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).

Trichodesmium spp. are diazotrophic marine cyanobacteria that play a key role in the tropical and subtropical regions of the oceans not only as major primary producers but also as suppliers of new nitrogen through N2 fixation (17, 34). Trichodesmium is responsible for an estimated input of at least 65 Tg fixed N2 per year. Nitrogenase is a two-component metalloenzyme with MoFe and Fe cofactors. The MoFe and Fe proteins contain metalloclusters (Fe-S cores) that are required for nitrogenase activity (18, 26). Because of the high Fe content of the nitrogenase enzyme, the iron requirement of nitrogen fixers has been estimated to be 2.5 to 5.2 times higher than that of other phytoplankton (40). The mechanisms by which Trichodesmium acquires and utilizes Fe are thus of great interest. In this study we focused on the question of Fe storage, which is critical because of the episodic nature of Fe input into many tropical and subtropical regions of the oceans.

Free iron in cells is extremely toxic as it catalyzes the generation of reactive oxygen species, such as the hydroxyl radical (·OH), which causes oxidative damage (Haber-Weiss/Fenton reactions).

Thus, iron storage in cells is effected by ferritins, a superfamily of proteins that store iron in a readily available yet nontoxic form (4). Mammalian-type ferritins (i.e., ferritins proper), bacterioferritins, and Dps proteins (DNA binding proteins from starved cells) belong to this family of proteins. Ferritins and bacterioferritins are composed of 24 subunits that form a spherical protein shell with a hollow center in which Fe can be stored (up to 4,500 Fe atoms in ferritins and 2,000 Fe atoms in bacterioferritins) (4, 20). Bacterioferritins differ from mammalian ferritins in that they have up to 12 noncovalently bound heme groups (4, 39). The heme centers have bis-methionine ligation (12, 13). Dps proteins have not been as thoroughly characterized as ferritins or bacterioferritins yet. These proteins are composed of only 12 subunits and can accommodate correspondingly fewer Fe atoms (~500 atoms) (22). Iron is incorporated into the central cavity of ferritins by oxidation of Fe2+, followed by formation of a microcrystalline ferrirhydrite-phosphate core. The conversion of Fe2+ to Fe3+ is catalyzed by a ferroxidase center that is found in the subunits in ferritins and bacterioferritins (4) and between subunits in Dps proteins (22, 35, 38). Studies with various organisms have confirmed that the role of all ferritins is to store Fe and prevent its toxicity (41). In addition, it has been found that Dps protects DNA against oxidative agents by cocrystallization of the nucleic acid with the protein (3, 31, 51). DNA binding appears not to be sequence specific since no DNA binding motifs have been identified in Dps proteins yet. The protection of DNA appears to occur through oxidation and sequestration of Fe2+ ions, avoiding Fenton chemistry (31, 51).

Despite the current interest in the use of iron by autotrophs and particularly by nitrogen fixers in the oceans, no protein belonging to the ferritin family has been isolated or characterized from a marine microorganism previously. Here we describe the identification, isolation, and characterization of a Dps protein in Trichodesmium erythraeum.
METHODS AND MATERIALS

Bacterial strains. The DH5α 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 (Siga-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′-GAAATAAATCATATGTTTAGG-3′ (upper primer) and 5′-TCTGTGCTTTTCACTTGCGAGG-3′ (lower primer). NdeI and BglII restriction sites were added to the PCR primers.

The upper primer was designed to recognize restriction endonuclease 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 resulting vector was named pCR-dps. The resulting PCR product was cloned into the pBR322 vectors (Invitrogen) by utilizing the NdeI and BglII restriction sites, which resulted in pRSET-dps. DNA sequencing reactions were performed by using the plasmid template pRSET-dps with ABI Prism BigDye 3.1 sequencing kits (Applied Biosystems) and an ABI PRISM 3100 genetic analyzer (Applied Biosystems). For protein production 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 to an optical density at 600 nm of 0.7 in 1 liter of Luria-Bertani broth containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol, IPTG (0.4 mM) was added to the cultures, and this was followed by incubation for 4 h for production of recombinant protein. The cells were collected by centrifugation at 10,000 × g for 15 min and dialyzed overnight in 50 mM Tris-HCl (pH 7.9)–50 mM NaCl. Final purification of the protein was performed by using a Source 30 Q anion-exchange column and a Superdex-30 size exclusion column.

Preparation of 55Fe-loaded Dps. Preparation of 55Fe-loaded Dps was described in the previous publication of *T. erythraeum* IM101 (http://www.jgi.doe.gov) and identified with bacterioferritin sequences, the protein encoded with bacterioferritin sequences, the protein encoded with bacterioferritin sequences, the protein encoded.

Identification of iron storage proteins. We searched the previously published genome of *T. erythraeum* IM101 (http://www.jgi.doe.gov) 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 *Azoto bacter vinelandii* (13%), *Pseudomonas putida* (17%), *Synechocystis* sp. strain PCC 6803 (14%), and *Desulfobirrio desulfuricans* (18%). Analysis of an alignment of the sequence encoded by *Trichodesmium* genome A as a Dps-like protein gene (http://www.jgi.doe.gov).

RESULTS AND DISCUSSION

Identification of iron storage proteins. We searched the previously published genome of *T. erythraeum* IM101 (http://www.jgi.doe.gov) 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 *Azoto bacter vinelandii* (13%), *Pseudomonas putida* (17%), *Synechocystis* sp. strain PCC 6803 (14%), and *Desulfobirrio 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-thionine 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 bacte-
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 Dpers. 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 Dpers 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 Dpers (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 Dpers. After overexpression of the putative Dpers 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-

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 Dpers 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 Dpers (Tricho_Dps) Crocosphaera watsonii putative Dps (Crocosphaera), Synechococcus sp. strain PCC 7942 DpsA (Symp7_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 dimer (~132 kDa) (Fig. 4). 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 Dps<sub>tery</sub>. 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 Dps<sub>tery</sub>, as expected. No ~55Fe was observed in any of the protein bands from the control cells, demonstrating that the result obtained for Dps<sub>tery</sub> 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 Dps<sub>tery</sub>.

The iron storage capacity of Dps<sub>tery</sub> was quantified by incubating the protein with ferrous ammonium sulfate in the presence of O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub>. We found 260 ± 20 Fe atoms/protein molecule in the presence of O<sub>2</sub> and 270 ± 10 Fe atoms/protein molecule in the presence of H<sub>2</sub>O<sub>2</sub>. Because the maximum incorporation of Fe by Dps proteins has been reported to be 500 atoms per molecule, a solution of Dps<sub>tery</sub> 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, H<sub>2</sub>O<sub>2</sub> has been shown to be a more effective oxidant of Fe(II) than O<sub>2</sub>, but in Dps<sub>tery</sub> 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 Dps<sub>tery</sub> 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 Dps<sub>tery</sub>, obtained from overexpression in E. coli was found to contain 10 ± 1 P<sub>i</sub> molecules/protein molecule. When Dps<sub>tery</sub> 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<sub>i</sub> molecules/protein molecule were obtained, showing that the core has a P<sub>i</sub>/Fe ratio of about 1:4. In horse spleen ferritin a P<sub>i</sub>/Fe ratio of 1:8 has been reported, while in bacterioferritins P<sub>i</sub>/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 Dps<sub>tery</sub> 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 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 Dpstery binds DNA, we performed a gel mobility shift assay using the E. coli pUC19 plasmid as a template. Incubation of pUC19 DNA with Dpstery 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. For determination of the apparent dissociation constant (kd) of DNA with Dpstery, pUC19 (0.15 nM) was titrated with Dpstery, the products were resolved by agarose gel electrophoresis, and the bands were quantified using IMAGE J (1). The apparent kd of Dpstery 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 E. coli (kd, 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 T. erythraeum and E. coli.

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 Dpstery 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 Dpstery produced an intense band that remained in the loading well. Thus, the DNA-Dpstery 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 Dpstery iron cores. The spectra of ferritin and Dpstery 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-
FIG. 9. Near-edge spectra of the iron K-edge of horse spleen ferritin (spectrum a) and \( \text{Dps}_{\text{ery}} \) (spectrum b). The pre-edge feature of ferritin at \(-7.100\) eV is characteristic of ferric iron octahedrally coordinated to oxygen, and a similar pre-edge feature was observed for \( \text{Dps}_{\text{ery}} \).

Potential role of Dps proteins in the marine environment.

The \( \text{Dps}_{\text{ery}} \) that we isolated is homologous to previously described Dps proteins and has a similar molecular mass. \( \text{Dps}_{\text{ery}} \) 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 Oxalobacter formigenes, a cyanobacterium, and our XANES data also demonstrated that the core compositions of \( \text{Dps}_{\text{ery}} \) and ferritin are similar (21).

Dps PROTEIN FROM *T. ERYTHRAEUM* 2923

out oxygenic photosynthesis in the presence of intense sunlight.

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REFERENCES


