# Enzymic and molecular characterization of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Synechococcus* PCC 7942: resistance of the enzyme to hydrogen peroxide

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NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been purified to electrophoretic homogeneity from *Synechococcus* PCC 7942 cells. The native enzyme had a molecular mass of 160 kDa and consisted of four subunits with a molecular mass of 41 kDa. The activity was 6-fold higher with NADPH than with NADH; the apparent $K_m$ values for NADPH and NADH were $62 \pm 4.5$ and $420 \pm 10.5$ $\mu$M respectively. The gene encoding NADP-dependent GAPDH was cloned from the chromosomal DNA of *Synechococcus* 7942. A 1140 bp open reading frame, encoding an enzyme of 380 amino acid residues (approx. molecular mass of 41.3 kDa) was observed. The deduced amino acid sequence of the gene had a greater sequence similarity to the NADP-dependent and chloroplastic form than to the NAD-dependent and cytosolic form. The *Synechococcus* 7942 enzyme lacked one of the cysteines involved in the light-dependent regulation of the chloroplast enzymes of higher plants. The recombinant enzyme expressed in *Escherichia coli* as well as the native enzyme purified from *Synechococcus* 7942 cells were resistant to 1 mM $H_2O_2$.

## INTRODUCTION

Organisms possess two distinct glyceraldehyde-3-phosphate dehydrogenases (GAPDHs), the NAD-dependent enzyme involved in cytosolic glycolysis and an NADP-dependent enzyme of the photosynthetic reduction of carbon (PRC) cycle in chloroplasts [1]. In chloroplasts of higher plants, the GAPDH (EC 1.2.1.13) exists as either a homotetramer consisting of a subunit of GapA (36–37 kDa) or a heterotetramer consisting of two subunits of GapA and GapB (39–40 kDa) [2,3]. The GapA subunit has a slightly lower molecular mass than the GapB subunit by virtue of a highly charged 30-amino-acid C-terminal extension in the latter [1,4]. The cytosolic NAD-dependent GAPDH (EC 1.2.1.12) in higher plants is a homotetramer of identical GapC subunits (36–37 kDa) [1,2].

We have previously demonstrated that the photosynthesis of eukaryotic algae such as *Euglena* and *Chlamydomonas* and of cyanobacteria such as *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 is insensitive to $H_2O_2$ up to 1 mM, in comparison with that of chloroplasts of higher plants [5]. The resistance of thiol-modulated enzymes fructose 1,6-bisphosphatase, NADP-dependent GAPDH and ribulose-5-phosphate kinase to $H_2O_2$ was also observed in algal chloroplasts and cyanobacteria. These results suggest that the insensitivity of photosynthesis to $H_2O_2$ is in part caused by the resistance of the algal thiol-modulated enzymes to $H_2O_2$. In the present paper, we describe the purification and some properties of the *Synechococcus* 7942 GAPDH and the cloning and nucleotide sequence of the gene encoding GAPDH. We also investigate the resistance of the purified enzyme and the recombinant enzyme expressed in *Escherichia coli* to 1 mM $H_2O_2$.

## MATERIALS AND METHODS

### Materials

3-Phosphoglyceric phosphokinase, 3-phosphoglyceric acid and $H_2O_2$ were obtained from Sigma Chemical Co., U.S.A. The molecular biology reagents and enzymes were obtained from Takara Shuzo Co., Japan. Geneclean® II Kit was obtained from BIO 101 Inc., U.S.A. Other chemicals were reagent grade and obtained from Nacalai Tesque, Inc., Japan.

### Organism and culture

*Synechococcus* 7942 was cultured in Allen's medium at 26 °C for 5 days under illumination (240 $\mu$E/m² per s) with bubbling of sterile air at 8 l/min [6]. *E. coli*, strains JM109 and BL21(DE3)plysS, were cultured at 37 °C in Luria broth [7].

### Enzyme assays

The NADP-dependent GAPDH activity was spectrophotometrically determined by the absorbance change following NAD(P)H oxidation at 340 nm. The reaction mixture contained 100 mM Tris/HCl buffer, pH 8.0, 10 mM $MgCl_2$, 10 mM GSH, 5 mM ATP, 0.2 mM NADPH, 2 units of 3-phosphoglyceric phosphokinase, 2 mM 3-phosphoglyceric acid and the enzyme.

### Purification

All the purification steps were performed at 4 °C. *Synechococcus* 7942 cells (25 g wet wt.) were harvested by centrifugation, resuspended in 50 ml of 50 mM potassium phosphate buffer,

Abbreviations used: DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl-$\beta$-D-thiogalactoside; PRC, photosynthetic reduction of carbon.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the accession number D61379.

pH 7.5, containing 2.5 mM dithiothreitol (DTT), 1 mM GSH and 10 % (w/v) sucrose, 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. The obtained crude enzyme was loaded on to a DEAE-cellulose column (2.8 cm × 40 cm) equilibrated with the potassium phosphate buffer and developed with a 300 ml linear gradient of KCl (0 to 0.5 M). The active fractions were combined and adjusted to 30 % satn. with $(NH_4)_2SO_4$. The supernatant was chromatographed on a phenyl-Sepharose HR 5/5 column (FPLC system, Pharmacia) equilibrated with 30 % $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose. The column was eluted with 100 ml of a linear gradient of 30 to 0 % $(NH_4)_2SO_4$ at an elution rate of 1 ml/min. The active fractions were combined and fractionated with $(NH_4)_2SO_4$, and the pellet precipitating between 30 and 70 % satn. was dissolved in 2 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose. The enzyme solution was chromatographed on to a Superdex 200 HiLoad 16/60 column equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH, 10 % sucrose and 0.15 M NaCl. The active fractions were concentrated to a final volume of 0.5 ml by ultrafiltration (Amicon PM-30) and loaded on to a 2′,5′-ADP–Sepharose column (0.8 cm × 4 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose. The column was eluted with 1 ml of the potassium phosphate buffer containing 1 mM $NADP^+$ and then with 10 ml of potassium phosphate buffer. The enzyme fractions were collected and chromatographed on to a Sephadex G-25 column (2 cm × 15 cm) equilibrated with 100 mM Tris/HCl buffer, pH 8.0, to remove the $NADP^+$. The purified enzyme was stored at −20 °C without extensive inactivation for several weeks.

## PAGE

Disc gel electrophoresis with 7.5 % (w/v) polyacrylamide at pH 9.4 was performed as described previously [8]. Electrophoresis was carried out at a constant current (2 mA/gel) with Bromophenol Blue as the migration marker. SDS/PAGE was performed on a 12.5 % (w/v) polyacrylamide slab gel as described previously [9]. Proteins in the gel were stained with Coomassie Brilliant Blue R-250 and destained in 7 % acetic acid.

### Estimation of molecular mass

The molecular mass of GAPDH was estimated by using a Superdex 200 HiLoad 16/60 column (2.4 cm × 90 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH, 10 % sucrose and 0.15 M NaCl, and calibrated with Blue Dextran (2000 kDa), bovine thyroglobulin (66.9 kDa), apoferritin from horse spleen (44.3 kDa), β-amylase from sweet potato (20 kDa), alcohol dehydrogenase from yeast (15 kDa), BSA (67 kDa) and carbonic anhydrase from bovine erythrocytes (30 kDa). For the determination of the molecular mass of a subunit by SDS/PAGE [9], phosphorylase *b* from rabbit muscle (94 kDa), BSA (67 kDa), ovalbumin from egg white (43 kDa), carbonic anhydrase from bovine erythrocytes (30 kDa), trypsin inhibitor from soybean (20.1 kDa) and α-lactalbumin from bovine milk (14.4 kDa) were used as standards.

### Analysis of amino acid sequence

The amino acid sequence of the N-terminal region of the enzyme was determined by an automated Edman degradation using a Model 477A gas-phase protein sequencer [10].

### Isolation and nucleotide sequence analysis of the GAPDH gene

The chromosomal DNA was isolated from the *Synechococcus* 7942 cells (wet weight 1.5 g) as described previously [11]. The oligonucleotide primers corresponding to the most highly conserved regions {residues 13–32 [5′-GT(AG)GC(AC)ATCAA-TGG(AT)TTTGG] and 452–474 [5′-GTTGGTGGTGCAAGA-AGCATTGC]} of NADP- and NAD-dependent GAPDHs from various organisms [2,3,12] were designed and synthesized by JBioS Co., Japan. The PCR products using these primers were used as probes to screen the full-length DNA fragment including the gene of GAPDH from the *Synechococcus* 7942 chromosomal DNA digested with *Eco*RI, *Bam*HI, *Hin*dIII, *Xba*I, *Bst*PI or *Sty*I (Takara Shuzo Co., Japan) at 5 units/μg of DNA. The southern blot hybridization was completed according to the instructions of Amersham. As a result (results not shown), there was only one fragment hybridizing with the probe in each lane. A 6.6 kb *Bam*HI fragment including the GAPDH gene was isolated from the agarose gel using the Geneclean® II Kit (BIO 101 Inc., U.S.A.), subcloned into a pBluescript II SK(+) vector, and sequenced using the dideoxy chain primer method modified for double-stranded plasmid DNA. The overlapping insert DNA fragments were obtained by subcloning after restriction digestion.

### Expression of the GAPDH gene in *E. coli* and purification of recombinant enzyme

For the construction of the plasmid to express the cloned GAPDH gene, the full-length protein-encoding DNA fragments were amplified by PCR. Two oligonucleotide primers contained *Nde*I restriction sites with the ATG translation initiation codon and *Bam*HI restriction sites (bold sequence), 5′-AGGGTAT-AGG**CATATG**ACGATTCG-3′ (nucleotide residues −6–17) and 5′-AGCATTT**GGATCC**CTGAACC-3′ (nucleotide residues 1172–1191). The PCR products were digested with the *Nde*I and *Bam*HI restriction enzymes, and ligated into a *Nde*I/*Bam*HI-digested pET3a expression vector followed by its transformation into the *E. coli* strain, BL21(DE3)pLysS. The *E. coli* cells were grown with shaking at 37 °C to an $A_{600}$ of 0.6–0.7 in LB medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The expression was then induced by adding isopropyl β-D-thiogalactoside (IPTG) to 0.4 mM and shaking for 6 h at 37 °C. The cells (2.2 g wet weight) were harvested by centrifugation at 500 ***g*** for 10 min, suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose, and disrupted by sonication (10 kHz) for 3 min. This lysate was centrifuged at 180000 ***g*** for 20 min. The obtained crude enzyme was used for analysis of total soluble protein of *E. coli* by SDS/PAGE and then adjusted to 30 % satn. with $(NH_4)_2SO_4$. The supernatant was chromatographed on a phenyl-Sepharose HR 5/5 column equilibrated with 30 % $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose. The column was eluted with 100 ml of a linear gradient of 30 to 0 % $(NH_4)_2SO_4$ at an elution rate of 1 ml/min. The active fractions were combined and fractionated with $(NH_4)_2SO_4$, and the pellet precipitating between 30 and 70 % satn. was dissolved in 2 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH and 10 % sucrose. The enzyme solution was chromatographed on to a Superdex 200 HiLoad 16/60 column equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH, 10 % sucrose and 0.15 M NaCl. The active fractions were combined, adjusted to 0.05 M

NaCl and loaded on to a Mono Q HR 10/10 column equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 2.5 mM DTT, 1 mM GSH, 10% sucrose and 0.05 M NaCl. The column was eluted with 100 ml of a linear gradient of 0.05 to 1 M NaCl at an elution rate of 1 ml/min. The active fractions were combined and concentrated to a final volume of 1 ml by ultrafiltration (Amicon PM-30).

To completely remove the DTT from the enzyme purified from the recombinant cells, as well as the enzyme purified from the *Synechococcus* 7942 cells, the extracts were chromatographed on a column (1.5 cm × 10 cm) of Sephadex G-25 equilibrated with 100 mM Tris/HCl buffer, pH 8.0, containing 16 mM $MgCl_2$. The protein fractions were placed into vials saturated with $N_2$ gas to give a concentration of approx. 12 $\mu$g of protein/ml. The purified recombinant and native enzymes were incubated with $H_2O_2$ for 10 min in the dark as described previously [5].

## RESULTS AND DISCUSSION

### Characterization of NADP-dependent GAPDH

The purification scheme of *Synechococcus* 7942 GAPDH using the five-step procedure is summarized in Table 1. The purification procedure yielded a GAPDH preparation purified approx. 173-fold over the crude enzyme with a yield of 14.9%. This purification was repeated three times with similar results. During purification of GAPDH from *Synechococcus* 7942, the enzyme activity was eluted from each column as a sharp and single peak, indicating that *Synechococcus* 7942 contains only one type of GAPDH. The PAGE and SDS/PAGE of the purified enzyme showed only one detectable protein band (Figure 1). The specific activity of purified enzyme with NADPH was 150.5 $\mu$mol/min per mg of protein, which was the same order of magnitude as that of GAPDH from higher plants [13]. The enzyme also showed specific activity with NADH, which was 26.9 $\mu$mol/min per mg of protein. The enzyme reaction with NADPH and NADH followed Michaelis–Menten-type kinetics. From the double-reciprocal plots, the apparent $K_m$ values of the enzyme for NADPH and NADH were $62 \pm 4.5$ and $420 \pm 10.5$ $\mu$M respectively. These results indicate that NADPH is the physiological electron donor for *Synechococcus* 7942 GAPDH.

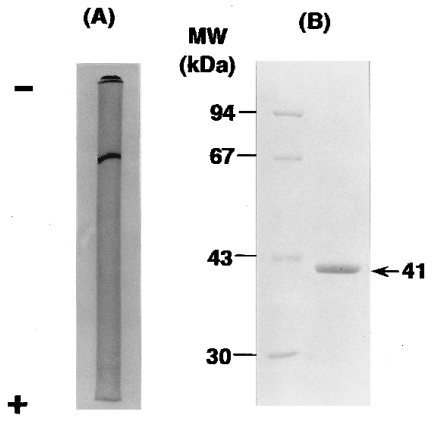


**Figure 1 PAGE and SDS/PAGE of GAPDH**

The procedures for electrophoresis of the purified enzyme were carried out as described in the Materials and methods section. PAGE (**A**) and SDS/PAGE (**B**) of purified enzyme (3 $\mu$g) stained with Coomassie Brilliant Blue R-250.

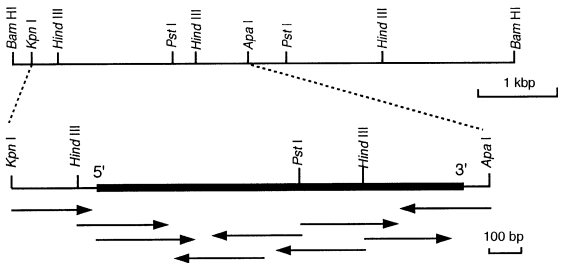


**Figure 2 Restriction map of the 6.6 kb *Bam*HI genomic region and sequence strategy of GAPDH gene**

The thick bar represents the location of the GAPDH gene, with 3′ and 5′ ends indicated. The subfragment containing GAPDH gene was subjected to sequence analysis with the strategy indicated in the lower portion.

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

Gel filtration on a Superdex 200 HiLoad 16/60 column of GAPDH with several standard proteins indicated a molecular mass of 160 kDa. The subunit molecular mass of the enzyme was estimated to be 41 kDa from SDS/PAGE, indicating that the enzyme is a tetramer with a subunit molecular mass of 41 kDa (Figure 1). The amino acid sequences of the N-terminus to the 24th residue of the purified enzyme was determined by an automated Edman degradation, and the sequence was as follows: T-I-R-V-A-I-N-G-F-G-R-I-G-R-N-F-L-R-C-W-F-G-R-Q-.

### Isolation and characterization of DNA encoding GAPDH

The restriction map and sequencing strategy for the 6.6 kb *Bam*HI fragment containing the GAPDH gene are shown in

**Table 1 Purification of GAPDH from *Synechococcus* 7942**

| Step | Total protein (mg) | Total activity ($\mu$mol/min) | Specific activity ($\mu$mol/min per mg of protein) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude | 1156.2 | 1008.5 | 0.87 | 1.0 | 100.0 |
| DEAE-cellulose | 100.2 | 757.2 | 7.56 | 8.7 | 75.1 |
| 30% $(NH_4)_2SO_4$ | 66.8 | 643.8 | 9.60 | 11.0 | 63.8 |
| Phenyl-Sepharose | 11.3 | 499.1 | 44.2 | 50.8 | 49.5 |
| Superdex 200 | 4.21 | 322.9 | 76.9 | 88.4 | 32.0 |
| 2′,5′-ADP–Sepharose | 1.00 | 150.5 | 150.5 | 173.0 | 14.9 |

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      -49 AAGCTTAATAGGTATTTACACCTGAACGCACGTACGAGGGTATAGGGCA   -1
                                          SD

GTGACGATTCGAGTTGCGATCAATGGCTTTGGCCGTATTGGCCGGAATTTTCTCCGTTGC   60
M T I R V A I N G F G R I G R N F L R C                      20

TGGTTTGGACGGCAGAACACCGATCTAGAGGTTGTGGCCATTAACAACACCTCGGATGCA  120
W F G R Q N T D L E V V A I N N T S D A                      40

CGGACGGCTGCTCACCTGCTGGAGTACGACTCTGTTCTCGGCCGGTTCAACGCCGACATC  180
R T A A H L L E Y D S V L G R F N A D I                      60

AGCTACGACGAGAATTCGATCACCGTCAACGGCAAGACGATGAAAATCGTCTGCGATCGC  240
S Y D E N S I T V N G K T M K I V C D R                      80

AACCCCCTCAACCTGCCTTGGAAAGAGTGGGATATCGATCTCGTCATTGAATCTACAGGT  300
N P L N L P W K E W D I D L V I E S T G                     100

GTGTTCGTCACCGCTGAAGGCGCATCCAAGCACATCCAAGCCGGGGCCAAGAAAGTTCTG  360
V F V T A E G A S K H I Q A G A K K V L                     120

ATCACGGCTCCTGGTAAAGCCGAAGGTGTCGGCACCTACGTCATCGGTGTCAACGATTCG  420
I T A P G K A E G V G T Y V I G V N D S                     140

GAATACCGCCACGAAGACTTCGCAGTCATCAGCAATGCAAGCTGCACCACCAACTGCTTA  480
E Y R H E D F A V I S N A S C T T N C L                     160

GCACCGGTCGCCAAAGTTCTGCATGACAACTTTGGCATCATCAAAGGCACGATGACCACC  540
A P V A K V L H D N F G I I K G T M T T                     180

ACCCACAGCTACACGCTGGACCAGCGCATCTTGGACGCCAGCCACCGTGATCTACGTCGG  600
T H S Y T L D Q R I L D A S H R D L R R                     200

GCTCGGGCTGCAGCCGTTAACATCGTTCCCACCACGACCGGCGCTGCTAAAGCCGTTGCT  660
A R A A A V N I V P T T T G A A K A V A                     220

TTGGTGATCCCCGAGCTGAAAGGCAAACTAAACGGGATTGCGCTGCGCGTTCCTACGCCA  720
L V I P E L K G K L N G I A L R V P T P                     240

AACGTGTCTGTCGTTGACTTGGTGGTTCAAGTCGAGAAACCGACGATCACTGAGCAGGTC  780
N V S V V D L V V Q V E K P T I T E Q V                     260

AATGAAGTCCTGCAAAAAGCTTCTCAAACGACGATGAAGGGCATCATCAAGTACTCGGAT  840
N E V L Q K A S Q T T M K G I I K Y S D                     280

CTGCCCTTGGTCTCTTCCGACTTCCGGGGTACTGACGAGTCTTCGATCGTTGACTCCAGC  900
L P L V S S D F R G T D E S S I V D S S                     300

CTGACCTTGGTAATGGATGGCGATCTCGTCAAAGTAATTGCTTGGTACGACAACGAGTGG  960
L T L V M D G D L V K V I A W Y D N E W                     320

GGCTACAGCCAACGAGTTGTCGACTTGGCTGAACTGGCCGCTCGCAAATCGGGCCGCTTA 1020
G Y S Q R V V D L A E L A A R K S G R L                     340

GGACTTGTTCAAACCCTCTTAATTTCTAGTCAAGATAGCCTCCCATTAACGGGAGGCTTT 1080
G L V Q T L L I S S Q D S L P L T G G F                     360

TTTGTGGCCAAGAGAGAACACCCCAGGAGCCAAGATTCGATCAATCCATCAGGCCCCGAT 1140
F V A K R E H P R S Q D S I N P S G P D                     380

TAGGGTGAAATGACGGATTTTCAATCCCCTAGGTTCAGGAATCCAAATGCTATTAGGGCT 1200
*

GAATTTCAGTTGGCTGAAAGCAGAGCCAATCAGCTCGCCACCTATAATTCAGAGAAGTGA 1260
CGATTAGGAGCGATCGCCGCATGGCTTTGCTGGAGACCCGCACCGAGCCAATGGTGCTCA 1320
ACATGGGCCC 1330
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**Figure 3 Nucleotide sequence of the GAPDH gene and deduced amino acid sequence, including upstream and downstream elements**

The amino acid sequence of the N-terminal region, which is identical to the sequence determined using the automated Edman degradation of the purified enzyme, is underlined. The numbers stand for those from the N-terminal first residues on each line. The putative ribosome-binding site, the Shine–Dalgarno sequence, is doubly underlined.

Figure 2. The nucleotide sequence of the GAPDH gene consisted of an 1140 bp open reading frame, encoding for a peptide of 380 amino acid residues (Figure 3). The initiation codon is GTG instead of ATG, similar to the situation observed for *Phytophthora infestans gpdA* gene [14]. The calculated molecular mass of the cloned sequence in the gene of GAPDH was 41 304 Da, which was closed to the 41 kDa of the GAPDH subunit determined by SDS/PAGE. The deduced amino acid sequence coincided with the N-terminus of the NADP-dependent GAPDH purified from *Synechococcus* 7942. Upstream from the initiation codon (by 16 bp) is a putative ribosomal-binding site, the Shine–Dalgarno sequence, ACGAGG. The G+C content of the GAPDH gene was 54.1 %, which was similar to the percentage of G+C base pairs found in the genomic DNA from *Synechococcus* 7942.

## Comparative analysis of amino acid sequences of GAPDH from various sources

Figure 4 compares the deduced amino acid sequences of the *Synechococcus* 7942 GAPDH gene with other GAPDH genes from various organisms. The deduced amino acid sequence of the *Synechococcus* 7942 GAPDH gene was 66.9 %, 67.0 % and 45.9 % identical to types GapA, GapB, and GapC of *Arabidopsis* respectively [3]. Similar results were also obtained with GapA (65.7 %), GapB (66.1 %) and GapC (44.6 %) of tobacco [15]. There was a relatively low similarity between the *Synechococcus* 7942 GAPDH and the *E. coli* NAD-dependent form (48.8 % identity) [16]. These results showed that the amino acid sequence of the *Synechococcus* 7942 GAPDH gene had a greater sequence similarity with the NADP-dependent and chloroplastic form than with the NAD-dependent and cytosolic form. This view is also supported by the donor specificity for NADPH; the specific activity and the $K_m$ values for NADPH were as described above.

The subunits of *Arabidopsis* GapA and GapB had approx. 14- and 35-amino-acid N-terminal extensions respectively, which indicate features common to the transit peptides of cytosolic precursors of proteins imported across the chloroplastic envelope [2,3]. Unlike GapA and GapB, the *Synechococcus* 7942 GAPDH gene had no transit peptides. Furthermore, it is interesting to note that the C-terminal of the *Synechococcus* 7942 GAPDH gene, as well as the GapB gene, was longer than those of the chloroplastic and cytosolic forms. The 42-residue C-terminal region of the gene from *Synechococcus* 7942 GAPDH had a very low sequence similarity with a 28-residue C-terminal region of GapB and other sequences in the Swiss-Prot database.

Both GapA and GapB are characterized by the five Cys residues at the conserved positions as follows: 33, 167, 171, 291 and 302 in *Arabidopsis* GapA; however, GapC lacked the three Cys residues corresponding to 33, 291 and 302 [3]. In the *Synechococcus* 7942 GAPDH gene, the two Cys residues corresponding to 291 and 302 were absent, but four Cys residues at 20, 78, 155 and 159 were present.

## The resistance of recombinant and native enzymes of *Synechococcus* 7942 to $H_2O_2$

After induction with IPTG, the recombinant proteins from the GAPDH gene were expressed in *E. coli* cells. As shown in Figure 5, the recombinant protein correlated well with the calculated molecular mass of the native enzyme purified from *Synechococcus* 7942 cells. The *E. coli* recombinant enzyme showed an NADPH-dependent GAPDH activity of 13.4 $\mu$mol/min per mg of protein. In contrast, the *E. coli* as a control showed an NADH-dependent GAPDH activity of 2.9 $\mu$mol/min per mg of protein, but no NADPH-dependent GAPDH activity. The purification procedure yielded a recombinant GAPDH preparation purified approx. 11.2-fold over the crude enzyme, giving a final 57.0 % recovery of the activity. The SDS/PAGE of the purified enzyme showed only one detectable protein band (results not shown). The specific activity of the purified recombinant enzyme with NADPH was 149.8 $\mu$mol/min per mg of protein, which was in agreement with that of the purified native enzyme.

We have previously demonstrated that the thiol-modulated

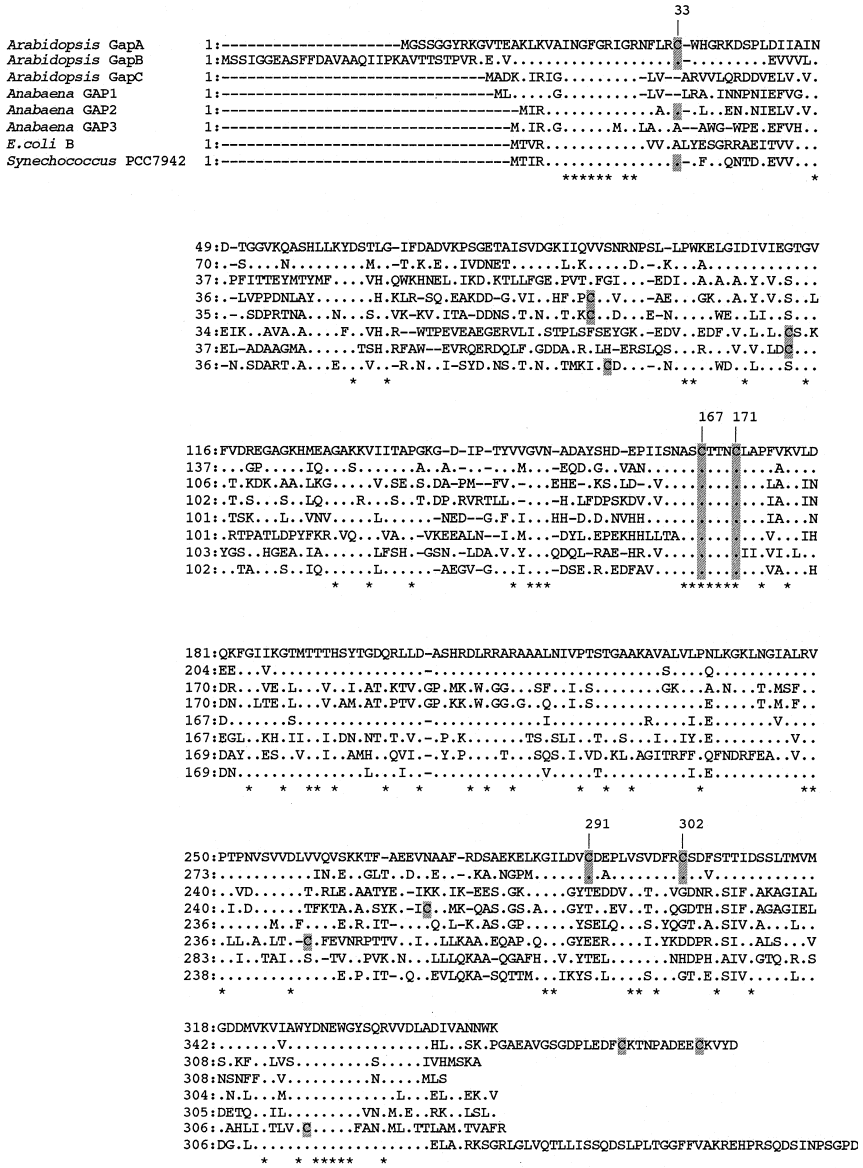

**Figure 4 Comparison of the predicted amino acid sequence of the *Synechococcus* 7942 GAPDH protein with the sequences of GAPDH from *Arabidopsis* (GapA, GapB and GapC), *Anabaena* (GAP1, GAP2 and GAP3) and *E. coli***

The differences in the GAPDH protein sequences can be identified with the substitution of another amino acid below the *Arabidopsis* GapA sequence. Dashes are included to maximize the alignment, and the dots indicate homology with the *Arabidopsis* GapA sequence. The asterisks show the consensus amino acids.

enzymes, fructose 1,6-bisphosphatase, NADP-dependent GAPDH and ribulose-5-phosphate kinase in chloroplasts of *Euglena* and *Chlamydomonas* and in crude extracts of cyanobacteria, *Synechococcus* 7942 and *Synechocystis* PCC 6803 are resistant to $H_2O_2$ [5]. Here we studied the effects of $H_2O_2$ on the activities of the native GAPDH purified from *Synechococcus* 7942 cells and the recombinant GAPDH purified from *E. coli* cells. As shown in Figure 6, both the native and recombinant GAPDHs were only slightly inhibited by 1 mM $H_2O_2$, indicating the absence of susceptibility of the GAPDHs to $H_2O_2$ up to 1 mM. In contrast, the spinach chloroplast GAPDH was completely inhibited at 0.1 mM $H_2O_2$, which was in agreement with the result reported previously [5].

The fructose 1,6-bisphosphatase, NADP-dependent GAPDH and ribulose-5-phosphate kinase are activated by reducing equivalents that can be photochemically generated via a thioredoxin–ferredoxin system and may be involved in the metabolic regulation in the PRC cycle of higher plant chloroplasts [17,18]. Kaiser [19] has reported that $H_2O_2$ at low levels (10 $\mu$M) inhibits the thiol-modulated enzymes of the PRC cycle, because $H_2O_2$ readily oxidizes the reduced thiol groups. Indeed, the thiol-modulated enzymes are major targets of attack by $H_2O_2$. The resistance of the *Synechococcus* 7942 GAPDH to $H_2O_2$ suggests that there might be considerable structural differences in the vicinity of the thiol groups involved in the light activation between the GAPDH of *Synechococcus* 7942 and the chloroplastic forms (GapA and GapB) of higher plants. It has been reported that the light activation of GAPDH in peas involves a

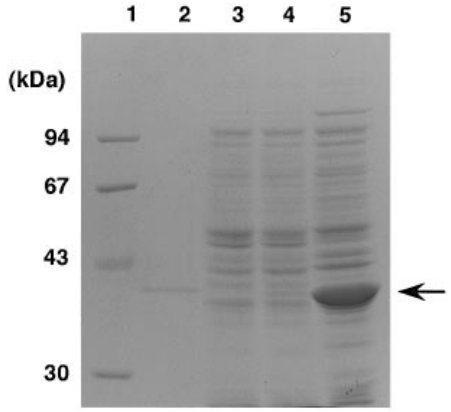


**Figure 5 Production of GAPDH in *E. coli* BL21(DE3)pLysS by the GAPDH gene**

Protein standards (lane 1), purified enzyme (lane 2), the lysate of *E. coli* (lane 3), the lysate of *E. coli* transformed with the GAPDH gene and incubated in the absence (lane 4) and presence (lane 5) of IPTG were subjected to SDS/PAGE. Each lane was stained with Coomassie Brilliant Blue R-250 and destained with 7% acetic acid. The arrow indicates the GAPDH.

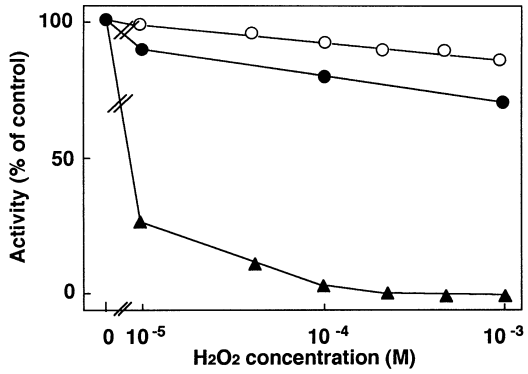


**Figure 6 Effects of $H_2O_2$ on activities of recombinant and native enzymes in *Synechococcus* 7942**

Recombinant GAPDH was purified from *E. coli* which was transformed with the GAPDH gene and incubated in the presence of IPTG. The native GAPDH was purified from *Synechococcus* 7942 cells as described in the Materials and methods section. Spinach intact chloroplasts were prepared as previously described [5]. After incubation with $H_2O_2$ at the indicated concentrations for 10 min in the darkness, the NADP-dependent GAPDH was subjected to an assay as described in the Materials and methods section. The control activities of native (●), recombinant (○) and spinach chloroplast (▲) GAPDHs are shown as 100%, which are $150.5 \pm 8.9$, $149.8 \pm 6.5$ and $1.3 \pm 0.3$ $\mu$mol/min per mg of protein, respectively. Data are represented as the results of three assays $\pm$ S.D.

reductive cleavage of a disulphide bond, and it has been suggested that in the reductively activated chloroplast enzyme, the formation of a disulphide bond between the Cys-18 corresponding to Cys-33 in *Arabidopsis* GAPDH (on the nucleotide-binding domain) and Cys-285 corresponding to Cys-302 in *Arabidopsis* GAPDH (on the carbon-substrate-binding domain) interferes with the interdomain movement, thereby restricting catalysis [20]. As previously described, a Cys residue corresponding to the Cys-285 in pea GapA was absent in the GAPDH gene of *Synechococcus* 7942; however, Cys-20 was present. Consequently, it seems likely that the resistance of the GAPDH in *Synechococcus* 7942 to $H_2O_2$ is due in part to the lack of one of the cysteines found in the chloroplast GAPDH (GapA and GapB) of higher plants. In addition, the lack of Cys-285 in *Synechococcus* 7942 GAPDH raises questions as to whether it is insensitive to treatment with the reducing agent DTT *in vitro*, and is not regulated by light activation *in vivo*. Recently, the GAPDHs from *Anabaena variabilis* and *Synechocystis* PCC 6803 have been reported to lack one of the cysteines typically found in higher plant enzymes [21]. Additionally, these GAPDHs could not be reductively activated [21]. We are confirming the detailed relationship between the resistance of GAPDH to $H_2O_2$, the lack of thiol groups, and the absence of light regulation in *Synechococcus* 7942.

Received 23 October 1995/29 January 1996; accepted 1 February 1996

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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