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NADPH-dependent glutathione peroxidase-like proteins (Gpx-1, Gpx-2) reduce unsaturated fatty acid hydroperoxides in *Synechocystis* PCC 6803

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Abstract Here we isolated and characterized two genes (*slr1171*, *slr1992*) designated *gpx-1* and *gpx-2*, respectively, encoding glutathione peroxidase (GPX)-like proteins (Gpx-1, Gpx-2) from *Synechocystis* PCC 6803. The deduced amino acid sequences for *gpx-1* and *gpx-2* showed high similarity to those of GPX-like proteins from higher plants and mammalian GPXs, respectively. Surprisingly, both recombinant proteins in *Escherichia coli* were able to utilize NADPH, but not reduced glutathione, as an electron donor and unsaturated fatty acid hydroperoxides or alkyl hydroperoxides as an acceptor. It seems accurate to refer to Gpx-1 and Gpx-2 as NADPH-dependent GPX-like proteins that serve as a new defense system for the reduction of unsaturated fatty acid hydroperoxides.

Key words: Glutathione peroxidase-like protein; NADPH-dependent; Unsaturated fatty acid hydroperoxide; *Synechocystis* PCC 6803

1. Introduction

To date, cDNAs encoding proteins similar to animal glutathione peroxidases (GPXs), especially phospholipid hydroperoxide GPX (PHGPX), have been isolated from higher plants including *Citrus sinensis* and *Nicotiana sylvestris* [1–6], *Chlamydomonas* [7,8], and yeast [9]. However, these genes have been found to carry a codon for a cysteine residue at the putative catalytic site instead of the codon TGA for the selenocysteine (Sec) of animal GPXs. The activity of the selenium-independent GPX-like proteins in *C. sinensis* was detected towards phospholipid hydroperoxides; however, the GPX-like protein expressed by the corresponding gene showed an activity 500 times lower toward lipid hydroperoxide than the pig heart PHGPX [10,11]. These facts thus lead to a limited understanding of the potential physiological role of GPX-like proteins in photosynthetic organisms.

Cyanobacteria contain catalase peroxidase and thioredoxin peroxidase (2-cysteine-peroxiredoxin) as the scavenging system of H_2O_2 [12–15]. It has been reported that in *Synechocystis* PCC 6803, thioredoxin peroxidase is able to reduce H_2O_2 and alkyl hydroperoxide and the inactivation of the enzyme by the gene disruption leads to increased stress sensitivity [14,15]. Furthermore, the authors could not detect any in vitro GPX activity using reduced glutathione (GSH) as an electron donor in *Synechocystis* PCC 6803 despite the fact that two genes (*slr1171* and *slr1992* according to Cyanobase), which are designated *gpx-1* and *gpx-2* respectively and encode GPX-like proteins (Gpx-1, Gpx-2), are present in its genome [12,13]. Here, we isolated and characterized the two genes and constructed their expression systems in *Escherichia coli*. Next, we analyzed their molecular properties including the substrate specificity of the recombinant proteins. Interestingly, both Gpx-1 and Gpx-2 were able to utilize NADPH, but not GSH as an electron donor and α-linolenic acid hydroperoxide as an electron acceptor. We also discuss the physiological role of both enzymes as a new defense system against oxidative damages in *Synechocystis* PCC 6803.

2. Materials and methods

2.1. Materials

Hydroperoxides of unsaturated fatty acids, phosphatidylcholine, and digalactosyl diacylglycerol (Sigma Chemical Co., USA) were prepared by oxygenation with 2,2′-azobis-(2-amidinopropane) dehydrochloride and purified by reverse-phase HPLC in a mobile phase by the method reported previously [16]. The amount of hydroperoxide was calculated from the UV absorption at 234 nm ($\varepsilon = 27\,400\ M^{-1}\ cm^{-1}$). *Synechocystis* PCC 6803 cells were cultured photoautotrophically in Allen's medium at 27°C for 5 days under illumination (240 μmol $m^{-2}\ s^{-1}$) with the bubbling of sterile air at 8 l min^{-1} [17].

2.2. Expression of gpx-1 and gpx-2 genes in E. coli

The chromosomal DNA was isolated from *Synechocystis* PCC 6803 by the method of Williams [18]. Two DNA fragments containing open reading frame slr1171 (*gpx-1*) or slr1992 (*gpx-2*) were amplified by PCR with the following primers: 5′-GCTAAAT**CATATG**ACTGCCC-3′ (*gpx-1*F), 5′-AGAAAATTACAACAATTTCT-3′ (*gpx-1*R), 5′-CTTAACA**CATATG**CCATTAC-3′ (*gpx-2*F), and 5′-ATAGCACACAATGTTTGTGC-3′ (*gpx-2*R). The forward primers were designed to introduce an *Nde*I site with an ATG codon for the initiation of translation (bold sequence). Amplified DNA fragments were cloned into a pT7Blue-T vector (Novagen, Madison, WI, USA) and sequenced with an automated DNA sequencer (ABI310A, Applied Biosystems, Japan). For the construction of the plasmids to express *gpx-1* and *gpx-2*, the plasmids were digested with *Nde*I and each 0.5-kb DNA fragment was cloned into a pET3a vector (Novagen) digested with the same restriction enzyme. The resulting constructs, designated pET/*gpx-1* and pET/*gpx-2*, respectively, were introduced into the *E. coli* strain BL21(DE3)pLysS. The recombinant enzymes in *E. coli* were produced by the method described previously [17].

2.3. Enzyme assays

The recombinant *E. coli* cells or the algal cells were harvested by centrifugation, suspended in 50 mM Tris–HCl, pH 8.2, containing

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Abbreviations: GPX, glutathione peroxidase; GSH, reduced glutathione; PHGPX, phospholipid hydroperoxide glutathione peroxidase; Sec, selenocysteine

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10% (w/v) sorbitol, 1 mM EDTA, and 10 mM GSH (solution A), and sonicated at 10 kHz for a total of 80 s with four intervals of 20 s each. These lysates were centrifuged at 12 000 × *g* for 15 min. Each crude enzyme obtained was used for the assay. The GPX activity with GSH and H_2O_2 or hydroperoxides was assayed spectrophotometrically in the presence of glutathione reductase, which catalyzes the reduction of oxidized glutathione formed by GPX, according to Takeda et al. [19]. The reaction mixture contained 100 mM Tris–HCl, pH 8.2, 1 mM GSH, 0.4 mM NADPH, 0.2 mM H_2O_2 or hydroperoxides, 1 U glutathione reductase and the enzyme in a total volume of 1 ml. The reduction of hydroperoxides was measured in the same assay mixture, except for the replacement of H_2O_2 with 0.2 mM unsaturated fatty acid or lipid hydroperoxide and 0.1% Triton X-100. The activity using NADPH was assayed in the reaction mixture (1 ml) containing 100 mM Tris–HCl, pH 8.2, 0.4 mM NADPH, 0.2 mM H_2O_2 or hydroperoxide, and the enzyme at 37°C. The oxidation of alternative electron donors was measured in the same assay mixture, but NADPH was replaced with 1 mM ascorbate (290 nm, 3.3 mM^{-1} cm^{-1}), 0.4 mM NADH (340 nm, 6.22 mM^{-1} cm^{-1}), and 60.5 μM of reduced cytochrome *c* (550 nm, 19 mM^{-1} cm^{-1}). Protein was determined by the method of Bradford [20] with bovine serum albumin as a standard. SDS–PAGE was performed on 15% (w/v) polyacrylamide slab gels as previously described [21].

3. Results

3.1. Isolation and characterization of gpx-1 and gpx-2 genes

We isolated and characterized two genes (*gpx-1*, *gpx-2*) encoding GPX-like proteins (Gpx-1 and Gpx-2) from *Synechocystis* PCC 6803. The *gpx-1* consisted of 507 bp encoding 169 amino acids with a calculated molecular mass of 18 451 Da, while *gpx-2* contained 462 bp encoding 154 amino acids with about 16 645 Da. The deduced amino acid sequence of *gpx-1* shared 47% identity with that of *gpx-2* (Fig. 1). The deduced amino acid sequences for *gpx-1* and *gpx-2* showed 37–55% identity to those of GPX-like proteins from higher plants, eukaryotic algae, yeast, and mammalian GPXs. The Gpx-1 and Gpx-2 proteins contained a Cys residue instead of the Sec at the catalytic site in mammalian GPXs. The Gpx-1 and Gpx-2 proteins also had two highly conserved amino acid residues (Gln-71, Trp-135 for Gpx-1 and Gln-64, Trp-117 for Gpx-2), which coincided with GPX-like proteins of higher plants and GPXs for animals.

3.2. Expression of gpx-1 and gpx-2 genes in E. coli

We examined the optimum conditions for the expression of *gpx-1* and *gpx-2* that were subcloned into the pET3a expression vector. After induction with IPTG, each recombinant enzyme was expressed at a high level in *E. coli* cells. As shown in Fig. 2, the protein bands corresponding to the recombinant Gpx-1 and Gpx-2 were correlated with the molecular mass (18.4 kDa and 16.6 kDa, respectively) calculated from the deduced amino acid sequence of each clone. Both recombinant Gpx-1 and Gpx-2 proteins accounted for nearly 20% of the total protein in *E. coli* cells.

3.3. Detection of enzyme activities of Gpx-1 and Gpx-2

In the crude enzymes from the recombinant *E. coli*, we could not detect the activities of either Gpx-1 or Gpx-2 using GSH as an electron donor and H_2O_2, cumene hydroperoxide, α-linolenic acid hydroperoxide, or phosphatidylcholine hydroperoxide as an electron acceptor, which was in agreement with

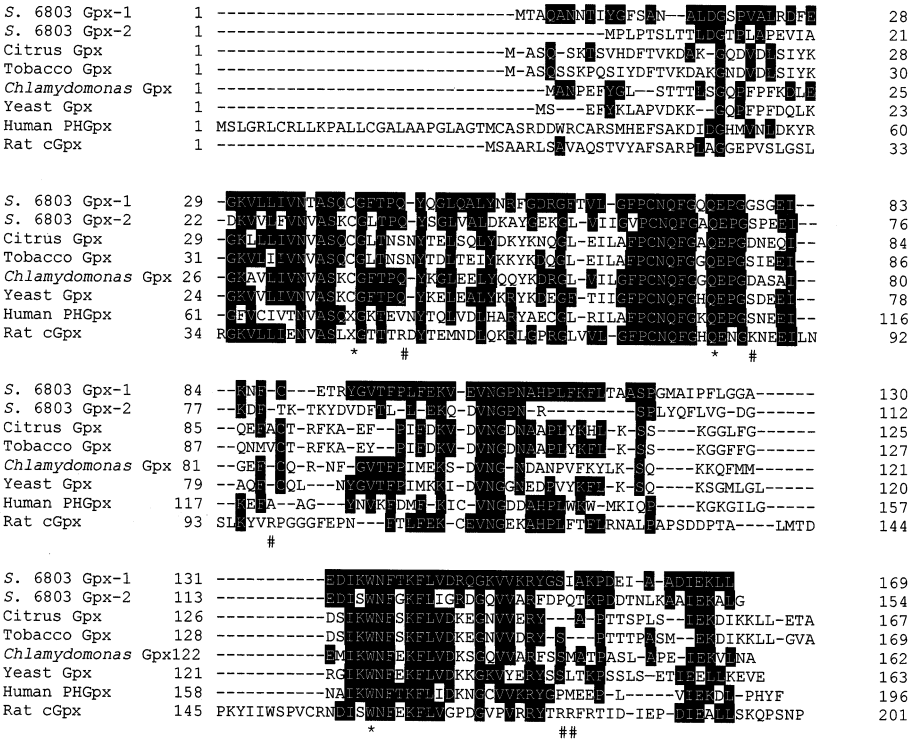

Fig. 1. Comparison of the deduced amino acid sequences of *gpx-1* and *gpx-2* with those of GPX-like proteins from higher plants, eukaryotic algae, and yeast and GPXs from animals. The deduced sequences of *Synechocystis* PCC 6803 GPXs (*S.* 6803 Gpx-1 and Gpx-2) are aligned with those of citrus GPX [2], tobacco GPX [1], *Chlamydomonas reinhardtii* GPX [8], yeast (*Saccharomyces cerevisiae*) GPX [9], human PHGPX [29], and rat cGPX [30] using the single-letter code. Residues found at the same position as *Synechocystis* PCC 6803 Gpx-1 are shown as white letters on black. The asterisks show the catalytic triad (Cys, Gln, and Trp; X indicates Sec). Arg-52, 98, 178 and 179 and Lys-86 (position numbers of rat cGPX) involved in GSH binding by cGPX are indicated by sharps.

previous reports [12,13]. Then, we studied the utilization of other electron donors. As a result, the activities of both recombinant proteins could be detected when NADPH was used as the electron donor and α-linolenic acid hydroperoxide or cumene hydroperoxide as the electron acceptor. Furthermore, we found a combined activity of 6.3 ± 0.2 nmol/min/mg protein of both Gpx-1 and Gpx-2 for NADPH and α-linolenic acid hydroperoxide in the crude homogenate from *Synechocystis* PCC 6803 cells.

The *Synechocystis* PCC 6803 cells were cultured in Allen's medium containing sodium selenite (3 mg/l). As a result, we could not detect the GPX activity in the extract from the algal cells using GSH and H_2O_2, cumene hydroperoxide, α-linolenic acid hydroperoxide, or phosphatidylcholine hydroperoxide. The combined activity of both Gpx-1 and Gpx-2 for NADPH and α-linolenic acid hydroperoxide was the same as that in the crude extract from the cells grown in the absence of sodium selenite. When sodium selenite was added to the crude extract from the algal cells grown in the absence of the selenium compound, the activity for GSH and H_2O_2 was not detected and the activity for NADPH and α-linolenic acid hydroperoxide did not alter. Accordingly, it is unlikely that *Synechocystis* PCC 6803 is able to put in Sec at the post-translational modification of a Cys residue

3.4. Purification of recombinant Gpx-1 and Gpx-2

The specific activities of recombinant Gpx-1 and Gpx-2 in each crude extract from *E. coli* were 16.1 ± 1.2 and 23.0 ± 2.4 nmol/min/mg protein, respectively. Considering the utilization of NADPH as the electron donor, each crude enzyme (3.2 mg protein for Gpx-1 and 2.6 mg protein for Gpx-2) was subjected to an ADP-Sepharose affinity column (0.8 × 3 cm) equilibrated with 50 mM Tris–HCl, pH 8.2, containing 10% sorbitol and 10 mM GSH (solution B). Both enzymes, which were able to bind to the affinity column, were eluted with solution B containing 0.5 mM $NADP^+$. These data suggest the presence of a specific NADPH binding domain in the deduced amino acid sequences of both *gpx-1* and *gpx-2* genes. The SDS–PAGE analysis of the purified recombinant Gpx-1 and Gpx-2 showed a single band of 18.4 kDa and 16.6 kDa, respectively (Fig. 2). The purification procedure yielded Gpx-1 and Gpx-2 preparations purified approx. 8.2- and 6.5-fold over the crude enzyme with a recovery of 88 and 92%, respectively. The specific activities of both Gpx-1 and Gpx-2 with NADPH and α-linolenic acid hydroperoxide were 133 ± 8.2 and 150 ± 7.9 nmol/min/mg protein, respectively.

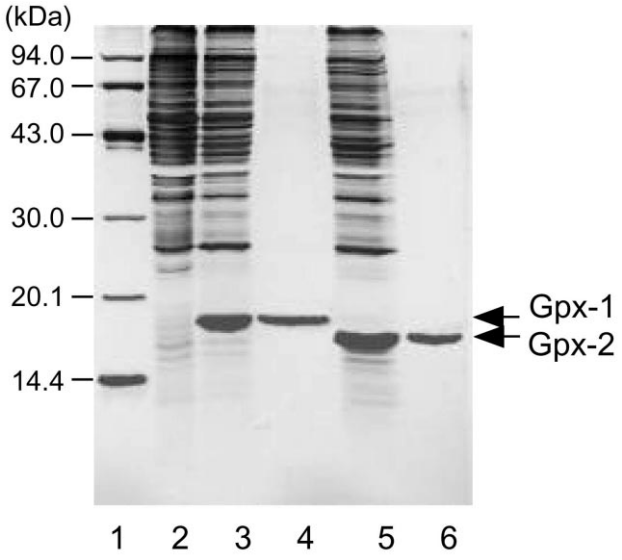

Fig. 2. Analysis by SDS–PAGE of the recombinant Gpx-1 and Gpx-2 expressed in *E. coli* cells and purified by an ADP-Sepharose affinity chromatography. *E. coli* cells were incubated with 0.4 mM IPTG for 6 h at 37°C. Each crude extract (2 μg of protein) and the purified recombinant enzyme (0.2 μg of protein) were analyzed by 15% SDS–PAGE. Lane 1, molecular mass protein standards, LMW kit E from Pharmacia; lane 2, pET3a-transformed *E. coli*; lane 3, pET/*gpx-1*-transformed *E. coli*; lane 4, purified recombinant Gpx-1; lane 5, pET/*gpx-2*-transformed *E. coli*; lane 6, purified recombinant Gpx-2. The protein of each lane was stained with silver stain kits. The arrows indicate Gpx-1 and Gpx-2. Positions and sizes in kDa of molecular mass protein standards are shown on the left side of the panel.

3.5. Enzymatic properties of recombinant Gpx-1 and Gpx-2

We studied the specificities of electron donors and acceptors for both purified recombinant enzymes (Table 1). We could not detect the activities of either Gpx-1 or Gpx-2 using GSH as an electron donor and H_2O_2, alkyl hydroperoxides (cumene hydroperoxide and *t*-butyl hydroperoxide), several unsaturated fatty acid hydroperoxides or lipid hydroperoxides as an electron acceptor, which was in agreement with the data of the crude enzymes. Next, we confirmed the activities of both purified enzymes with NADPH and several unsaturated fatty acid hydroperoxides or alkyl hydroperoxides. Among unsaturated fatty acid hydroperoxides, α-linolenic acid hydroperoxide was the best electron acceptor. There were no activities of either Gpx-1 or Gpx-2 using NADPH and H_2O_2, phosphati-

Table 1
Substrate specificities of purified recombinant enzymes of Gpx-1 and Gpx-2

	Activity (nmol/min/mg protein)				
	Gpx-1		Gpx-2		Citrus PHGPX[a]
	NADPH	GSH	NADPH	GSH	GSH
Linoleinic acid-OOH	133 ± 8.2	0	150 ± 7.9	0	n.d.
Linoleic acid-OOH	87 ± 11.3	0	106 ± 9.7	0	44
Oleic acid-OOH	44 ± 12.8	0	53 ± 10.7	0	n.d.
Cumene-OOH	71 ± 14.3	0	80 ± 11.3	0	50
t-Butyl-OOH	83 ± 6.5	0	85 ± 5.3	0	24
H_2O_2	0	0	0	0	0
PC-OOH	0	0	0	0	40
DGDG-OOH	0	0	0	0	n.d.

Values are shown as the average of three independent experiments ± S.D.
n.d., not determined; PC-OOH, phosphatidylcholine hydroperoxide; DGDG-OOH, digalactosyl diacylglycerol hydroperoxide.
[a]See [10].

dylcholine hydroperoxide or digalactosyl diacylglycerol hydroperoxide. When NADPH was replaced with NADH as an electron donor, we could not detect the activity with H_2O_2, alkyl hydroperoxides, unsaturated fatty acid hydroperoxides or lipid hydroperoxides. Neither cytochrome *c* nor ascorbate substituted for NADPH as an electron donor. The molecular masses of Gpx-1 and Gpx-2 were 18.4 and 16.6 kDa, respectively, by SDS–PAGE (Fig. 2) and the gel filtration (data not shown), indicating that Gpx-1 and Gpx-2 exist in a monomer form in their native state. Neither recombinant enzyme was inhibited by 1 mM cyanide and 1 mM azide. The activity of Gpx-1 was inhibited 50% by mercaptosuccinate at 0.8 mM (inhibitor for Cys or Sec). *p*CMB at 0.3 mM and *N*-ethylmaleimide at 1 mM (inhibitors for the SH group) inhibited the Gpx-1 activity by 30% and 60%, respectively. A similar result was also obtained in the Gpx-2 activity. These data were in agreement with those of bovine GPX [22]. Metal ions, Cu^{2+} at 100 μM, Zn^{2+} and Ag^{+} at 1 mM completely inhibited the activities of Gpx-1, Gpx-2, and bovine GPX. The activities of both Gpx-1 and Gpx-2 were maximal at pH 8.2 and 37°C. The recombinant Gpx-1 and Gpx-2 obeyed Michaelis–Menten-type kinetics toward NADPH (0.02–0.5 mM) and α-linolenic acid hydroperoxide (0.025–0.3 mM). The apparent K_m value for NADPH of Gpx-1 at 0.2 mM α-linolenic acid hydroperoxide was determined to be 83.1 ± 6.9 μM. The K_m value for α-linolenic acid hydroperoxide was 215 ± 5.9 μM when the concentration of NADPH was 0.4 mM. The apparent K_m values of Gpx-2 for α-linolenic acid hydroperoxide and NADPH were 82.1 ± 8.6 μM and 57.3 ± 10.7 μM, respectively.

4. Discussion

Two genes (*gpx-1* and *gpx-2*) with significant similarity to the cDNAs of GPX-like proteins from higher plants and GPX from animals were present in *Synechocystis* PCC 6803 cells. The deduced amino acid sequences for *gpx-1* and *gpx-2* indicated that both proteins resemble those of the mammalian PHGPX more than its cGPX (Fig. 1). Interestingly, both enzymes showed activities with NADPH and unsaturated fatty acid hydroperoxides or alkyl hydroperoxides (Table 1). No activity toward GSH and H_2O_2 or alkyl hydroperoxide was detected in *Synechocystis* PCC 6803 cells even though the cyanobacterium contained millimolar levels of GSH, which was in agreement with the data previously reported [13,23]. Jacobson et al. [24] reported that bacterial alkyl hydroperoxide reductase consisting of two separable subunits can convert linoleic acid hydroperoxide and alkyl hydroperoxide to the corresponding alcohols using either NADH or NADPH. The NH_2-terminal sequences for the first 25 amino acid residues for both subunits were completely different from those of Gpx-1 and Gpx-2. Furthermore, Coves et al. [25] demonstrated that *E. coli* cells had NAD(P)H:H_2O_2 oxidoreductase activity designated as NAD(P)H peroxidase; however, its enzymological properties were clearly distinct from those of either Gpx-1 or Gpx-2. Thus, both peroxidases in *Synechocystis* PCC 6803 were named NADPH-dependent GPX-like proteins.

The activities of purified recombinant Gpx-1 and Gpx-2 with NADPH and α-linolenic acid hydroperoxide were less than 1% of the activity of purified native PHGPXs from mammalians with GSH and phospholipid hydroperoxide [11,26] and approximately 3.5-fold higher than the activity of purified recombinant GPX-like protein from citrus with GSH and phospholipid hydroperoxide [10]. It seems likely that the relatively low activities of Gpx-1 and Gpx-2 as well as GPX-like proteins from higher plants compared to those of mammalian PHGPXs are due to the presumed presence of a Cys catalytic residue in these enzymes in contrast to the Sec residue in the mammalian GPXs. In fact, it has been demonstrated that the replacement of the catalytic Sec by Cys in pig heart PHGPX by a point mutation of the cDNA resulted in a drastic decrease in the enzyme activity to a value of only 0.4% of that of the native enzyme [26].

From the alignment and the molecular model of mammalian cGPXs, Arg-52, 98, 178 and 179 and Lys-86 (position numbers of rat cGPX; Fig. 1) seem to be involved in the binding site of GSH as donor substrates [27,28]. These amino acids are specific for all of cGPX species but are not completely conserved in other GPX families such as PHGPX, extracellular plasma GPX, and cytosolic gastrointestinal GPX. Among the five amino acid residues, only Arg-90 was conserved in Gpx-1, while none of the five amino acid residues was detectable in Gpx-2 (Fig. 1), which may support the fact that the GPX-like protein cannot utilize GSH as a donor substrate.

According to cyano2Dbase, the protein (GPX-2) corresponding to *gpx-2* was detected in the crude extract prepared from *Synechocystis* PCC 6803 cells. Recently, we prepared an antibody raised against the recombinant protein of *gpx-1* which reacted specifically with the corresponding enzyme but not with the recombinant GPX-2 (unpublished data). By immunoblotting, we detected the native protein band corresponding to the protein of *gpx-1* in the crude extract. These data indicate that both proteins are constitutively expressed in the *Synechocystis* PCC 6803 cells.

Synechocystis PCC 6803 contains catalase peroxidase and thioredoxin peroxidase as the scavenging system of H_2O_2 and/or alkyl hydroperoxide [13–15]. We demonstrated that in *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942, some enzymes involved in the Calvin cycle are resistant to high levels of H_2O_2, resulting in the insensitivity of the photosynthetic CO_2 fixation to H_2O_2. These observations indicate that cyanobacteria have a battery of defense and tolerance systems against H_2O_2 and/or hydroperoxides. The present data clearly indicate that NADPH-dependent GPX-like proteins serve as a new defense system for the reduction of unsaturated fatty acid hydroperoxides, leading to the protection of cellular membrane integrity from oxidative damages. Using a gene-disrupted mutant of *Synechocystis* PCC 6803, Yamamoto et al. [15] have recently reported that the activity of thioredoxin peroxidase is coupled to the photosynthetic electron transport system. We are investigating the supply of NADPH as the electron donor for Gpx-1 and Gpx-2 activities from the photosynthetic electron transport system and/or the oxidative pentose phosphate pathway using *gpx-1* and/or *gpx-2* disrupted mutants.

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