Iron is used in numerous metabolic reactions and is essential in most living organisms. However, the low solubility of iron in oxygenated waters limits its bioavailability to aquatic organisms. This is the case in the surface waters of the ocean, where Fe is found primarily as particulate oxyhydroxides and Fe input from the atmosphere and deep seawater can be limited (8, 24). Recent studies have shown that low availability of Fe to phytoplankton directly limits primary production in some regions of the ocean (5, 14, 15, 30). Other studies have suggested that Fe may limit nitrogen fixation by marine cyanobacteria in other oceanic regions and thus control primary production by limiting the input of fixed nitrogen (17, 34).

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METHODS AND MATERIALS

Bacterial strains. The DH5 strain of Escherichia coli was used for cloning, and the BL21(DE3)pLysS strain (Novagen) was used for protein production. Genomic DNA from T. erythraeum IMS101 was used for PCR amplification (kindly provided by Eric Webb, Woods Hole Oceanographic Institution). For DNA damage assays, pUC19 (Sigma-Aldrich) from E. coli was used.

Cloning of the dps gene. The T. erythraeum IMS101 dps gene was amplified by PCR with oligonucleotide primers 5'-GAAATATCATGATGTCAGCGC-3' (upper primer) and 5'-CATTTCCTTATAGCTTGCGAGC-3' (lower primer). The lower primer was designed to recognize restriction endonucleases NdeI, and the lower primer was designed to recognize BglII. The resulting PCR product was cloned into the pCR 2.1 vectors (Invitrogen) by the TA cloning method and the lower primer was designed to recognize BglII. The resulting PCR product was cloned into the pCR 2.1 vectors (Invitrogen) by the TA cloning method and the lower primer was designed to recognize BglII.

DNA sequencing kits (Applied Biosystems) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems) were used for DNA sequencing. The recombinant plasmid pRSET-dps was transferred to the BL21(DE3)pLysS host, which is inducible with isopropyl-β-D-thiogalactopyranoside (IPTG).

Overexpression and purification of Dps. E. coli BL21(DE3)pLysS containing pRSET-dps was grown in LB medium containing 50 μg/ml ampicillin, and 1 mM IPTG was added to the cultures, and this was followed by incubation for 2 h for production of recombinant protein. The cells were collected by centrifugation at 10,000 × g for 15 min in a Centricon 10 tube. The supernatant was separated by centrifugation at 10,000 × g for 15 min and dialyzed overnight in 50 mM Tris-HCl (pH 7.9)–50 mM NaCl.

Lowry et al. (29).

Purification of the protein was achieved by using a Source 30 anion-exchange column and a Superdex-30 size exclusion column. To concentrate the purified protein, a Centriprep YM-50 MW centrifugal filter was used. The purity of the overexpressed Dps protein was checked by sodium dodecyl sulfide (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie brilliant blue R250. The protein concentration was determined by the method of Lowry et al. (29).

Preparation of 55Fe-loaded Dps. To obtain radiolabeled cultures, E. coli cells harboring the pRSET-dps and pRSET vectors were grown in LB medium containing [55FeCl3], and purified as described above. The native PAGE gel containing the Dps protein was then exposed to a phosphor screen and scanned with a STORM 860 Phosphorimager (Molecular Dynamics) to detect 55Fe.

Gel retardation assays. dpsret was mixed with pUC19 at DNA:protein molar ratios 1:500, 1:800, and 1:1050 in 50 mM Tris-HCl (pH 7.9) and incubated for 30 min at 30°C. The complexes obtained were resolved on 1% agarose gel containing 10 μg/ml of 0.5 M sodium acetate buffer were added, and the solution was left at room temperature for 30 min. The absorbance at 625 nm was determined with a UV-visible spectrophotometer. The absorbance at 562 nm of the samples was determined with a UV-visible spectrophotometer.

Fe and phosphate analysis. The iron concentration was determined chemically by the method of Stookey, modified for protein use (28). Protein (20 μl) was digested with 50 μl of 1 M HCl for 1 h and neutralized with 50 μl of 1 M NaOH. Then 1 ml of the malachite green reagent was added, and the preparation was incubated at room temperature for 30 min. The absorbance at 625 nm was determined with a UV-visible spectrophotometer.

Fe K-edge X-ray absorption. Fe K-edge near-edge X-ray absorption (XANES) spectra were collected at beamline 11-2 at the Stanford Synchrotron Radiation Laboratory, Stanford, CA, using an Si (220) monochromator crystal. The incident photon flux was measured using an ion chamber filled with N2. The XANES spectra of all samples and standards were collected in fluorescence mode using a 30-element Ge detector. The standards were also examined in transmission mode to minimize saturation effects in the fluorescence yield spectra. All spectra were normalized to the incident photon flux, and their energies were calibrated with respect to the pre-edge peaks in goethite with the high-energy pre-edge transition set to 7.101 eV. The slits were set to 6 × 1 mm to obtain high-resolution XANES spectra.

RESULTS AND DISCUSSION

Identification of iron storage proteins. We searched the publicly available genome of T. erythraeum IM101 (http://aem.asm.org/) and identified two genes, designated genes A and B, that encode amino acid sequences homologous to known bacterioferritin sequences (Fig. 1). Gene A exhibits sequence similarity with bacterioferritin genes from Azotobacter vinelandii (13%), Pseudomonas putida (17%), Synechocystis sp. strain PCC 6803 (14%), and Desulfovibrio desulfuricans (18%). Analysis of an alignment of the sequence encoded by Trichodesmium gene A with bacterioferritin sequences revealed conservation of the amino acid residues that act as ligands to the ferroxidase center (5). Amino acids Glu-18, Glu-50, His-54, Glu-94, Glu-127, and His-130, which form the ferroxidase center in A. vinelandii, are conserved in the gene A product as Glu-23, Glu-55, His-59, Glu-100, Glu-132, and His-135 (5). However, the gene A product lacks the heme-methionine ligand that is conserved in all bacterioferritins, which indicates that it is likely a bacterial ferritin instead.

Gene B codes for a sequence consisting of 180 amino acids (molecular mass, ~20.23 kDa) with sequence identity to known bacterioferritin sequences of Synechocystis (19%), P. putida (19%), D. desulfuricans (15%), and A. vinelandii (13%). Although identified with bacterioferritin sequences, the protein encoded by this gene does not contain all the amino acids residues involved in the di-iron ferroxidase coordination site in bacter-
A comparison of the gene B product with known Dps proteins revealed high levels of similarity with members of the Dps family (Fig. 2). Gene B thus codes for a putative Dps protein, which we designated Dpstery; this protein exhibits 69% primary amino acid sequence identity with *Synechococcus* DpsA, 32% primary amino acid sequence identity with *E. coli* Dps, and 30% primary amino acid sequence identity with *Listeria innocua* Flp.

Sequence alignment of Dpstery and other proteins revealed conservation of the amino acid motifs that are thought to be involved in the formation of the intersubunit dinuclear ferritin-like ferroxidase center in Dps proteins (Fig. 2). In the crystal structure of the Flp protein from *L. innocua*, a member of the Dps family, 12 iron atoms have been observed occupying the putative ferroxidase centers. The amino acids involved in the coordination of iron are His-31, His-43, Asp-47, Asp-58, and Glu-62 (22). Equivalent amino acid residues were found in Dpstery (His-53, His-65, Glu-70, Glu-81, and Glu-84). Recent work in vivo has shown that the amino acids involved with the putative ferroxidase center are crucial for the incorporation of iron. Site-directed mutagenesis of the negatively charged amino acids, Asp-74 and Glu-78, with Ala prevented iron incorporation by a Dps homologue in *Streptococcus suis* (37).

**Amplification and cloning of Dpstery.** After overexpression of the putative Dpstery in *E. coli*, cell extracts were subjected to SDS-PAGE analysis. Figure 3 shows the presence of the expected 20-kDa band after induction of *E. coli* harboring pR-

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**FIG. 1.** Sequence alignment for bacterioferritins and a homologous protein in *T. erythraeum* (encoded by gene A). The residues involved in metal binding are indicated by stars, and the methionine heme ligand is underlined. The bacterioferritin sequences of *A. vinelandii* (BFR_Azotobacter), *P. putida* (BFR_Pseudomonas), *Synechocystis* sp. strain PCC 6803 (BFR_Synechocystis), and *D. desulfuricans* (BFR_Desulfuricans) were used to identify the homologous protein in *Trichodesmium* (BFR_Trichodesmium). Residues identical in all sequences are shown with a black background, and conserved residues are indicated by a gray background.

**FIG. 2.** Alignment of the Dpstery sequence with known and putative Dps sequences. Residues identical in all the sequences are indicated by a black background, and conserved residues are indicated by a gray background. Amino acid residues that are red are the residues known to be involved in metal binding in *Listeria* ferritin (Listeria_flp), and these residues are aligned with homologous positions in Dpstery (Tricho_Dps) *Crocosphaera watsonii* putative Dps (Crocosphaera), *Synechococcus* sp. strain PCC 7942 DpsA (Synp7_DspA), and *E. coli* Dps (Ecoli_Dps).
SET-dps with IPTG. Examination of an 8% native PAGE gel containing the recombinant protein purified by using a ferritin purification protocol revealed a protein whose molecular mass is less than that of horse spleen ferritin (~450 kDa) but more than that of the bovine serum albumin monomer (~132 kDa) (Fig. 3). This result indicates that the native recombinant protein is not composed of 24 subunits and likely consists of 12 subunits (~240 kDa). On the basis of the amino acid sequence, we calculated a molecular mass of 20.23 kDa for one subunit and a molecular mass of ~242.76 kDa for the native protein.

Fe binding. The iron binding ability of Dps proteins (or Dps homologs) has been confirmed with proteins isolated from *E. coli*, *L. innocua*, *Mycobacterium smegmatis*, *Campylobacter jejuni*, and *Streptococcus mutans* (19, 23, 42, 52, 54). Thus, we investigated the iron binding ability of DpsEry. *E. coli* cells with the pRSET (but not Dps) vector were grown in the same medium as a negative control. Upon exposure of the native PAGE gel to a phosphorimager, we observed the radiolabel iron in DpsEry, as expected. No 55Fe was observed in any of the protein bands from the control cells, demonstrating that the result obtained for DpsEry was not due to the presence of Dps from *E. coli* or to nonspecific iron binding (Fig. 5). The intense 55Fe band seen in the phosphorimage reflected the high iron binding capacity of DpsEry.

The iron storage capacity of DpsEry was quantified by incubating the protein with ferrous ammonium sulfate in the presence of O2 or H2O2. We found 260 ± 20 Fe atoms/protein molecule in the presence of O2 and 270 ± 10 Fe atoms/protein molecule in the presence of H2O2. Because the maximum incorporation of Fe by Dps proteins has been reported to be 500 atoms per molecule, a solution of DpsEry that contained 260 Fe atoms per molecule was reincubated with a ferrous ammonium sulfate solution (7, 53). The additional incubation did not increase the incorporation of Fe by the protein (260 ± 40 Fe atoms/protein molecule). In *E. coli* Dps, H2O2 has been shown to be a more effective oxidant of Fe(II) than O2, but in DpsEry the maximum iron capacity remained the same regardless of the oxidant used (21). We do not know if the lower iron capacity that we observed in DpsEry than in Dps from *E. coli* corresponds to an intrinsic difference between the proteins or to a lack of optimization in our Fe loading protocol.

Incorporation of phosphate. Although phosphate incorporation into the iron core of ferritins and bacterioferritins has been reported, until now the possibility that Dps proteins may contain phosphate has not been studied (46, 48). Purified DpsEry obtained from overexpression in *E. coli* was found to contain 10 ± 1 P ions/molecule/protein molecule. When DpsEry was incubated with both ferrous ammonium sulfate and 1 mM or 5 mM potassium phosphate loading values of 66.4 ± 0.5 and 50 ± 4 P ions/molecule/protein molecule were obtained, showing that the core has a P/Fe ratio of about 1:4. In horse spleen ferritin a P/Fe ratio of 1:8 has been reported, while in bacterioferritins P/Fe ratios between 1 and 2 have been observed (4, 46). Phosphate content is currently thought to affect the structure and size of the iron core in ferritins and bacterioferritins, and there is evidence that phosphate influences the availability of iron; however, the overall role of phosphate in the biochemistry of the protein is poorly understood (25, 45, 46). Nevertheless, the incorporation of phosphate into the iron core in DpsEry provides additional evidence for the ferritin-like properties of the core in Dps proteins.

DNA binding. An unusual feature of Dps compared with other ferritin family proteins is that it binds DNA. This has been shown in *E. coli*, *Porphyromonas gingivalis*, *Synechococcus*, and *M. smegmatis* (3, 19, 36, 47). (Dps-like proteins composed of 12 subunits that do not appear to bind DNA, such as the protein found in *L.
innocua, may logically be classified as a separate type of ferritin.)

To determine whether Dps<sub>tery</sub> binds DNA, we performed a gel mobility shift assay using the <i>E. coli</i> pUC19 plasmid as a template. Incubation of pUC19 DNA with Dps<sub>tery</sub> at 37°C for 30 min decreased the mobility of all the DNA bands on the agarose gel, and this effect was exaggerated at higher concentrations of protein (Fig. 6). Most strikingly, a large fraction of the DNA remained stationary and did not enter the agarose gel.

For determination of the apparent dissociation constant ($k_d$) of DNA with Dps<sub>tery</sub>, pUC19 (0.15 nM) was titrated with Dps<sub>tery</sub>, the products were resolved by agarose gel electrophoresis, and the bands were quantified using IMAGE J (1). The apparent $k_d$ of Dps<sub>tery</sub> calculated by measuring the protein concentration that resulted in 50% binding of the DNA was about 16 nM (Fig. 7), which is nearly 100-fold higher than the constant reported for the Dps protein from <i>E. coli</i> ($k_d$, 172 to 178 nM) (6, 10). Until more is known about the affinities of various Dps proteins for DNA, it is difficult to speculate on the meaning of this large apparent difference between the proteins from <i>T. erythraeum</i> and <i>E. coli</i>.

DNA protection. Dps-DNA complexes have been shown to be extremely stable; in addition, DNA binding stabilizes the Dps structure (3). It has been shown that once a DNA-Dps complex is formed, the DNA is protected from attack by various nucleases, such as DNase I (19). To test the ability of Dps<sub>tery</sub> to protect DNA, we incubated pUC19 DNA with Dps for 30 min and then added DNase I. Nonincubated pUC19 DNA and pUC19 incubated with DNase I were used as controls. All samples were separated on a 1% agarose gel (Fig. 8). As Fig. 8, lanes 1 and 2, show, incubation of pUC19 DNA with DNase I resulted in complete degradation of the nucleic acid, and there were no visible bands on the gel. In contrast, DNA that was preincubated with Dps<sub>tery</sub> produced an intense band that remained in the loading well. Thus, the DNA-Dps<sub>tery</sub> complex appeared to be effectively protected from degradation by DNase I.

Fe K near-edge X-ray absorption spectra. Despite identification of Dps proteins in numerous microorganisms, little is known about the structure of the Fe core. Figure 9 shows the X-ray absorption spectra of horse spleen ferritin and Dps<sub>tery</sub> iron cores. The spectra of ferritin and Dps<sub>tery</sub> are nearly identical and have similar pre-edge features. The weak pre-edge observed at ~7,100 eV corresponds to the 1s → 3d quadrupole-allowed, dipole-for-
Mutants of Synechocystis may be part of an effective protective mechanism described by Dps proteins and has a similar molecular mass. Dps provides UV-absorbing compounds, the Dps protein identified in the ocean during blooms. Along with carotenoids and other protective stress, the Dps protein identified in Synechococcus sp. H11011 is similar to that of horse spleen ferritin, we concluded that Dps is similar to that of horse spleen ferritin, which concluded that the iron core in Dps is composed of ferric iron that is octahedrally coordinated. Prior work has demonstrated that the chemistry of the Dps core from E. coli is similar to that of ferritin, and our XANES data also demonstrated that the core compositions of Dps and ferritin are similar (21).

Potential role of Dps proteins in the marine environment. The Dps protein that is isolated is homologous to previously described Dps proteins and has a similar molecular mass. Dps also appears to have all the properties ascribed to such proteins; it binds iron, binds DNA, and protects DNA from degradation. Genomic analysis revealed that genes encoding Dps homologues are present in the genomes of Prochlorococcus sp. strain MIT 9313 and Crocosphaera watsonii, two of the few marine microorganisms that have been sequenced so far. All three of the functions that we demonstrated for Dps may be useful to photrophs that live in the surface ocean. Such organisms must survive in environments where Fe inputs are low or episodic and they are subjected to oxidative stress because of the presence of molecular oxygen and intense sunlight. These organisms may also need to protect their genetic material when they survive in some dormant form during low-nutrient periods or when they are advected out of the photic zone.

The problem of photodamage may be particularly severe for Trichodesmium, which accumulates at the surface of the ocean during blooms. Along with carotenoids and other UV-absorbing compounds, the Dps protein identified in Trichodesmium may be part of an effective protective mechanism (11, 44). Mutants of Synechocystis, Synechococcus, and E. coli that lack the dps genes are extremely sensitive to photodamage and peroxide (16, 27, 32). The protection provided by Dps against degradation by reactive oxygen species probably contributes to the survival of Trichodesmium, which must carry out oxygenic photosynthesis in the presence of intense sunlight.

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REFERENCES


