Oxidative Stress in *Synechococcus* sp. Strain PCC 7942: Various Mechanisms for H$_2$O$_2$ Detoxification with Different Physiological Roles

Alexander Perelman, Avraham Uzan, Dalia Hacohen, and Rakefet Schwarz*

*Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel*

Received 2 October 2002/Accepted 21 March 2003

Cells of photosynthetic organisms possess substantial sources of reactive oxygen species (ROS) in addition to the ROS-producing processes common to all living cells. This stems from the need to harvest light energy for their phototrophic metabolism. Accordingly, these organisms face the challenge of capturing light energy efficiently while avoiding oxidative damage caused by a surplus of absorbed light. Excessive excitation stems from an imbalance between energy absorption and dissipation rates (11, 19, 22). Although high photon flux causes excess excitation, light intensity is not the only effective factor. Any environmental parameter that would slow down anabolism (nonoptimal temperature, nutrient limitation, etc.) would decrease photochemical dissipation and therefore may lead to oxidative stress caused by excess absorbed light.

Certain excited pigment molecules may produce ROS, i.e., singlet chlorophyll while decaying through triplet chlorophyll, causes the formation of singlet oxygen (11, 19). The photosynthetic electron transport chain may produce damaging oxygen species as well. For example, production of ROS may occur on the acceptor side of photosystem II through electron flow from phaeophytin or semiquinone. In addition, it is commonly accepted that under excess of absorbed light, photosystem I can reduce molecular oxygen to superoxide anion (Mehler reaction [1]), which can be converted by superoxide dismutase to H$_2$O$_2$. Apart from being potentially harmful by itself, in the presence of reduced metal ions H$_2$O$_2$ may be converted to hydroxyl radical, a highly reactive and damaging entity. A recent study (13) provided evidence for the involvement of A-type flavoproteins in photoreduction of O$_2$ by electron transfer from photosystem I (the Mehler reaction) in *Synechocystis* sp. strain PCC 6803 (referred to here as *Synechocystis* strain 6803). One of the flavoproteins essential for photoreduction of O$_2$ has been shown to reduce O$_2$ directly to water in vitro (35). It has therefore been suggested that in contrast to eukaryotes, the Mehler reaction in cyanobacteria does not produce ROS.

To avoid the damaging consequences of hydrogen peroxide production, cells possess various enzymes that detoxify this compound (9, 11, 21, 24, 27, 32, 33). Hydrogen peroxide-detoxifying enzymes are traditionally classified as catalases or peroxidases (27). Enzymes of the former group convert H$_2$O$_2$ to water and molecular oxygen, whereas the peroxidases rely on reduced thioredoxin (6, 10, 15–18) which in a photosynthetic organism would decrease photochemical dissipation and therefore may lead to oxidative stress caused by excess absorbed light.

To gain better understanding of the role of different routes of hydrogen peroxide detoxification, we inactivated *tplA* (thioredoxin-peroxidase-like), which we recently identified. In addition, we inactivated the gene encoding catalase-peroxidase and examined the ability to detoxify H$_2$O$_2$ and to survive oxidative stress in both of the single mutants and in the double mutant. Surprisingly, we observed that the double mutant survived H$_2$O$_2$ concentrations that the single catalase-peroxidase mutant could not tolerate. This phenotype correlated with an increased ability of the double mutant to detoxify externally added H$_2$O$_2$ compared to the catalase-peroxidase mutant. Therefore, our studies suggested the existence of a hydrogen peroxide detoxification activity in addition to catalase-peroxidase and thioredoxin-peroxidase. The rate of detoxification of externally added H$_2$O$_2$ was similar in the wild-type and the *TplA* mutant cells, suggesting that, under these conditions, catalase-peroxidase activity was essential for this process and *TplA* was dispensable. However, during excessive radiation, conditions under which the cell might experience oxidative stress, *TplA* appears to be essential for growth, and cells lacking it cannot compete with the wild-type strain. Overall, these studies suggested different physiological roles for various cellular hydrogen peroxide detoxification mechanisms in *Synechococcus* sp. strain PCC 7942.

* Corresponding author. Mailing address: Faculty of Life Sciences, Bar-Ilan University, 52900 Ramat-Gan, Israel. Phone: (972) 3-5317790. Fax: (972) 3-5351824. E-mail: schwarr2@mail.biu.ac.il.
synthetic cell may be reduced by photosystem I through ferredoxin (5).

During studies on acclimation of *Synechococcus* sp. strain PCC 7942 (referred to here as *Synechococcus*) to nutrient limitation and high-light stress, we identified an open reading frame (ORF) that is highly homologous to thioredoxin-peroxidases (*tplA*, for thioredoxin-peroxidase-like). To obtain a comprehensive picture of the relevance of *TplA*, as well as of the catalase-peroxidase, for hydrogen peroxide detoxification and cell survival, we inactivated the genes encoding for these enzymes and characterized each of the single mutants, as well as the double mutant.

**MATERIALS AND METHODS**

Culture conditions and competition experiments. *Synechococcus* and its mutants were cultured as previously described (7). For growth experiments under high-light conditions the cultures were illuminated with 800 μmol of photons m\(^{-2}\) s\(^{-1}\) (provided by halogen lamps cooled with water jackets) and bubbled with air. The same conditions were applied for “competition tests” in which a mixture of equal amounts of wild-type and a certain mutant (optical density at 750 nm [OD\(_{750}\)] of 0.02) served to initiate the experiment. The mixed culture was diluted daily to the original OD; thus, the absorbed light remained fairly constant throughout the experiment. The generation time under these conditions was about 12 h. The number of wild-type and mutant cells in the culture at a given time was assessed by diluting and plating the cultures on solid medium, followed by restreaking of single colonies on plates containing the appropriate antibiotic for the selection of a specific mutant.

Construction of mutant strains, DNA manipulation, and isolation of RNA. It is often observed that genes, the products of which are involved in cell growth and survival under certain environmental conditions, are clustered. Therefore, we sequenced further downstream of *nblR*, the gene encoding for a response regulator that is essential for cell survival during high-light illumination and nutrient starvation (30). This led to identification of an ORF highly homologous to thioredoxin-peroxidases located ca. 2 kb downstream of *nblR*. A 2-kb EcoRV/EcoRI subclone was used for interposon inactivation of *pldA* by insertion of a spectomycin resistance cassette at an *XcmI* site.

The gene encoding for catalase-peroxidase of *Synechococcus*, *katG*, was amplified from genomic DNA by using primers designed according to the published sequences (23) (5'-CCAAACACCAACAGAGA-3' and 5'-GTGGCGGATACTCCTTGAA-3'). The PCR product was cloned into a pGEM-T vector (Promega). Digestion by *ClaI* was used to delete a 609-bp fragment, which was replaced by a kanamycin resistance cassette. Each of the plasmids containing either the inactivated *pldA* or *katG* was used to transform the wild-type strain to yield the *tplA* mutant (*TplI*) and the *katG* mutant (*KatI*), respectively.

The double mutant, *KatTplI*, characterized throughout the present study was obtained by transforming the plasmid bearing the inactivated *katG* into *TplI*. Where indicated, a double mutant constructed by transforming *KatI* with inactivated *pldA* was analyzed. PCR on genomic DNA isolated from the transformants by using specific primers confirmed the complete segregation and replacement of the native gene with the inactivated one.

Molecular techniques were performed according to standard procedures (29). For transcript analyses, cultures were illuminated with 70 or 400 μmol of photons m\(^{-2}\) s\(^{-1}\) provided by fluorescent lamps. RNA isolation and Northern blot analyses were performed as described earlier (8). The 0.5-kbp EcoRI/XmnI fragment containing part of *pldA* was used as a probe.

**RESULTS**

Identification of a thioredoxin-peroxidase-like gene. During studies of the acclimation of *Synechococcus* to nutrient limitation and high-light stress, we have identified an ORF highly homologous to thioredoxin-peroxidases (also termed peroxiredoxins), enzymes conserved from microorganisms to mammals. For example, the *Synechococcus* *TplA* exhibits 88% similarity to s10755 of *Synechocystis* strain 6803 (http://www.kazusa.or.jp/cyanobase/), 82% similarity to BAS1 from barley (2), 75% similarity to human thioredoxin-peroxidase (31), and 55% similarity to AhpC from *Escherichia coli* (4). The highest homology was observed between *TplA* and a subfamily of thioredoxin-peroxidases designated 2-Cys peroxiredoxins. These enzymes function as homodimers in which a disulfide bridge between two conserved cysteins is formed during catalysis (6). Sequence alignment indicated that *TplA* possesses the two conserved cystein residues essential for catalysis (not shown). Although the physiological role of these enzymes is not completely understood, it has been established that peroxiredoxins reduce hydrogen peroxide or alkyl hydroperoxides by using electrons from thioredoxin (10, 12, 14, 25).

Viability of wild-type and mutant strains after H\(_2\)O\(_2\) treatment. To study the physiological role of *TplA* in *Synechococcus*, we inactivated its gene and characterized the mutant with respect to its ability to survive externally added H\(_2\)O\(_2\), to detoxify H\(_2\)O\(_2\), and to grow during high-light illumination, conditions under which the cells might experience oxidative stress. In order to gain better understanding of the ability of the cell to cope with oxidative stress, we also inactivated the gene encoding for catalase-peroxidase and characterized this mutant, as well as the double mutant lacking both genes.

The various strains were exposed to H\(_2\)O\(_2\) in liquid cultures for 24 h, followed by transfer of aliquots onto solid growth medium (Fig. 1). Strains possessing catalase-peroxidase (wild type and *TplI*) survived exposure to 50- to 100-fold-higher concentrations of H\(_2\)O\(_2\) than strains lacking this activity (KatI and KatTplI) (Fig. 1A and C, respectively). Apparently, catalase-peroxidase is required for detoxification of high concentrations of externally added H\(_2\)O\(_2\).

The ability to detoxify H\(_2\)O\(_2\) likely contributes to cell survival during oxidative stress and, therefore, we expected the double mutant to be the most sensitive to the application of H\(_2\)O\(_2\). Surprisingly, the double mutant survived higher H\(_2\)O\(_2\) concentrations than did the catalase-peroxidase mutant (Fig. 1C). Since the mutants were fully segregated (see Materials and
with the indicated concentrations of H₂O₂. Yellow represents the absence and purple represents the presence of H₂O₂. WT, wild type; TplΩ, tplA mutant; KatΩ, catalase-peroxidase mutant; KatTplΩ, double mutant. In the specific experiment shown, viability and residual H₂O₂ were determined simultaneously, and thus cells were prepared as described for the determination of H₂O₂ content (see Materials and Methods).

Methods), the data suggested that inactivation of both katG and tplA resulted in the induction of H₂O₂ detoxification activity that is supplementary to catalase-peroxidase and thioredoxin-peroxidase.

**Detoxification of H₂O₂ by wild-type and mutant cultures.** Qualitative determination of the residual H₂O₂ remaining after 24 h of incubation was performed on the cultures that served to assess the viability (Fig. 1). These analyses showed that the higher ability of the double mutant to survive H₂O₂ (compared to the catalase-peroxidase mutant) coincided with its increased capacity to detoxify these levels of H₂O₂ (Fig. 1C and D). These experiments also indicated that catalase-possessing strains were able to detoxify 100- to 200-fold-higher concentrations of H₂O₂ than catalase-lacking strains (Fig. 1B and D). It is interesting that in catalase-lacking strains (KatΩ and KatTplΩ), cell survival under a certain concentration of H₂O₂ correlated with the ability to decompose it (Fig. 1C and D). On the other hand, catalase-possessing strains (wild type and TplΩ) could detoxify H₂O₂ concentrations that caused cell death (Fig. 1A and B). Presumably, KatG remains active in H₂O₂-damaged cells, whereas peroxidases can no longer function if their electron source is exhausted.

The contribution of the different enzymes to H₂O₂ detoxification was assessed by monitoring the rate of H₂O₂ decomposition by the various strains. Comparisons between the rates of detoxification by illuminated and darkened cultures were performed since photosynthetic electron transport may be essential for replenishment of the reducing equivalents required for peroxidase activity.

The KatΩ and the double mutant decomposed relatively low concentrations of H₂O₂ (15 or 30 μM) at a similar rate under either illumination or darkness (Fig. 2A and B). Challenging these strains with higher H₂O₂ levels (50 and 100 μM) revealed a substantial difference between these mutants (Fig. 2C and D). The KatTplΩ mutant completely eliminated 50 μM within 12 min of incubation in the light and was capable of partially reducing even 100 μM H₂O₂ within 40 min of illumination, unlike KatΩ, which did not reduce such concentrations (Fig. 2C and D). These experiments confirmed our finding (Fig. 1) that the ability of the double mutant to survive high H₂O₂ levels originated from its ability to detoxify them.

In the dark, incubation with H₂O₂ did not reveal a difference between KatΩ and the double mutant; both strains were unable to reduce either 50 or 100 μM H₂O₂ (Fig. 2C and D). These two mutants, however, detoxified lower H₂O₂ concentrations similarly, although at a slower rate compared to cultures incubated in the light. The dependence of H₂O₂ detoxification in these strains on light suggests a requirement for reductants produced by the photosynthetic electron transport chain. This was further supported by the lack of light-dependent H₂O₂ detoxification upon addition of DCMU or illumination with a nonphotosynthetic (green) light (not shown). Therefore, reduction of relatively low concentrations of H₂O₂ in the dark (Fig. 2A and B) may depend on the availability of cellular pools of reduced compounds.

In accordance with their ability to survive high levels of H₂O₂, wild type and TplΩ were capable of detoxifying relatively high concentrations of H₂O₂ compared to KatΩ and the double mutant; 1 and 4 mM H₂O₂ were completely decomposed within 15 and 65 min, respectively. High concentrations of H₂O₂, such as 7 and 10 mM, were only partially reduced within 65 min (Fig. 3), presumably due to accumulating damage to enzyme activities. In addition, the rate of detoxification of H₂O₂ in the wild type and the tplA mutant were essentially identical (Fig. 3), suggesting that catalase activity was not affected by the inactivation of tplA.

Identical rates of H₂O₂ detoxification were observed in illuminated (Fig. 3) or darkened cultures (not shown) of the wild-type and TplΩ cells. Since peroxidases but not catalases rely on reduced constituents originating from photosynthetic electron transfer, this result indicates that the catalase function of the catalase-peroxidase provides the dominant activity required for detoxification of the high H₂O₂ levels. Catalase also appears to be the principal means for decomposition of lower concentration of H₂O₂; TplΩ detoxified 5 μM H₂O₂ much faster than did KatΩ (not shown).

**Response of mutant strains to high-light conditions during growth.** In addition to detoxification of externally supplied H₂O₂ (Fig. 1 to 3), it was important to assess the ability of the various strains to cope with environmental conditions that might result in oxidative stress, such as growth under intensive radiation. Mutants where tplA was inactivated (TplΩ and KatTplΩ) grew very poorly under these conditions, whereas KatΩ grew similarly to the wild-type cells (Fig. 4). Further-

![FIG. 1. Cells spotted on solid growth medium (A and C) and qualitative assay for remaining H₂O₂ (B and D) after incubation for 24 h with the indicated concentrations of H₂O₂. Yellow represents the absence and purple represents the presence of H₂O₂. WT, wild type; TplΩ, tplA mutant; KatΩ, catalase-peroxidase mutant; KatTplΩ, double mutant.](image-url)
more, growth experiments performed under high-light irradiance demonstrated that Tpl\(\alpha\) and the double mutant were outcompeted by the wild type, specifically under these conditions (Fig. 5), but not when the light intensity was 100 \(\mu\text{mol}\) of photons m\(^{-2}\) s\(^{-1}\) (not shown). Taken together, these data may indicate a crucial role for Tpl\(\alpha\) under a high-light regime. Furthermore, the novel H\(_2\)O\(_2\) detoxification activity observed in the double mutant cannot compensate for the lack of Tpl\(\alpha\) under these conditions. An additional support for the importance of Tpl\(\alpha\) for high-light growth was provided by the five-fold rise in the abundance of the \(tплA\) transcript (Fig. 6) after the high-light treatment.

**DISCUSSION**

A novel H\(_2\)O\(_2\) detoxification activity in *Synechococcus*. The double mutant, KatTpl\(\alpha\), survived and detoxified H\(_2\)O\(_2\) concentrations that were not tolerated or reduced by the Kat\(\alpha\) (Fig. 1 and 2). These observations indicated a novel H\(_2\)O\(_2\) detoxifying activity, in addition to that of Tpl\(\alpha\) and catalase-peroxidase. Presumably, the supplementary H\(_2\)O\(_2\) detoxification activity is expressed in the absence of both Kat\(\alpha\) and Tpl\(\alpha\). This novel activity relies on reductants produced by the photosynthetic electron transport chain, as suggested by its dependence on photosynthetic light and inhibition by DCMU. It is plausible that inactivation of both *tplA* and *katG* caused the induction of the novel H\(_2\)O\(_2\) detoxification activity. An alternative possibility is that a spontaneous mutation present in Tpl\(\alpha\) contributed to the phenotype of the double mutant since the latter was raised by inactivation of *katG* in Tpl\(\alpha\). However, a TplKat\(\alpha\) mutant that was raised by inactivation of *tplA* in Kat\(\alpha\) also exhibited higher resistance to H\(_2\)O\(_2\) compared to Kat\(\alpha\) (not shown), thus supporting the hypothesis that the lack of both Kat\(\alpha\) and Tpl\(\alpha\) causes the induction of a supplementary H\(_2\)O\(_2\)-decomposing activity.

Interestingly, the activity observed in the *katG* mutant (most likely originating from Tpl\(\alpha\)) is more susceptible to H\(_2\)O\(_2\) treatment than is the activity exhibited by the double mutant. For example, the lack of detoxification of 50 or 100 \(\mu\text{M}\) H\(_2\)O\(_2\) by the *katG* mutant implies rapid oxidation of the reduced substrates required for H\(_2\)O\(_2\) detoxification and/or damage to the enzyme itself. On the other hand, the activity present in the double mutant was not impaired under these conditions (Fig. 2), although the addition of 200 \(\mu\text{M}\) of H\(_2\)O\(_2\) eliminated this activity as well (not shown).

Although originally classified as lacking ascorbate peroxidase (21), *Synechococcus* was shown to possess an ascorbate peroxidase-like activity (28). This activity, which appeared to be cytosolic, copurified with a small non-heme iron-containing compound and was heat stable. This ascorbate-peroxidase-like activity probably does not account for the H\(_2\)O\(_2\)-detoxifying activity.
activity observed in the double mutant since initial characterization of this activity indicated that it is membrane associated and heat inactivated, unlike the ascorbate-dependent activity. Currently, we are characterizing the novel H$_2$O$_2$-detoxifying activity observed in the double mutant to clarify its nature and physiological significance.

**Differential role for catalase-peroxidase and TplA.** Analyses of viability (Fig. 1) and of H$_2$O$_2$ detoxification (Fig. 1 to 3) indicated that catalase-peroxidase is essential for survival and the elimination of relatively high concentrations of externally added H$_2$O$_2$. Studies of Synechocystis strain 6803 and its katG mutant also suggested such a role (34). Despite its importance for the elimination of relatively high concentrations of H$_2$O$_2$, catalase-peroxidase seems to be dispensable for growth under high-light illumination (Fig. 4 and 5), conditions under which the cell might experience severe oxidative stress (26). Further, although it reduced externally added H$_2$O$_2$ as efficiently as the wild type (Fig. 3), a mutant lackingTplA grew very poorly and was outcompeted by wild-type cells (Fig. 4 and 5, respectively) activity observed in the double mutant to clarify its nature and physiological significance.

**FIG. 3.** Time courses of decomposition of H$_2$O$_2$ by illuminated cultures of wild type (A) and tplA mutant (B). At zero time, 1 mM (○), 4 mM (■), 7 mM (▲), and 10 mM (□) H$_2$O$_2$ was added to the cultures. Identical curves were obtained with darkened cultures.

**FIG. 4.** Growth as measured by the change in OD$_{750}$ of wild type (○), KatΩ (●), TplΩ (▲), and the double mutant KatTplΩ (■) during illumination with high-light intensity.

**FIG. 5.** Percentage of CFU of wild type (shaded), KatΩ (hatched), TplΩ (dotted), and the double mutant KatTplΩ (check pattern) at time zero and after 5 days of growth in a mixed culture illuminated with 800 µmol of photons m$^{-2}$ s$^{-1}$ (see Materials and Methods for details of the competition tests).

**FIG. 6.** Northern blot hybridization with a tplA specific probe to RNA isolated from Synechococcus grown at 70 µmol of photons m$^{-2}$ s$^{-1}$ (lane 1) or exposed to 400 µmol of photons m$^{-2}$ s$^{-1}$ for 45 min (lane 2) or 2 h (lane 3). The probe hybridized to a single band of ca. 800 bp. Each lane was loaded with 5 µg of RNA.
during excessive radiation. Under the latter conditions, the double mutant exhibited a phenotype similar to that of TplΔ (Fig. 4 and 5). This indicated that TplA was crucial for growth under high-light conditions and the supplementary H₂O₂-detoxifying activity observed in the double mutant could not replace it. Evidence for the importance of thioredoxin-peroxidase for high-light growth is also provided by the effect of high-light treatment on the quantum yield in *Synechocystis* strain 6803 and its thioredoxin-peroxidase mutant; the latter exhibited a lower quantum yield compared to wild-type cells (18). In addition to the high-light-sensitive phenotype of the TplA-lacking mutants (Fig. 4 and 5), transcription induction of *tplA* observed in wild-type cells, following high-light illumination (Fig. 6), also supports the physiological relevance of this gene product under high-light conditions. The *tplA* transcript appears to be monocistronic (the probe hybridized to a 800-bp fragment) mutant (37). Production of organic hydroperoxides during excessive radiation. Under the latter conditions, the addition of *fl* gene product under high-light conditions. The plastid A (18). In addition to the high-light-sensitive phenotype of the strain 6803 and its thioredoxin-peroxidase mutant; the latter high-light treatment on the quantum yield in *Synechocystis* strain 6803 showing that when linear electron transduction of H₂O₂ by *Anacystis nidulans* strain 50:1–50:2, disruption of 2-cysteine-peroxiredoxin in *Synechococcus* sp. strain PCC 7942: not all bleaching is the same. *J. Bacteriol.* 174:4718–4726.


**ACKNOWLEDGMENTS**

A.P. and A.U. contributed equally to this study.

This work was supported by United States-Israel Binational Science Foundation (grant 9800146) and by The Israel Science Foundation (grant 210/99).


