The catalase–peroxidase of *Synechococcus* PCC 7942: purification, nucleotide sequence analysis and expression in *Escherichia coli*

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Synechococcus PCC 7942, a cyanobacterium, possesses catalase–peroxidase as the sole hydrogen peroxide-scavenging system. The enzyme has been purified to electrophoretic homogenenity from the cells. The native enzyme had a molecular mass of 150 kDa and was composed of two identical subunits of molecular mass 79 kDa. The apparent K_m value of the catalase activity for H_2O_2 was 4.2 ± 0.27 mM and the k_{cat} value was 2.6×10^4 s^{-1}. The enzyme contained high catalase activity and an appreciable peroxidase activity with *o*-dianisidine and pyrogallol. The catalase activity was not inhibited by 3-amino-1,2,4-triazole but by KCN and NaN_3 (apparent K_i values 19.3 ± 0.84 and 20.2 ± 0.95 μM respectively). The enzyme showed an absorption spectrum of typical protohaem and contained one protohaem molecule per dimer. The gene encoding catalase–peroxidase was cloned from the chromosomal DNA of *Synechococcus* PCC 7942. A 2160 bp open reading frame (ORF), coding a catalase–peroxidase of 720 amino acid residues (approx. 79.9 kDa), was observed. The deduced amino acid sequence coincided with that of the N-terminus of the purified enzyme and showed a remarkable similarity to those of a family of catalase–peroxidases of prokaryotic cells. *Escherichia coli* BL21(DE3)plysS, harbouring a recombinant plasmid containing the catalase–peroxidase gene, produced a large amount of proteins that co-migrated on SDS/PAGE with the native enzyme. The recombinant enzyme showed the same ratio of catalase activity to peroxidase activity with *o*-dianisidine and the same K_m for H_2O_2 as the native enzyme.

INTRODUCTION

In photosynthetic organisms, the generation of active oxygen species such as the superoxide radical, H_2O_2 and hydroxyl radicals is inevitable under normal conditions and causes significant cellular stress and damage [1,2]. To neutralize potentially lethal reactive oxygen molecules, these organisms have evolved several enzymic and non-enzymic mechanisms [2]. For the detoxification of H_2O_2, three classes of haem proteins are involved: catalase, catalase–peroxidase and electron-donor-specific peroxidase. Catalases, which catalyse the dismutation of H_2O_2 into dioxygen and water, are common to animals, plants and micro-organisms [3]. Among the bacteria, an atypical hydroperoxidase, which differed from the typical catalase, was first purified from *Escherichia coli* by Claiborne and Fridovich [4]. Nadler et al. [5] have proposed the definition of a new class of hydroperoxidase, designated catalase–peroxidase, with both catalase and peroxidase activities and sharing characteristics with the typical catalases and peroxidases from higher organisms. These enzymes have been observed in photosynthetic bacteria, facultative anaerobes and strict anaerobes [6].

Miyake et al. [7] have reported a scavenging system of H_2O_2 in cyanobacteria with respect to the acquisition of ascorbate peroxidase during the evolution of these organisms. Some species of cyanobacteria lack ascorbate peroxidase as a H_2O_2-scavenging enzyme. Thus cyanobacteria can be divided into two groups: the first scavenges H_2O_2 with ascorbate peroxidase and catalase, and the second scavenges H_2O_2 only with catalase. We have previously investigated the H_2O_2-removing enzymes in *Synechococcus* PCC 7942: we failed to detect ascorbate peroxidase activity, but did detect catalase activity [8]. Here we report the purification and characterization of a catalase protein from *Synechococcus* PCC 7942 and the gene coding for its presence. Interestingly, *Synechococcus* PCC 7942 contained a catalase–peroxidase. We also discuss the H_2O_2-scavenging system in *Synechococcus* PCC 7942.

MATERIALS AND METHODS

Materials

H_2O_2, 3-amino-1,2,4-triazole and *o*-dianisidine were obtained from Sigma. The molecular biology reagents and enzymes were of reagent grade and obtained from commercial sources. *Synechococcus* PCC 7942 and *E. coli* ME8303, the catalase-deficient mutant, were obtained from Dr. T. Omata, Nagoya University, Nagoya, Japan, and Dr. K. Tanaka, Tokyo University, Tokyo, Japan, respectively.

Organisms and culturing

Synechococcus PCC 7942 was cultured in Allen's medium at 26 °C for 5 days under illumination (240 μE/s per m^2) with bubbling of sterile air at 8 l/min [9]. *E. coli* strains JM109, ME8303 and BL21(DE3)plysS were cultured at 37 °C in Luria broth (LB) [10].

Enzyme assays

Catalase activity was determined spectrophotometrically by following the rate of H_2O_2 disappearance at 240 nm, taking ϵ_{240} as 43.6 $M^{-1} \cdot cm^{-1}$ [4]. The reaction mixture contained 50 mM

Abbreviation used: ORF, open reading frame; LB, Luria broth.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D61378.

potassium phosphate buffer, pH 7.0, 10.5 mM H_2O_2 and the enzyme. The reaction was run at 27 °C for 5 min and the initial linear rate was used to calculate the activity. One unit of enzyme activity was defined as the amount of enzyme that decomposes 1 μmol of H_2O_2 per min. Peroxidase activity was monitored spectrophotometrically at 27 °C in a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM H_2O_2, an electron donor and the enzyme. The standard assay was carried out with 1 mM *o*-dianisidine by following the rate of oxidation at 460 nm (ϵ_{460} 11.3 $mM^{-1} \cdot cm^{-1}$). The activities with other electron donors were assayed in the same reaction mixture as used for *o*-dianisidine, but *o*-dianisidine was replaced with 20 mM pyrogallol (ϵ_{430} 2.47 $mM^{-1} \cdot cm^{-1}$), 5 mM guaiacol (ϵ_{470} 26.6 $mM^{-1} \cdot cm^{-1}$), 0.4 mM ascorbate (ϵ_{290} 2.8 $mM^{-1} \cdot cm^{-1}$) and 0.2 mM NAD(P)H (ϵ_{340} 6.22 $mM^{-1} \cdot cm^{-1}$).

Purification

All the purification steps were performed at 4 °C. *Synechococcus* PCC 7942 cells (25 g wet wt.) from the stationary phase were harvested by centrifugation, resuspended in 50 ml of 10 mM potassium phosphate buffer, pH 7.0, and sonicated (10 kHz) for a total of 10 min with four intervals of 2 min each. This lysate was centrifuged at 12000 ***g*** for 15 min to obtain the crude enzyme. The supernatant was subjected to ultracentrifugation at 100000 ***g*** for 1 h. The supernatant was loaded onto a DEAE-cellulose column (2.4 cm × 44 cm) equilibrated with the phosphate buffer and developed with 300 ml of a linear gradient of 0–0.6 M KCl. The active fractions were combined and fractionated with ammonium sulphate; the pellet precipitating between 30 and 70 % saturation was dissolved in 2 ml of the phosphate buffer. After dialysis for 6 h against 2 litres of 10 mM phosphate buffer, pH 7.0, the enzyme solution was chromatographed on a Mono Q HR 5/5 column (FPLC system; Pharmacia) equilibrated with the phosphate buffer. The column was eluted with a 100 ml linear gradient of KCl (0–0.6 M). The active fraction was concentrated to a final volume of 0.5 ml by ultrafiltration (Amicon PM-30) and chromatographed on to a Superdex 200 Hiload 16/60 column equilibrated with the phosphate buffer. The combined active fractions were subjected to chromatography on a phenyl-Superose HR 5/5 column equilibrated with 30 % ammonium sulphate in the phosphate buffer. The column was eluted with 100 ml of a linear gradient of 30–0 % ammonium sulphate at an elution rate of 0.15 ml/min. The purified enzyme was collected and stored at −20 °C.

Polyacrylamide gel electrophoresis

Disc gel electrophoresis with 7.5 % (w/v) polyacrylamide at pH 9.4 was performed as described previously [11]. A double staining for catalase–peroxidase was employed using 3,3′-diaminobenzidine, followed by the ferricyanide negative stain to reveal bands in the polyacrylamide gel by the method of Wayne and Diaz [12]. SDS/PAGE was performed on 12.5 % (w/v) polyacrylamide slab gels as previously described [13]. Proteins in the gel were stained with silver-staining reagent.

Other procedures

The amino acid sequence of the N-terminal region of the enzyme was determined by automated Edman degradation on a Model 477A gas-phase protein sequencer [11].

The molecular mass of native catalase–peroxidase was determined by gel filtration on a Sephacryl S-300 column (2.4 cm × 90 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0, and calibrated with molecular mass markers (MW-GF-1000) from Sigma. In determining the molecular mass of a subunit by SDS/PAGE [13], LMW kit E from Pharmacia was used for standards.

The optimum pH was determined at 35 °C in 100 mM Tris/HCl buffer (pH 7.5–9.0) and 50 mM potassium phosphate buffer (pH 6.0–8.5). The pH stability was determined by assaying activity after treatment of the purified enzyme at various pH values for 15 min at 58 °C. The thermal stability was measured with the enzymes pretreated at various temperatures up to 70 °C for 15 min at pH 6.5. The kinetic parameter shows the mean ± S.D. for three assays. Protein was measured by the method of Bradford [14] with BSA as the standard.

Isolation and nucleotide sequence of genomic DNA

Chromosomal DNA was isolated from the *Synechococcus* PCC 7942 cells (1.5 g wet wt.) by the method of Williams [15]. Finally the DNA (approx. 165 μg) was dissolved in 500 μl of TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 7.8). Oligonucleotide primers corresponding to the most highly conserved regions {residues 226–240 [5′-TGGTGGCC(GC)GC(CT)GAC-3′] and 1429–1445 [5′-GC(GT)CCGCCGCGCTT(AG)TC-3′]} of catalase–peroxidase from prokaryotic cells were designed and synthesized by JBioS, Co., Japan. An initial DNA fragment as a probe of Southern blot hybridization was amplified from genomic DNA by PCR with primers. The conditions of the PCR were as follows: 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. The PCR products were size-fractionated on a 1.5 % (w/v) agarose gel and the band corresponding to the expected size (1.2 kbp) was cut out. The DNA fragment was isolated from the agarose gel using the GENECLEAN®II Kit (BIO 101 Inc.), cloned into a pBluescript II SK(+) vector and sequenced by the dideoxy chain primer method with M13 forward and reverse sequencing primers. To screen the full-length DNA fragment including the catalase–peroxidase gene, Southern blotting of the chromosomal DNA (approx. 2 μg) digested with each restriction enzyme was performed with the PCR products as a probe labelled with digoxigenin. Hybridization was done in accordance with the instructions of Amersham. The membranes were washed twice in 0.1 × SSC/0.1 % SDS (where SSC is 0.15 M NaCl and 0.015 M citrate, pH 7.0) at 68 °C for 20 min. In consequence (results not shown) only one fragment hybridized with this probe in each lane. A 3.5 kbp *Hin*d III fragment was isolated from the gel using the GENECLEAN®II Kit (BIO 101 Inc and subcloned into a pBluescript II SK(+) vector. Colony hybridization with the screening probe was performed to isolate a positive clone with a 3.5 kbp *Hin*d III fragment, which was designated pSCP1. The nucleotides of the pSCP1 were sequenced. Overlapping insert DNA fragments were obtained by subcloning after restriction digestion and partial digestion by exonuclease III.

Expression of catalase–peroxidase gene in *E. coli*

pSCP1 was transformed into the *E. coli* ME8303 catalase-deficient mutant. Cells grown on 250 ml of LB to a D_{600} of approx. 1 were harvested by centrifugation, suspended in 3 ml of 10 mM phosphate buffer, pH 7.0, and disrupted by sonication (20 kHz for 5 min). The supernatant obtained by centrifugation at 20000 ***g*** for 10 min at 4 °C was used as the crude extract. Catalase activity was assayed by the method described above. Catalase activity (97.5 units/mg of protein) was observed in the crude extract of *E. coli* ME8303 with pSCP1, but was not detected in that of *E. coli* without the recombinant plasmid. Colonies on the plate were flooded with 1 mM H_2O_2 solution,

thereby causing them to bubble dioxygen from H_2O_2 (results not shown).

Because there were no convenient restriction sites with which to subclone the catalase–peroxidase gene into the pET3a expression vector, an oligonucleotide-directed PCR was used to produce the full-length protein coding region. The PCR products were ethanol-precipitated, dissolved in restriction enzyme buffer, and digested with the *Nde*I restriction enzyme, and then the fragment was ligated into an *Nde*I-digested pET3a vector. The plasmid obtained was designated pSCP2. Competent *E. coli* strains BL21(DE3)pLysS were transformed with pSCP2 and grown in LB at 37 °C containing 50 μg/ml ampicillin and 34 μg/ml chroramphenicol. Cultures were grown until a D_{600} of 0.7 was reached, and the induction of catalase–peroxidase activity was started by the addition of isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM. After incubation for 6 h, samples of the cells were removed for analysis of total catalase–peroxidase accumulation by SDS/PAGE, and for quantification of the enzyme activity.

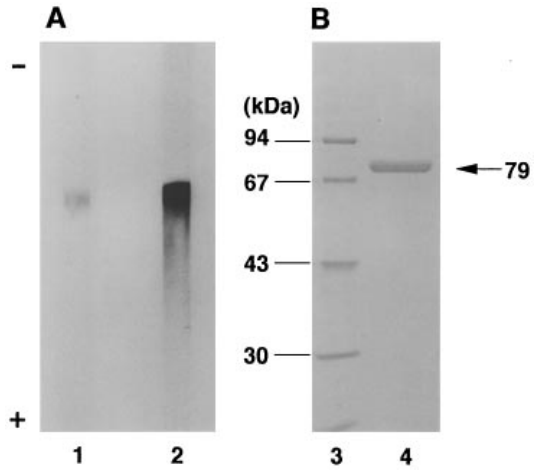

Figure 1 PAGE and SDS/PAGE of catalase–peroxidase

The procedures for electrophoresis and activity staining of purified enzyme were performed as described in the Materials and methods section. (**A**) PAGE of purified enzyme (5 μg) stained by silver (lane 1), and ferricyanide and 3,3′-diaminobenzidine (lane 2) (**B**) SDS/PAGE of Pharmacia molecular mass standards (lane 3) and purified enzyme (lane 4).

RESULTS AND DISCUSSION

Purification of catalase–peroxidase

Synechococcus PCC 7942 lacked ascorbate peroxidase activity [8], as did *Synechococcus* PCC 7002 and *Synechococcus* PCC 6311 [7,16]. However, we detected catalase activity in cyanobacterial cells. The six-step purification scheme of *Synechococcus* PCC 7942 catalase–peroxidase is summarized in Table 1. During purification of catalase–peroxidase from *Synechococcus* PCC 7942, the catalase activity was eluted from each column as a sharp and single peak. These results indicate that *Synechococcus* PCC 7942 cells contain only one type of catalase protein. The purification procedure yielded a catalase–peroxidase preparation purified approx. 909-fold over the crude enzyme, giving a final 12.8 % recovery of the catalase activity. This purification was repeated three times with similar results. PAGE of the purified enzyme showed only one detectable protein band, which was in agreement with that detected by a double-staining method (Figure 1A). SDS/PAGE of the purified enzyme gave a single band (Figure 1B).

Catalytic properties

The catalase activity of the purified enzyme was 6670 units per mg of protein, which was much higher than those of the hydroperoxidase from *Escherichia coli* [3] and catalase–peroxidase from *Bacillus* NY-2000 [17], but the same order of magnitude as that of catalase–peroxidase from *Rhodopseudomonas capsulata* [18]. Whether the peroxidase reaction has a physiological role in destroying H_2O_2 would depend on the availability of suitable hydrogen donors [6]. The enzymes from *R. capsulatus*, *E. coli* and *Klebsiella pneumoniae* could use both nicotinamide nucleotides, suggesting that NADH and NADPH might be physiological substrates of the enzymes [6,18]. *Synechococcus* PCC 7942 catalase–peroxidase showed peroxidase activity with *o*-dianisidine and pyrogallol of 9.5 and 162.2 units/mg respectively. No reaction was detected with guaiacol, ascorbate, NADH or NADPH. These results imply that the catalase activity of catalase–peroxidase may function to remove H_2O_2 generated in *Synechococcus* PCC 7942.

The enzyme reaction followed Michaelis–Menten kinetics with H_2O_2 concentrations of 1, 1.3, 2, 2.5, 3.3, 4.5, 5, 10 and 20 mM, although the enzyme's activity was inhibited by H_2O_2 at concentrations greater than 30 mM. From the double-reciprocal plots, the apparent K_m of the enzyme for H_2O_2 was 4.2 ± 0.27 mM, which is similar to that of catalase–peroxidases from other micro-organisms [17]. From the estimation of V_{max}, the k_{cat} of the *Synechococcus* PCC 7942 enzyme was 2.6×10^4 s^{-1}, which was the same order of magnitude as those of other catalase–

Table 1 Purification of catalase–peroxidase from *Synechococcus* PCC 7942

Step	Total protein (mg)	$10^{-3} \times$ Total activity (units)	Specific activity (units per mg of protein)	Purification (fold)	Yield (%)
Crude extract	2125	15.6	7.34	1.0	100.0
Ultracentrifuge	1496	14.9	9.96	1.4	95.5
DEAE-cellulose	552	14.4	26.1	3.6	92.3
30–70% $(NH_4)_2SO_4$	223	13.0	58.3	7.9	83.3
Mono Q	38.4	10.1	263	36	64.7
Superdex 200	13.2	8.0	606	83	51.3
Phenyl-Superose	0.3	2.0	6670	909	12.8

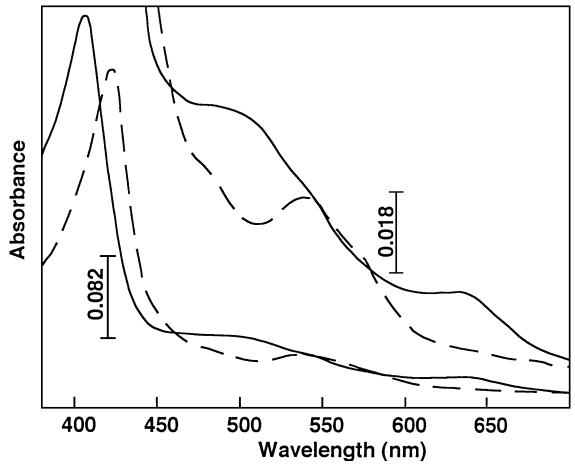

Figure 2 Absorption spectra of *Synechococcus* PCC 7942 catalase–peroxidase

The purified enzyme (218.4 μg) was dissolved in 1 ml of 10 mM potassium phosphate buffer, pH 8.0. Absorbance spectra for the purified enzyme were recorded with a Shimadzu spectrophotometer UV-2200A. The upper two traces show the visible portion of the spectra on an expanded scale. Solid line, native enzyme; broken line, enzyme plus 5 mM KCN.

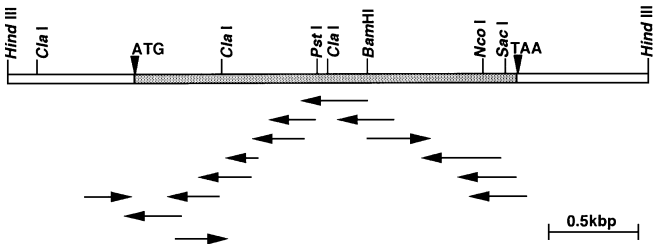

Figure 3 Sequencing strategy for the catalase–peroxidase gene

Plasmid pSCP2 is a hybrid plasmid consisting of pBluescript II SK(+) and the 3.5 kb *Hin*dIII fragment containing the catalase–peroxidase gene of *Synechococcus* PCC 7942. The ORF for catalase–peroxidase, which starts from the ATG codon and terminates at the TAA codon, is also shown. DNA was deleted by exonuclease III to obtain small fragments for sequencing. Arrows indicate the directions and extents of elongation in the sequencing reactions.

peroxidases [3,18], but was one or two orders of magnitude lower than those of plant catalases from *Nicotiana sylvestris* (CAT-1) [19] and pumpkin cotyledons [20]. However, the k_{cat}/K_m ratio of *Synechococcus* PCC 7942 catalase–peroxidase was of a similar order of magnitude to those of plant catalases because the K_m values for H_2O_2 of plant catalases are an order of magnitude higher than those of catalase–peroxidases in micro-organisms, including *Synechococcus* PCC 7942.

Catalase–peroxidases differ from typical catalases in several properties: the pH optima of catalase activity are between 6.0 and 7.5, they have both catalase and peroxidase activities, and they are insensitive to 3-amino-1,2,4-triazole, an irreversible inhibitor of the typical catalase. The catalase activity of *Synechococcus* PCC 7942 catalase–peroxidase was maximal at pH 6.5 and was not inhibited by 3-amino-1,2,4-triazole. The haem protein ligands KCN and NaN_3 caused inhibition of the enzyme: 50% inhibition was attained at 19.3 ± 0.84 μM KCN and 20.2 ± 0.95 μM NaN_3. Catalase–peroxidase was stable for 30 min at up to 50 °C and lost 50% of its activity at 58 °C. The optimum pH was 6.5. The enzyme retained full activity between pH 4.5 and 8.5.

Spectroscopic properties

Typical catalases are found in the ferric state and their spectra show a strong Soret band at 402–406 nm and a series of bands in the visible region of decreasing intensity at 500–505, 535–540 and 620–635 nm [18]. The absorption of the catalase–peroxidase purified from *Synechococcus* PCC 7942 showed a Soret peak at 406 nm, an absorption maximum at 633 nm and two shoulders at 494 and 545 nm (Figure 2). The addition of KCN to the enzyme resulted in a shift of the Soret band to 422 nm, abolition of the peak at 633 nm, and the appearance of a peak at 535 nm and shoulders at 494 and 545 nm. The A_{406}/A_{280} ratio of *Synechococcus* PCC 7942 catalase–peroxidase was 0.54. This is similar to those of catalase–peroxidases of *Streptomyces* sp. and *Bacillus stearothermophilus*, which contain 1 haem molecule per homodimer [21,22]. Therefore the similar low haem content might also be found in *Synechococcus* PCC 7942 catalase–peroxidase. Treatment of the enzyme with sodium dithionite did not cause any changes in its absorption spectrum (results not shown). Typical catalases are not reduced or are only slightly reduced by dithionite [23], but the catalase–peroxidases are readily reduced [17,18].

Molecular mass analysis and sequence of the N-terminal amino acid region

The molecular mass of the native enzyme was 150 kDa by gel filtration on Sephacryl S-300 (results not shown) and 79 kDa by SDS/PAGE (Figure 1B). These results indicate that catalase–peroxidase of *Synechococcus* PCC 7942 exists in a dimeric form in its native state, which is composed of two subunits of the same size. Typical catalases are generally composed of four subunits of equal size, with a combined molecular mass in the range 225–270 kDa [5]. Catalase–peroxidases also exhibit a tetrameric molecular mass in the range 236–337 kDa with equal-sized subunits [17]. The unusual existence of an active dimer of catalase–peroxidase from *Synechococcus* PCC 7942 has parallels only in catalase from spinach [24] and catalase–peroxidases from *Streptomyces* sp. [21], *B. stearothermophilus* [22] and *Comamonas compransoris* [25]. Purified catalase–peroxidase was subjected to Edman degradation to determine the amino acid sequence. The amino acid sequence from the N-terminus to the 14th residue was TATQGKXPVMHGGA-.

Isolation and characterization of the genomic gene coding for the catalase–peroxidase

The restriction sites and sequencing strategy for the 3.5 kbp *Hin*d III fragment including the catalase–peroxidase gene are shown in Figure 3. A 2160 bp open reading frame (ORF) from the start site at ATG (position 1) to the termination codon TAA (position 2161), coding for a peptide of 720 amino acid residues, was found (Figure 4). The molecular mass calculated from the deduced amino acid sequence was close to the 79 kDa of the catalase–peroxidase subunit determined by SDS/PAGE (Figure 1B). The identity with the amino acid sequence of the N-terminus (underlined in Figure 4) shows that this ORF encodes the catalase–peroxidase gene of *Synechococcus* PCC 7942. The tenth base pair upstream from the initiation codon is a putative

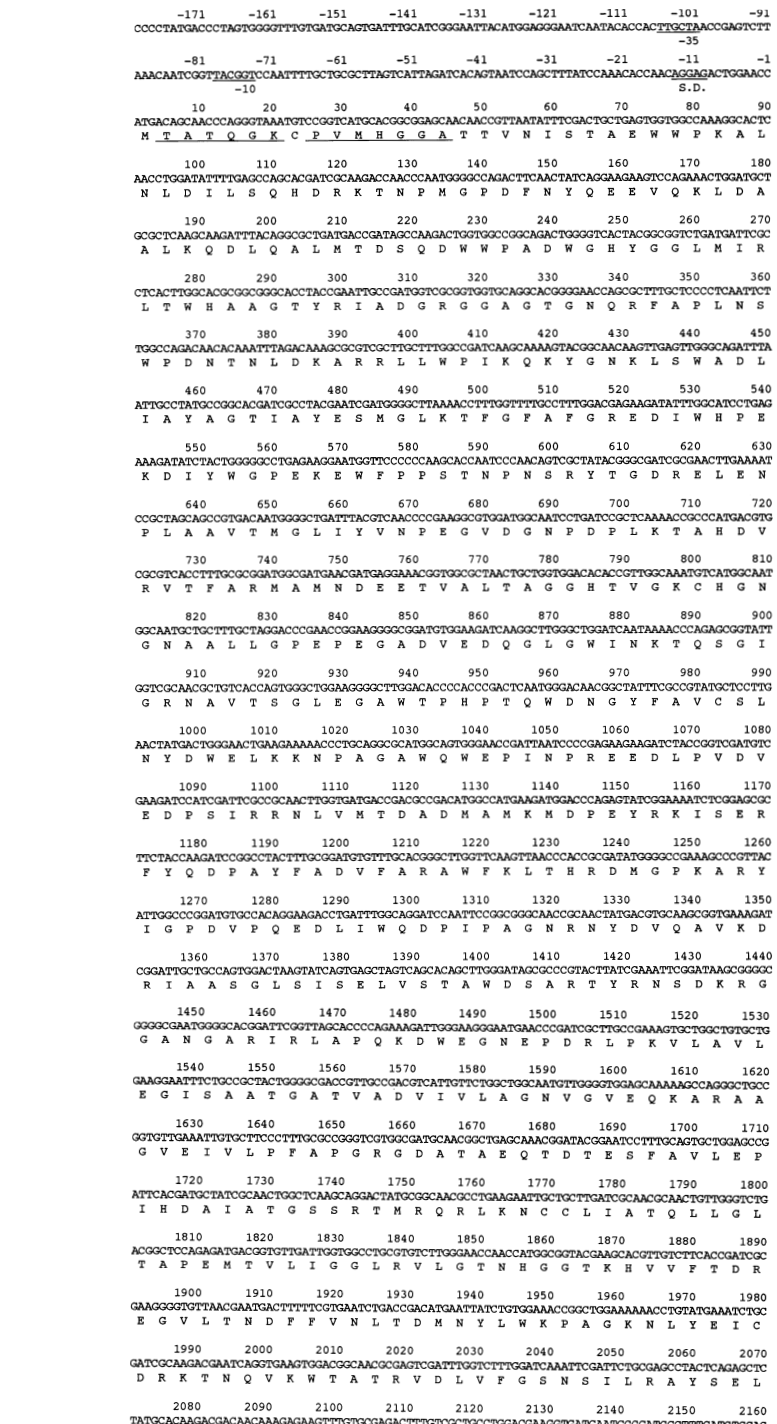

Figure 4 Nucleotide sequence of the catalase–peroxidase gene and deduced amino acid sequence, including upstream and downstream elements

Potential −35, −10 and Shine–Dalgarno sequences are indicated. The amino acid sequences of the N-terminal regions, which are identical to the sequences determined with an automated Edman degradation of the purified enzyme, are underlined. Numbering is from the N-terminal first residues on each line.

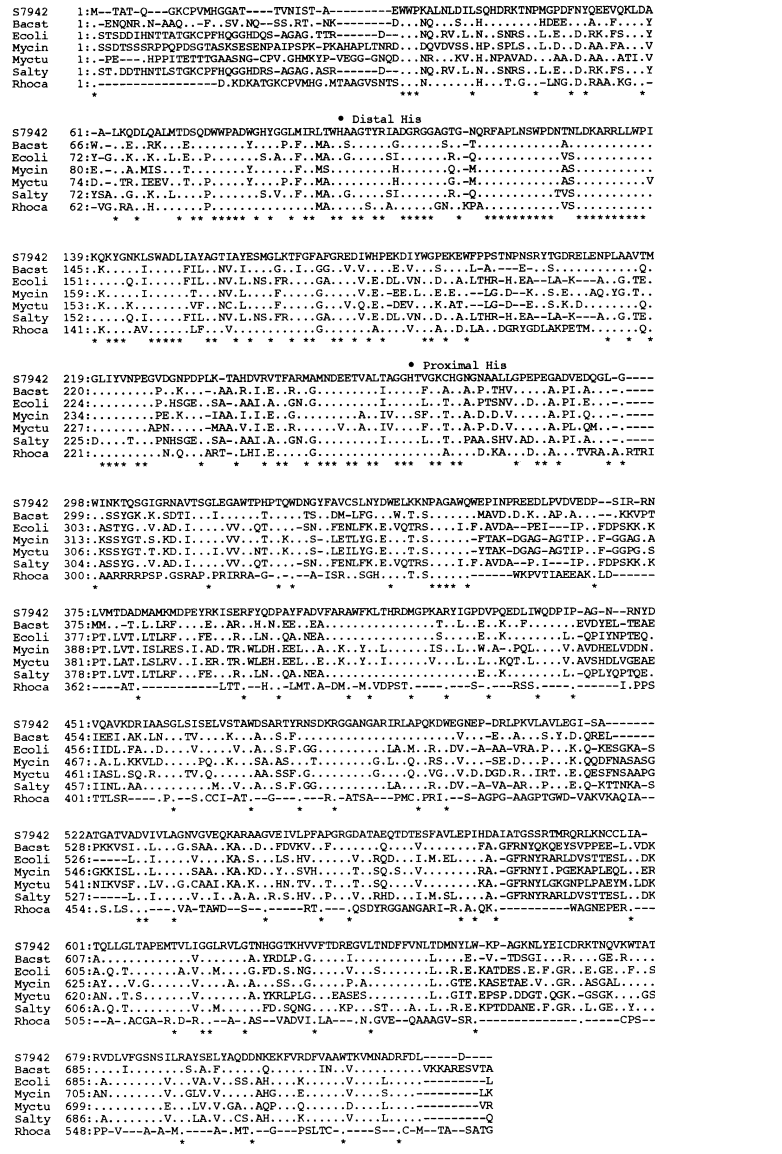

Figure 5 Comparison of the predicted amino acid sequence of the *Synechococcus* PCC 7942 catalase–peroxidase protein with the sequences of hydroperoxidases from *E. coli katG* (Ecoli) and *Synechococcus typhimurium* (Salty), catalase–peroxidase from *M. intracellulare* (Mycin), *M. tuberculosis* (Myctu) and *R. capsulatus* (Rhoca) and the peroxidase from *B. stearothermophilus* (Bacst)

The differences in the catalase–peroxidase protein sequences can be identified by the substitution of another amino acid below the *Synechococcus* PCC 7942 catalase–peroxidase sequence. Dashes are included to maximize alignment and dots indicate homology with the *Synechococcus* PCC 7942 catalase–peroxidase sequence. The asterisks show the consensus amino acids. The distal and proximal His residues are shown by bold dots.

Shine–Dalgarno site, the ribosomal binding sequence AGGAG. Possible promoter sequences at the −35 and the −10 regions were located upstream from the Shine–Dalgarno sequence. The expression of catalase–peroxidases (*katG* gene) in *E. coli* and of alkyl hydroperoxide reductase (*ahp* gene) in *Salmonella* is mediated by the *oxyR* gene in response to oxidative stress [26,27]. A comparison of the sequences upstream from the *Synechococcus* PCC 7942 catalase–peroxidase gene indicates similarity to the *oxyR*-regulated promoters upstream from the *katG*, *ahp* and *oxyR* genes [28]. Investigation of the presence and function of an *oxyR*-like regulon of *Synechococcus* PCC 7942 catalase–peroxidase gene in response to oxidative stress is now under way in our laboratory.

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Distal side
                          ●
 S7942    83:HYGGLMIRLTWHAAGTYRIADGRGGA
 Ecoli    95:SYAGLFIRMAWHGAGTYRSIDGRGGA
 Mycin   103:SYAGLFIRMSWHGAGTYRSHDGRGGA
 Myctu    97:SYAPLFIRMAWHGAGTYRSHDGRGGA
 Bacst    90:SYAPLFIRMAWHSAGTYRSGDGRGGA
 Salty    96:SYVGLFIRMAWHGAGTYRFIDGRGGR
 Rhoda    95:HYGGLMIRMAWHSACSYRAADGRGGG
 CCCP     41:GYGPVLVRLAWHTSGTWDKHDNTGGS
 SAP4     31:QCAPLMLRLAWHSAGTFDCTSKTGGP

Proximal side
                                ●
 S7942   247:WAMNDEETVALTAGGHTVGKCHGN
 Ecoli   252:MGMNDEETVALIAGGHTLGKTHGA
 Mycin   262:WAMNDEETAALIVGGHSFGKTHGA
 Myctu   255:WAMNDVETAALIVGGHTFGKTHGA
 Bacst   247:MGMNDEETVALIAGGHTFGKAHGA
 Salty   253:MGMNDEETVALIAGGHTLGKTHGP
 Rhoca   250:MGMNDEETVALTAGGHTVGKAHGN
 CCCP    161:RLNMDREVVALMCAHALGKTHLK
 SAP4    131:MGLTDQDIVALSGGHTLGRCHKD
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Figure 6 Comparison of catalase–peroxidase from *Synechococcus* PCC 7942 with other catalase–peroxidases in regions near the distal and proximal His residues

Amino acids that are identical with the gene of *Synechococcus* PCC 7942 are shaded. Heavy dots (●) indicate distal and proximal His residues. Abbreviations used: SAP4, spinach ascorbate peroxidase [36]; CCP, yeast cytochrome *c* peroxidase [37].

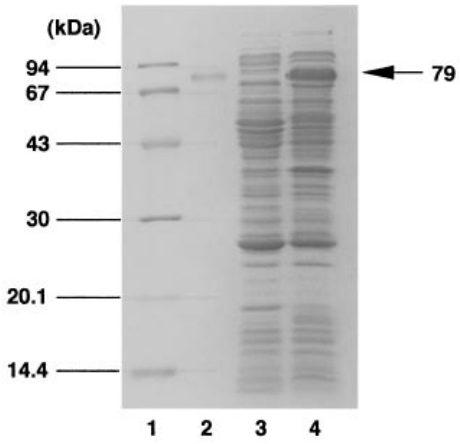

Figure 7 Production of the catalase–peroxidase in *E. coli* BL21(DE3)pLysS by pSCP2

Protein standards (lane 1), purified catalase–peroxidase (lane 2), the lysate of *E. coli* in the absence (lane 3) and presence (lane 4) of isopropyl β-D-thiogalactoside were subjected to SDS/PAGE. Each lane was stained with Coomassie Brilliant Blue R-250 and destained in 7% (v/v) acetic acid. The arrow indicates the catalase–peroxidase. Details of the procedure are described in the Materials and methods section.

Comparative analysis of amino acid sequences of catalase–peroxidases and hydroperoxidases from different micro-organisms

A comparison of the deduced amino acid sequence of the catalase–peroxidase from *Synechococcus* PCC 7942 with sequences of hydroperoxidase and catalase–peroxidases is shown in Figure 5. Catalase–peroxidase genes have shown a high homology but no obvious homologies to typical catalases [29,30]. The deduced amino acid sequence of the gene exhibited homologous sequences with those of hydroperoxidases from *E. coli* (*kat*G) (55.8%) [31] and *Synechococcus typhimurium* (55.5%) [32], catalase–peroxidase from *Mycobacterium intracellulare* (56.5%) [33], *Mycobacterium tuberculosis* (52.9%) [34] and *R. capsulatus* (58.3%) [35], and the peroxidase from *B. stearothermophilus* (65.1%) [36] respectively. The similarity seemed to be higher in the N-terminal half of the proteins and decreased towards the C-terminus. Catalase–peroxidases contain two His residues, which are proposed to be essential for activity, one distal (position 94) and the other proximal (position 262). Figure 6 shows a comparison of the amino acid sequences in regions near the distal and proximal His residues [31–38]. Welinder [39] has described the grouping of plant-type peroxidases into three classes that are distantly related structurally: class I contains bacterial peroxidases (catalase–peroxidases), yeast cytochrome *c* peroxidase and ascorbate peroxidase; class II contains fungal peroxidases such as lignin and manganese peroxidases; and class III contains classical plant peroxidases such as horseradish peroxidase. The cytochrome *c* peroxidase and ascorbate peroxidase are conserved around the distal His region (RLXWH). The sequence corresponding to this region of *Synechococcus* PCC 7942 catalase–peroxidase is RLTWH. Other catalase–peroxidases possessed Leu^{91} rather than Met^{91}; thus in this respect the catalase–peroxidase of *Synechococcus* PCC 7942 is more similar to cytochrome *c* peroxidase and ascorbate peroxidase than to other catalase–peroxidases. The proximal His regions (VALTAGGH) of the *Synechococcus* PCC 7942 enzyme are also highly conserved in other catalase–peroxidases. However, conservation of the proximal His regions between catalase–peroxidases and other class I enzymes is less than that of the distal His regions.

Expression of the *Synechococcus* PCC 7942 catalase–peroxidase gene in *E. coli*

Figure 7 shows an SDS/PAGE of total (soluble) protein in *E. coli* BL21(DE3)pLysS at 37 °C in LB. There was no expression of the enzyme protein in the absence of isopropyl β-D-thiogalactoside (lane 3). In lane 4 a band corresponding to the predicted size of the catalase–peroxidase subunit was accumulated. To evaluate the enzymic activity of the recombinant proteins, the lysate of *E. coli* was prepared as described in the Materials and Methods section. Catalase activity and peroxidase activity of the recombinant enzyme of *E. coli* with pSPC2 were 462.3 units/mg and 0.66 unit/mg respectively with *o*-dianisidine. In contrast, the *E. coli* as a control showed a catalase activity of 9.0 units/mg. The K_m of the catalase activity for H_2O_2 in the recombinant enzyme was 4.2 mM. The recombinant enzyme showed the same ratio of catalase activity to peroxidase activity and the same K_m for H_2O_2 as the native enzyme.

Conclusion

The mechanism of protection against H_2O_2 may be more efficient in photosynthetic organisms including algae and cyanobacteria than in other organisms because they not only consume oxygen during respiration but they also generate it during photosynthesis. Cyanobacteria were the first organisms that supplied dioxygen to the atmosphere by a system using the oxidation of water for electron donation to the reaction centre [40]. Accompanied by an increase in the atmospheric concentration of dioxygen, effective H_2O_2-scavenging systems consisting of catalase and peroxidase activities were required in cyanobacteria to protect the cells from oxidative damage. The production of H_2O_2 is inevitable in cyanobacterial cells since superoxide dismutase has been present [7]. The primitive scavenging system of H_2O_2 appears to be catalase (catalase–peroxidase), because many aerobic prokaryotes contain only catalase (catalase–peroxidase) and lack peroxide-

scavenging peroxidases. The absence of ascorbate in prokaryotes and fungi can account for the lack of ascorbate peroxidase in these organisms [40]. A similar situation may also occur in *Synechococcus* PCC 7942. Recently we demonstrated that the photosynthesis (CO_2 fixation) of *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 has found not to be susceptible to 1 mM H_2O_2, which was in part due to the resistance of the thiol-modulated enzymes fructose 1,6-bisphosphatase, NADP: glyceraldehyde 3-phosphate dehydrogenase and ribulose 5-phosphate kinase to H_2O_2 in photosynthetic carbon reduction in cyanobacteria [41]. Accordingly, it is conceivable that *Synechococcus* PCC 7942 acquired the catalase–peroxidase as a H_2O_2-scavenging protein and the resistance of photosynthesis to H_2O_2 during the evolution of cyanobacteria to adapt to oxidative stress from active oxygen species. Furthermore we have shown that H_2O_2 formed in *Euglena* chloroplasts and mitochondria diffuses from each organelle to the cytosol, and is then scavenged by the ascorbate–glutathione cycle including ascorbate peroxidase distributed in the cytosol [42]. Excretion of H_2O_2 has been shown in several cyanobacteria as well as in *Ulva rigida* C. Ag [43]. The diffusion of H_2O_2 from the cells to the outside also may be a protection system against H_2O_2 generated in *Synechococcus* PCC 7942. The fact that the k_{cat}/K_m ratio for *Synechococcus* PCC 7942 catalase–peroxidase was of a similar order of magnitude to those of plant catalases localized in microbodies may support this view.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 07251218) from the Ministry of Education, Science and Culture, Japan, and by the Japan Private School Promotion Foundation and the Environmental Science Research Institute.

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Received 6 November 1995/15 January 1996; accepted 24 January 1996