Localization and Role of Manganese Superoxide Dismutase in a Marine Diatom

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Superoxide dismutase (SOD) catalyzes the transformation of superoxide to molecular oxygen and hydrogen peroxide. Of the four known SOD isoforms, distinguished by their metal cofactor (iron, manganese [Mn], copper/zinc, nickel), MnSOD is the dominant form in the diatom *Thalassiosira pseudonana*. We cloned the MnSOD gene, sodA, using the expression vector pBAD, overexpressed the product in *Escherichia coli*, and purified the mature protein (TpMnSOD). This recombinant enzyme was used to generate a polyclonal antibody in rabbit that recognizes MnSOD in *T. pseudonana*. Based on quantitative immunoblots, we calculate that in vivo concentrations of TpMnSOD are approximately 0.9 amol cell⁻¹ using the recombinant protein as a standard. Immunogold staining indicates that TpMnSOD is localized in the chloroplasts, which is in contrast to most other eukaryotic algae (including chlorophytes and embryophytes) where MnSOD is localized exclusively in mitochondria. Based on the photosynthetic Mn complex in photosystem II, cellular Mn budgets cannot account for 50% to 80% of measured Mn within diatom cells. Our results reveal that chloroplastic MnSOD accounts for 10% to 20% of cellular Mn, depending on incident light intensity and cellular growth rate. Indeed, our analysis indicates that TpMnSOD accounts for 1.4% (+0.2%) of the total protein in the cell. The TpMnSOD has a rapid turnover rate with an apparent half-life of 6 to 8 h when grown under continuous light. TpMnSOD concentrations increase relative to chlorophyll, with an increase in incident light intensity to minimize photosynthetic oxidative stress. The employment of a Mn-based SOD, linked to photosynthetic stress in *T. pseudonana*, may contribute to the continued success of diatoms in the low iron regions of the modern ocean.

All aerobic organisms produce intracellular and extracellular reactive oxygen species (ROS) as metabolic byproducts (Haliwell, 1982; Asada, 1999; Apel and Hirt, 2004). Photoautotrophs also produce ROS through photosynthesis (Falkowski and Raven, 1997; Anderson et al., 1999; Wolfe-Simon et al., 2005). The ROS byproducts include superoxide (O₂⁻), hydrogen peroxide, and hydroxyl radical (Haliwell, 1982). In addition to being highly reactive, O₂⁻ is particularly destructive because it cannot diffuse across cell membranes and, therefore, must be destroyed at the site of production. Superoxide dismutases (SODs) are a polyphyletic family of enzymes that protect cells from O₂⁻. SODs come in four isoforms, recognized by their metal center cofactors (iron [Fe], manganese [Mn], copper [Cu]/zinc [Zn], and nickel [Ni]), and catalyze the destruction of O₂⁻ to hydrogen peroxide and O₂. This key antioxidant has been well studied in many eukaryotic systems, including metazoa and plants (Bowler et al., 1992; Scandalios, 1993; Fridovich, 1995; Raychaudhuri and Deng, 2000; Zelko et al., 2002). However, few studies on the intracellular regulation of SOD in diatoms are available, which is unfortunate, as these algae dominate the flux of carbon in the contemporary ocean (Falkowski et al., 2004b).

Diatoms appear to rely primarily on the Mn form of SOD (MnSOD; Peers and Price, 2004); therefore, understanding the regulation of MnSOD in diatoms is important, as this enzyme must be critical to the cells’ ability to cope with oxidative stress. The regulation and subcellular localization of MnSOD varies significantly among algal taxa (Wolfe-Simon et al., 2005). In cyanobacteria, MnSOD is found in the periplasm and is associated with the thylakoid membranes (Herbert et al., 1992; Chen et al., 2001; Li et al., 2002). In contrast, MnSOD is found in the mitochondria of embryophytes, chlorophytes, and dinoflagellates (Kliebenstein et al., 1998; Kitayama et al., 1999; Wu et al., 1999; Okamoto et al., 2001; Okomoto et al., 2001; Fink and Scandalios, 2002). Although some information has been shown regarding SOD in diatoms (Huang et al., 2005; Ken et al., 2005), no information on the subcellular localization of MnSOD and the associated kinetics in diatoms is available.

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Given our lack of understanding of MnSOD in diatoms, despite their global significance, we examined the expression and preliminary regulation of MnSOD in the bloom-forming diatom *Thalassiosira pseudonana* CCMP1335 (Ziemann et al., 1991; Levasseur et al., 1992; Zigone et al., 1995; Cabecadas et al., 1999). Our results indicate that MnSOD is localized in the chloroplast and has a rapid turnover rate mediated by incident light levels that are closely coupled to photosynthetic activity.

### RESULTS

#### Native Molecular Mass and Western Analyses

Western blots from denaturing PAGE of crude cell extracts (Fig. 1) probed with anti-TpMnSOD reveal a major band of approximately 23 kD, which corresponds well with the predicted subunit molecular mass of 22.8 kD for MnSOD based on sequence analysis. The anti-TpMnSOD cross reacted with other diatom species and weakly recognized MnSOD in several dinoflagellates (Table I). Interestingly, there was no anti-TpMnSOD cross reactivity with two other heterokonts: *Nannochloropsis oculata* and *Heterosigma akashiwo*. No reactivity was observed in the chlorophytes, cyanobacteria, prymnesiophytes, cryptophytes, and rhodophytes.

#### MnSOD and the Cellular Mn Budget in Diatoms

Based on quantitative immunoanalyses of MnSOD in nutrient-replete, exponentially growing cultures of *T. pseudonana*, this marine diatom maintains 0.91 amol MnSOD per cell when grown at moderate light levels. This quantity of MnSOD accounts for 1.4% (±0.2%) of the total cellular protein (Table II). This pool turns over rapidly; TpMnSOD is virtually undetectable after 16 h under continuous light when protein synthesis is blocked (Fig. 2), corresponding to a 5- to 8-h half-life. The turnover was mediated by light as the protein was blocked (Fig. 2), corresponding to a 5- to 8-h half-life. The turnover was mediated by light as the protein was detectable even after 27 h when cells were kept in darkness, regardless of whether protein synthesis was inhibited.

The total Mn associated with TpMnSOD ranges between 10% and 20% of the total cellular Mn (Fig. 3; see legend for calculation details). Raven (1990) estimated that between 2 and 4 μmol Mn mol C⁻¹ are needed to support the Mn requirement of the PSII Mn complex in *T. pseudonana*, which would account for approximately 60% of total Mn within a cell (Sunda and Huntsman, 1986, 1998; Raven, 1990). Based on immunquantitative analyses, MnSOD accounts for another 15% of the Mn budget. Thus, approximately 80% of the total Mn budget is associated with MnSOD and PSII.

### Table I. Antibody cross reactivity

We reverse transcribed, amplified, and cloned the gene for MnSOD from freshly extracted *T. pseudonana* mRNA. We then raised an antibody in rabbits to the recombinant protein and tested the antibody against a wide range of whole cell protein extracts from cyanobacteria, primary green, primary red, and secondary red algae. Here, we present data showing the specificity of this antibody. It primarily recognizes only diatoms and only weakly some dinoflagellates. It did not cross react with any other phylum or class of algae. All algae were grown in pure culture at optimal conditions as recommended by the Culture Collection of Marine Phytoplankton (CCMP; www.bigelow.org). +, Positive recognition; −, weak recognition; −−, no recognition.

<table>
<thead>
<tr>
<th>Taxa Identification</th>
<th>CCMP</th>
<th>Recognition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillariophyta</td>
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<td></td>
</tr>
<tr>
<td>Ditylum brightwellii</td>
<td>358</td>
<td>+</td>
</tr>
<tr>
<td>Navicula incerta</td>
<td>542</td>
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</tr>
<tr>
<td>Nitzschia brevirostris</td>
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<td>+</td>
</tr>
<tr>
<td>Stephanopyxis turris</td>
<td>815</td>
<td>+</td>
</tr>
<tr>
<td>Thalassiosira pseudonana</td>
<td>1010</td>
<td>+</td>
</tr>
<tr>
<td>Skeletonema costatum</td>
<td>1332</td>
<td>+</td>
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<td>Dinophyceae</td>
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<tr>
<td>Karlodinium micrum</td>
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<tr>
<td>Eustigmatophyceae</td>
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<td></td>
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<tr>
<td>Nannochloropsis oculata</td>
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<td>−</td>
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<td>Raphidophyceae</td>
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<td>Heterosigma akashiwo</td>
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<td>Dunaliella tertiolecta</td>
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<tr>
<td>Pyramimonas parkeae</td>
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<tr>
<td>Nannochloris atomus</td>
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<td>−</td>
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<tr>
<td>Tetraselmis marina</td>
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<td>−</td>
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<td>Emiliana huxleyi</td>
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<td>Cryptophyceae</td>
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<td>Rhodomonas salina</td>
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<td>Rhodophyta</td>
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<tr>
<td>Porphyridium sp.</td>
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<td></td>
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<tr>
<td>Cyanobacteria</td>
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<tr>
<td>Trichodesmium sp. IMS101</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Synechocystis sp. PCC6803</td>
<td>−</td>
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</table>

*Figure 1. Immunoblot of selected diatom species. Immunoblots showing the anti-TpMnSOD antibody we produced cross reacted with multiple diatom species. The phylum specificity of this antibody suggests that all diatoms have a MnSOD with similar structure. All lanes loaded with 30 μg total protein (except control loaded with 10 ng pure recombinant TpMnSOD): lane 1, Ditylum brightwellii CCMP358; lane 2, Navicula incerta CCMP542; lane 3, Nitzschia brevirostris CCMP551; lane 4, Stephanopyxis turris CCMP815; lane 5, T. pseudonana CCMP1010; lane 6, Skeletonema costatum CCMP1332; CON, control protein overexpressed and purified recombinant TpMnSOD. Note: T. pseudonana strain used in this immunoblot is CCMP1010 and different than CCMP1335, which is the one used for cloning and overexpression of sodA. Marker indicates molecular mass standards in kilodaltons.*
Immunolocalization of MnSOD in Plastids

Immunogold labeling measurements suggest that MnSOD is mainly confined to the chloroplast (Fig. 4). The immunogold label is predominantly associated with thylakoid membranes and the pyrenoid. It is not associated with the cytosol or the mitochondria. Because the chloroplast-localized MnSOD is regulated by the nuclear-encoded sodA gene, plastid/endoplasmic reticulum transit peptides must be present, but they have not yet been identified.

Impact of Light on TpMnSOD Expression

When acclimated to a range of irradiance levels (25, 50, 120, 350, and 800 μmol m⁻² s⁻¹), T. pseudonana cells show a 73% increase in growth rate (Fig. 5). Total chlorophyll a cell⁻¹ is constant at low light levels (25–50 μmol m⁻² s⁻¹) but decreases by 63% as the incident light intensity increases from 50 to 800 μmol m⁻² s⁻¹. (Cells were kept optically thin in semiconfined batch cultures to avoid self shading.) Over this range of irradiances, the amount of TpMnSOD per unit chlorophyll increased by 60% (Fig. 5), reflecting changes in the chlorophyll concentration, not MnSOD. Although the chlorophyll a-normalized MnSOD content of T. pseudonana increased with increasing light, the amount of MnSOD per total cellular protein was constant (data not shown). Thus, the demand for MnSOD per cell in these cells appears to be constant over these light levels despite declining chlorophyll. A similar relationship between light intensity, SOD, and reduced cellular chlorophyll was also seen for the chloroplastic CuZnSOD in bean and other higher plants (Gonzalez et al., 1998, and refs. therein).

To further examine the relationship between light and MnSOD, the time course of TpMnSOD expression was followed over 30 h in cells acclimated to a 12/12-h photoperiod. TpMnSOD expression did not vary significantly over the photoperiod when grown at 120 μmol m⁻² s⁻¹ incident light (control), but increased by 40% within 24 h and after one dark period when transferred to high light (>800 μmol m⁻² s⁻¹; Fig. 6, A and B). The maximum photosynthetic quantum yield (F_/Fm′; Kolber et al., 1998) initially decreased by 50% under high light but recovered and exceeded the control within 30 h (Fig. 6, A and B). After a period of recovery (the dark cycle) to reorganize their metabolic profile, the cells then effectively cope with the high light stress with increased MnSOD expression. The increase in MnSOD expression per unit protein in the high light treatment (12/12-h light/dark) is significant (P = 0.0007) and is not exhibited by cells exposed to continuous high light. MnSOD expression, normalized to cell protein, doubled after transfer from culture grown in continuous light to a 12/12-h light/dark cycle (data not shown). Thus, continuous light apparently results in greater oxidative stress in diatoms than does a diel light cycle.

DISCUSSION

Our results clearly indicate that in T. pseudonana MnSOD is localized in the chloroplasts. This subcellular location is in contrast with all other cellular MnSOD distributions in eukaryotic photoautotrophs, where MnSOD is found exclusively in the mitochondria (Grace, 1990; Moller, 2001; del Rio et al., 2003). The presence of MnSOD in the chloroplast results in cells having a high cellular Mn requirement, given the substantial need for Mn of the photosynthetic machinery. The localization of this nuclear-encoded gene in a secondary symbiont presumably facilitates the rapid destruction of SOD, which is inevitably photochemically generated from the reaction centers in both photosystems. For example, the D1 protein (PsbA) has a turnover rate of approximately 30 min, one of the fastest turnover protein rates on Earth (Kim et al., 1993; Sundby et al., 1993; Andersson and Aro, 1997; Neidhardt et al., 1998). This high turnover is due, in large part, to the production of radical oxygen on the donor side of PSII (Mattoo et al., 1984). Chloroplast

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**Table II. Statistics for MnSOD in T. pseudonana CCMP1335**

<table>
<thead>
<tr>
<th>Unit of Measure</th>
<th>Valuation</th>
</tr>
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<tbody>
<tr>
<td>Protein mass per cell</td>
<td>19.8 ± 3 fg MnSOD cell⁻¹</td>
</tr>
<tr>
<td>Molecules per cell</td>
<td>5.5 ± 0.9 × 10⁵ MnSOD cell⁻¹</td>
</tr>
<tr>
<td>Molecules per cell</td>
<td>2.7 ± 0.5 × 10⁵ HOLO-MnSOD cell⁻¹</td>
</tr>
<tr>
<td>Molecules per cell</td>
<td>0.9 ± 0.2 amol cell⁻¹</td>
</tr>
<tr>
<td>Cell volume</td>
<td>19.6 ± 0.8 fl</td>
</tr>
<tr>
<td>Percent of total protein</td>
<td>1.4% ± 0.2%</td>
</tr>
</tbody>
</table>

*Based on molecular mass of 21.798 kD. *Based on hypothetical homodimer with a molecular mass of 43.696 kD. *Based on scanning electron microscopy.

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**Figure 2.** Immunoblot of T. pseudonana CCMP1335 cells and cells treated with 10 mg/mL cycloheximide (L = light, D = dark, Lc = light + cycloheximide, Dc = dark + cycloheximide) to inhibit protein synthesis. After 27 h, the protein is below detection in the cells grown under light with protein synthesis inhibited. This suggests that the turnover of TpMnSOD is related to processes that occur when cells are exposed to light. Conversely, cells exposed to continuous darkness show evidence of TpMnSOD throughout the experiment. Each lane is loaded with 8 μg of total protein extracts. Antibody was specifically raised against recombinant protein in control lane.
specific SODs influence the D1 protein turnover due to their role in catalyzing the destruction of ROS in the chloroplast (Barber and Andersson, 1992; Aro et al., 1993; Andersson and Aro, 1997). If diatoms use MnSOD to suppress oxidative stress associated with photosynthesis in the chloroplast, we would expect diatoms to have higher Mn requirements than other classes of phytoplankton. Indeed, the measured cellular Mn quota of diatoms is significantly higher than that reported for other eukaryotic algae (Raven, 1990; Raven et al., 1999; Ho et al., 2003; Quigg et al., 2003).

Given the potentially large Mn requirement associated with photosynthesis, a great deal of effort has been focused on determining the cellular Mn budget in marine phytoplankton. Current cellular Mn budget estimates for diatoms have been based solely on the Mn associated with PSII (Raven, 1990). These budgets significantly underestimate measured cellular Mn concentrations (Sunda and Huntsman, 1986, 1998; Peers and Price, 2004); however, including MnSOD (10%–20% of total cellular Mn), up to 80% of the total cellular Mn can be accounted for, all of which is in the chloroplasts (Sunda and Huntsman, 1986, 1998; Raven, 1990; Peers and Price, 2004).

Mn does not appear to be biolimiting in the oceans. Concentration profiles from numerous ocean basins show that Mn is often at biologically accessible concentrations, while Fe is typically undetectable in surface waters (Li, 1991; Shiller, 1997; Nozaki et al., 1998; Whitfield, 2001). Measured values for Mn in the Atlantic basin range from about 8 nmol kg\(^{-1}\) in coastal regions to between 2 and 5 nmol kg\(^{-1}\) at open ocean stations (Shiller, 1997). Using average values of chlorophyll as a proxy for biomass (3 µg kg\(^{-1}\) and 1 µg kg\(^{-1}\) for coastal and oceanic regions, respectively [Falkowski and Raven, 1997] and an average of 77 µg MnSOD mg chlorophyll \(a\)^\(-1\) [based on our measurements; see Fig. 5]), we calculated that oceanic Mn concentrations support more than 1,000 turnovers of Mn in diatoms assuming growth rate between 1 and 2 d\(^{-1}\). Mn could thus serve as a possible metal replacement for Fe and other biolimiting metals in marine algae (Whitfield, 2001; Peers and Price, 2004).

The high Mn requirement of diatoms is significant to the ecology of these eukaryotic algae, as the role of trace metals has been shown to structure oceanic phytoplankton productivity and community composition in many regions (Saito et al., 2003; Coale et al., 2004). A major focus of research has been on Fe, which limits productivity and is only present in subnanomolar concentrations in most of the world’s oceans (Boyd et al., 2000; Coale et al., 2004). Therefore, it is not surprising that some photoautotrophs have evolved mechanisms to compensate for low Fe availability. For example, diatoms can use flavodoxin, instead of the Fe-requiring ferredoxin, under low Fe conditions to support electron transport in PSI (LaRoche et al., 1993; McKay et al., 1999). Similarly, some cyanobacteria and chlorophytes substitute the Cu-containing plastocyanin for the Fe-heme cytochrome \(c_6\) in PSI to transfer electrons between the cytochrome \(b_f f\) complex and P700\(^{+}\) (Quinn and Merchant, 1999). This strategy appears to have been selected on substituting a limiting metal in a pathway with a more available and accessible metal in the same biochemical role.

This strategic biochemical substitution suggests that phytoplankton living in chronically Fe-limited waters may gain a competitive advantage if they can use alternative metals. Cyanobacteria from oligotrophic areas contain either NiSOD alone or both Ni and MnSOD instead of FeSOD found in freshwater species (Partensky et al., 1999; Palenik et al., 2003; Wolfe-Simon et al., 2005). Thus, two of the most successful groups of marine phytoplankton (diatoms and cyanobacteria; Falkowski et al., 2004a, 2004b) use non-Fe SODs to cope with oxidative stress in the Fe-poor regions of the modern ocean. Modern chlorophytes, including embryophytes, do not use Fe enzyme replacements. Therefore, it is not surprising that these taxa are not
dominant in the oceans and are found primarily in terrestrial, freshwater, and estuarine systems that have abundant Fe concentrations (Sterner et al., 2004). Thus, utilization of MnSODs may be one more mechanism underlying the dominance of red alga taxa over the last 275 million years (Falkowski et al., 2004a, 2004b).

The ancient aquatic ecosystem is thought to have been chemically reduced, which would have made Fe abundantly available to evolving organisms (Canfield, 1998; Brocks et al., 1999; Anbar and Knoll, 2002). As oxygen increased and oxidized most of the Fe to an insoluble oxide form, organisms were forced to evolve alternative options for biochemical pathways. Fe is still biochemically utilized for many electron transfer reactions. However, at protein active sites where Mn could substitute for Fe with few genetic mutations, Mn was often appropriated. This is especially true for electron transfer reactions involving O₂.

Figure 4. Immunogold localization of MnSOD in T. pseudonana CCMP1335. A, Osmium tetroxide-stained electron micrograph of whole cell. B, A second different view of osmium tetroxide-stained cell. C, Immunogold labeling of the chloroplast with the anti-TpMnSOD antibody. D, Magnified view of delineated area in B. E, Immunogold labeling of the chloroplast in another cell of T. pseudonana. F, Magnified view of delineated area in E. Note the absence of labeling of mitochondrial and cytosolic regions. c, Chloroplast; p, pyrenoid; m, mitochondrion; n, nucleus; nc, nucleolus; v, vacuole. Arrows indicate black, electron-dense gold label corresponding to TpMnSOD. Faint and less electron-dense granules also apparent in the pyrenoid are crystalline formations of almost pure Rubisco (Falkowski and Raven, 2007). Scale bars are length as indicated.
Figure 5. Comparison of growth rate, total cellular chlorophyll, and MnSOD per unit chlorophyll of T. pseudonana CCMP1335 cells grown at different continuous light intensities. Immunoblot images above the graph are of protein samples loaded according to equal chlorophyll concentrations. Growth rate (black circles and solid line) increases by 2-fold over these light levels. Concurrently, cellular chlorophyll (black squares, dotted line) decreases. Although MnSOD is constant per unit protein (data not shown), MnSOD per unit chlorophyll (black triangles, dashed and dotted line; western blot above image) increases. This supports the strong association of the relative contribution of MnSOD in the chloroplast to protecting the photosynthetic machinery, especially as the light-harvesting pigments decrease. Values are means, n = 2 ± sd.

Chlorophytes (as well as some embryophytes) typically utilize FeSOD isoforms in the chloroplast (Sakurai et al., 1993; Chen et al., 1996; Kitayama et al., 1999). Consequently, the use of MnSOD in the chloroplast should lower a cell’s Fe demand because there is less FeSOD in use. These proteomic differences are reflected in the metal quotas of various marine phytoplankton taxa as diatoms have significantly lower Fe requirements than chlorophytes (Ho et al., 2003; Quigg et al., 2003). Furthermore, this biochemical difference may reflect the environments under which the diverse photosynthetic taxa evolved (Williams, 1981). Thus, the nutritional difference of Mn between chlorophytes and diatoms may contribute to the success of the diatoms in the low Fe modern marine environment (Falkowski et al., 2004a).

MATERIALS AND METHODS

Organisms, Culture Conditions, and Standard Protocols

Axenic cultures of Thalassiosira pseudonana CCMP1335 cells were used for all manipulations, including nuclear acid isolation and physiological studies. Cells were maintained in F/2 + S medium (Guillard and Ryther, 1962; Guillard, 1975) at a salinity of 35 practical salinity units and 20°C with aeration under fluorescent cool-white lamps with an incident light intensity of 120 μmol m⁻² s⁻¹ unless otherwise stated. Chlorophyll a was analyzed using standard 90% acetone extractions from glass fiber filtered culture (Jeffrey and Humphrey, 1975) measured on a spectrophotometer (Agilent 8453E; Agilent Technologies). Variable fluorescence (Fᵥ/Fᵥm) was acquired using a fast repetition rate fluorometer (Jeffrey and Humphrey, 1975) measured on a spectrophotometer (Agilent 8453E; Agilent Technologies). Total carbon (inorganic and organic, subset of total cellular chlorophyll, and MnSOD per unit chlorophyll (black triangles, dashed and dotted line; western blot above image) increases. This supports the strong association of the relative contribution of MnSOD in the chloroplast to protecting the photosynthetic machinery, especially as the light-harvesting pigments decrease. Values are means, n = 2 ± sd.

Cloning and Purification of Recombinant TpMnSOD

Total nucleic acids from mid-log growth T. pseudonana cells were extracted and treated with DNA-free (catalog no. 1906; Ambion) to remove DNA. First-strand cDNA was synthesized with total RNA using M-MLV Reverse Transcriptase (catalog no. 28025-013; Invitrogen). The cDNA was then used as a template for PCR to amplify sodA with the specific primers 5’-ATGAAAAATCCATCATGA-3’ and 5’-TCTGCCACCGGACCTCCTG-3’. The full copy of the gene was then cloned into the pBAD vector (catalog no. K4300-40; Invitrogen) and transformed into Escherichia coli for overexpression. The expression of the protein was controlled by varying the concentration of Ara to achieve ideal expressed product. The vector contains a poly His-tag as well as a V5 epitope region. Thus, the recombinant protein was purified using Ni-NTA resin (catalog nos. 30230 and 30410; Qiagen) using both gravity chromatography and FPLC.

Antibody Production against TpMnSOD

For the initial immunization, equal volumes of recombinant TpMnSOD protein in a 2 mg/mL concentration and Freund’s Complete adjuvant were emulsified using a micro-emulsifying needle. A total of 0.8 mL of the emulsified protein and adjuvant were injected subcutaneously into two New Zealand White rabbits in four sites (maximum 0.2 mL/site). A subsequent injection was given 30 d later and was prepared using equal volumes of the provided antigen in a 1 mg/mL concentration and Freund’s Incomplete adjuvant. The emulsified protein and adjuvant were injected subcutaneously with a maximum of 0.2 mL/site. Subsequent injections were given at 30- to 35-d intervals. Blood draws from the central ear artery were performed between 10 and 20 d after each subsequent injection. The maximum blood withdrawn did not exceed the standard recommendation of blood amount withdrawn of 15% of total blood volume, or 1% of body weight.

Immunoblot Analyses

Protein was extracted from cell pellets in 2% SDS, 0.05 M sodium carbonate, 7.5% glycerol, 0.025% bromothymol blue, 5 mm phenylmethylsulfonyl fluoride, and 0.1 M dithiothreitol. Sample protein concentration was quantified using either the bichinchoninic acid method (catalog no. 23227; Pierce Biotechnology) or a fluorescent method (catalog no. R33200; Invitrogen). Samples were then run on 12%, 15%, or 18% (w/v) polyacrylamide gels and then blotted onto polyvinylidene fluoride membrane (Towbin et al., 1979). The blots were then probe with anti-TpMnSOD, the antibody raised against the recombinant MnSOD in T. pseudonana. A horseradish peroxidase-conjugated secondary antibody (catalog no. 172-1019; Bio-Rad Laboratories) was used according to instructions and the blots were visualized with a chemiluminescent substrate system on film (catalog no. 34080; Pierce Biotechnology). For quantitative immunoblot, known concentrations of both cells (total no.) and protein (total micrograms) were run on three separate gels and then compared to unknown samples using densitometry.

Immunogold Staining

After fixation for 3 h in a modified electron microscopy fixative (3% sodium chloride, 0.1 M sodium cacodylate, 2.5% glutaraldehyde, pH 7.4), cell pellets were rinsed three times in Eppendorf tubes (2 × 15 min and 1 × overnight) in 3% sodium chloride, 0.1 M sodium cacodylate, pH 7.4 (cells for transmission
electron microscopy imaging only were also postfixed for 2 h in 1% buffered osmium tetroxide. After the washes, the cells were then dehydrated through a graded series of ethanol washes, starting with 50% ethanol to 100% ethanol. The pellets were then embedded in Dr. Spurr’s Low Viscosity Embedding Media within the Eppendorf tubes.

Sections were cut using a LKB 2088 ultramicrotome (LKB-Produkter, S–161 25) collected on 300-mesh gold grids and immunostained. Briefly, each grid was incubated for 1 h in Tris-buffered saline plus Tween 20 (TBST; 0.02M Tris, 0.15M NaCl, 0.1% Tween 20) + 0.5% bovine serum albumin, pH 7.6. The grids were then transferred to primary antibody diluted in TBST (50-μL drops). The grids were then incubated overnight in a humidified chamber at 4°C. The next morning, the grids and solutions were left to come to room temperature and the grids were washed 10 times, 1 min each time. Then the grids were transferred to the appropriate gold-labeled secondary antibody (1:20 or 1:15; catalog no. G7402; Sigma-Aldrich) diluted in TBST. The grids were then incubated overnight in a humidified chamber at 4°C. The next morning, the grids and solutions were left to come to room temperature and the grids were washed 10 times, 1 min each time. Then the grids were transferred to the appropriate gold-labeled secondary antibody (1:20 or 1:15; catalog no. G7402; Sigma-Aldrich) diluted in TBST. They were incubated in the secondary antibody for 1 h at room temperature. Control grids were stained only with the secondary gold-labeled antibody. The grids were washed 10 times, 1 min each in TBST, then the same amount of times in ultrapure water. The grids were then counter-stained with uranyl acetate and lead citrate and photographed in a JEM-100CXII electron microscope (JEOL) at 80 kV.

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LITERATURE CITED


Figure 6. Diel expression of MnSOD in T. pseudonana CCMP1335. A, This figure demonstrates the quantum yield (Fv/Fm) of cells exposed to 12/12-h light/dark cycle under high light (800 μmol m⁻² s⁻¹, black triangles and dashed line) and control light (120 μmol m⁻² s⁻¹, black circles and solid line) over time (x axis). Fv/Fm decreases in the high light over the first 12 h when compared to the control and then recovered during and after the dark period. The dark period is represented by the shaded area. B, Immunoblot densitometric analysis shows significant recovery after the dark period of TpMnSOD in the high light (hatched bars) treatment as compared to the expression of TpMnSOD in the control light (solid bars) cultures (values are means, n = 2 ±SD). *, Significant differences between treatments (P = 0.0105).

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