O2 as the oxidant (----) and ferrous core Dps (-----). For the ferric core two increments of 200 Fe(II)/Dps were added aerobically at 2 min intervals and the sample was analyzed after about 2 h. The ferrous core was prepared by adding eight increments of 50 Fe(II)/Dps anaerobically in the presence of 1 mM Na2S2O4, for a total of 400 Fe(II)/Dps. Conditions are as follows: ferric core, 1.0 µM Dps, 50 mM Mops, 200 mM NaCl, pH 7.0, 20 °C; ferrous core, 0.25 µM Dps, 50 mM Mops, 200 mM NaCl, 1 mM Na2S2O4, pH 7.0, 20 °C.

FIG. 9. X-band EPR signal of the EMPO-OH adduct in the presence and absence of Dps. A, addition sequence of DTPA + EMPO + H2O2 + Fe(II); B, addition sequence of EMPO + H2O2 + Fe(II); C, addition sequence of Dps + EMPO + H2O2 + Fe(II); D, addition sequence of Dps + EMPO + Fe(II) + H2O2. Conditions are as follows: 4.0 µM Dps or 96 µM DTPA, 25 mM EMPO, 96 µM FeSO4, 500 µM H2O2, 50 mM Mops, 200 mM NaCl, pH 7.0, at room temperature.

Magnetic Field (Gauss)
and suggest a molecular basis for the protective effect of Dps on DNA when the bacterium is under conditions of oxidative stress (1, 6).

The observed stoichiometries of 24 Fe(II)/Dps for both Fe(II) binding and oxidation (Fig. 1) are consistent with the binding of two Fe(II) at each of the 12 ferroxidase centers followed by Fe(II) oxidation. The significant quenching of the protein intrinsic fluorescence brought about by Fe(II) binding (Fig. 1A) supports this contention. In fact, the Trp⁵⁹ and Trp¹⁶⁰ residues in the 12-mer are all located within a radius of about 4 Å from the ferroxidase centers (7) and thus can function as reporter groups of the Fe(II) binding reaction. Because Fe(II) and its complexes do not readily hydrolyze at the pH 7.0 employed here (29), the production of ≈1 H⁺/Fe(II) upon Fe(II) binding is presumably derived from deprotonation of protein ligands, His⁵¹ of the putative ferroxidase site being a primary candidate. The large difference between rates of Fe(II) binding to Dps versus L. innocua ferritin and the slow oxidation of Fe(II) by O₂ in Dps ("Results") but not in L. innocua ferritin (12) may reside in their somewhat different ferroxidase centers. Lys⁸⁸ is coordinated to the metal in the Pb²⁺ derivative of Dps but this residue is absent in L. innocua ferritin (7, 10). Despite these differences, the mineralization reaction is faster in both Dps and L. innocua ferritin than the ferroxidation reaction, a property uniquely different from 24-mer ferritins where mineralization is always slower and is preceded by a rapid ferroxidation, the slower migrating bands correspond to lesser degrees of supercoiling, and to the circular plasmid.

And H₂O₂ are added in the proper order. In either case, hydroxyl radical production was significantly diminished by the presence of Dps.

The molar absorptivity corresponding to the ferroxidase center iron in Dps (λ_max = 297 nm, 29,800 M⁻¹ cm⁻¹ per iron) is about 10-fold larger than those of human H-chain ferritin (λ_max = 305 nm; 2,990 M⁻¹ cm⁻¹), horse spleen ferritin (λ_max = 305 nm; 3,540 M⁻¹ cm⁻¹) (16, 19) and E. coli bacterioferritin (λ_max = 300 nm; 3,380 M⁻¹ cm⁻¹) (14). Molar absorptivities of proteins and model complexes with μ-oxo or μ-hydroxyl bridges range from 2,000 to 12,000 M⁻¹ cm⁻¹ per iron dimer (30, 31). The value for Dps is anomalously high and may reflect the presence of more than one oxo/hydroxyl bridging group, which would lend stability to the complex and account for the slow turnover of Fe(II) at the ferroxidase center as evidenced by the stability of its spectrum (Fig. 5). The release of 3 H⁺ upon oxidation of the di-iron(II) center is also suggestive of formation of multiple μ-oxo or hydroxyl bridges (Equation 3). The fact that the H⁺/Fe(II) stoichiometry and the rate of iron oxidation are different for the first and second additions of 24 Fe(II)/Dps (Fig. 2) indicates a shift to a mineralization mechanism following saturation of the ferroxidase centers with 24 Fe(III).

The formation of a ferric core in Dps with H₂O₂ as the Fe(II) oxidant was demonstrated by the spectrophotometric titration (Fig. 7, curve a), the measured oxidation/hydrolysis reaction (Equation 4), the ultracentrifugation data (Fig. 8A), and the similarity between the molar absorptivity of the Dps core (λ_max = 300 nm, 3,200 M⁻¹ cm⁻¹) and those of known ferritins (14, 16). Although a ferric core was also formed in the presence of dioxygen, a poor oxidant with respect to H₂O₂ (Fig. 3), the O₂-driven mineralization most likely does not take place in E. coli. The bacterium contains three kinds of ferritin, namely E. coli ferritin type A, E. coli ferritin type B, and E. coli bacterioferritin, that act as iron storage and detoxification proteins in aerobic environments (36). The role of Dps is distinct. Dps is synthesized under conditions of nutritional and oxidative stress to protect DNA from hydroxyl radical damage by preventing the coordination of Fe(II) to the phosphodiester backbone or to the bases of DNA (1, 37). The unusual ferritin-like properties of Dps, which also permit H₂O₂ consumption, therefore have to be coupled to the physical association with DNA.

Of particular interest is the observation of ferrous core formation (Figs. 7, curve b, and 8, B) as previously reported for horse spleen ferritin (32–35). The hydrolysis chemistry suggests an FeOH⁻ species with associated chloride to maintain electroneutrality (Equation 5) to give a core composition FeOCl⁻. The hydrolysis rates of proteins in aerobic environments (36). The role of Dps is distinct. Dps is synthesized under conditions of nutritional and oxidative stress to protect DNA from hydroxyl radical damage by preventing the coordination of Fe(II) to the phosphodiester backbone or to the bases of DNA (1, 37). The unusual ferritin-like properties of Dps, which also permit H₂O₂ consumption, therefore have to be coupled to the physical association with DNA.

The biological effect of Dps is most evident when E. coli is under assault by H₂O₂. Killing of E. coli by H₂O₂ is bimodal (37, 38). Mode I is maximal at 1–3 mM H₂O₂, is approximately zero order with respect to H₂O₂ concentration, and requires...
active metabolism; mode II seems to be first-order with respect to H$_2$O$_2$ between 10 and 100 m M and does not require active metabolism. It is believed especially in mode I killing that DNA damage is mediated in part by Fenton chemistry (1, 37) where intracellular generation of HO$^-$ causes oxidative DNA lesions (2). Indeed, upon the exposure of the Fe(II)-DNA complex to H$_2$O$_2$ in vitro, DNA is completely degraded; in contrast, DNA saturated by Dps is fully protected (Fig. 10). From the present data, it is evident that there are at least two kinds of peroxide-consuming mechanism in Dps to cope with peroxide damage. As noted, both peroxidase site and mineralized core play an important role in consuming H$_2$O$_2$ (Figs. 2, 4, and 6). Furthermore, DpsA, a member of the Dps family, exhibits a weak and its capacity to bind Fe(II) and consume H$_2$O$_2$ without producing HO$^-$ radical, thereby ensuring survival of the genetic code of the organism.

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