

Tomoya Kitatani,^a Yoshihiro Nakamura,^a Kei Wada,^a Takayoshi Kinoshita,^b Masahiro Tamoi,^c Shigeru Shigeoka^c and Toshiji Tada^{b*}

^aDepartment of Applied Biochemistry, Graduate School of Agriculture and Life Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan, ^bDepartment of Biological Science, Graduate School of Science, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan, and ^cDepartment of Advanced Bioscience, Faculty of Agriculture, Kinki University, Nakamachi, Nara 631-8505, Japan

Correspondence e-mail:
tada@b.s.osakafu-u.ac.jp

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Structure of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Synechococcus* PCC7942 complexed with NADP

The crystal structure of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP-GAPDH) from *Synechococcus* PCC 7942 (*S.* 7942) in complex with NADP was solved by molecular replacement and refined to an *R* factor of 19.1% and a free *R* factor of 24.0% at 2.5 Å resolution. The overall structure of NADP-GAPDH from *S.* 7942 was quite similar to those of other bacterial and eukaryotic GAPDHs. The nicotinamide ring of NADP, which is involved in the redox reaction, was oriented toward the catalytic site. The 2'-phosphate O atoms of NADP exhibited hydrogen bonds to the hydroxyl groups of Ser194 belonging to the S-loop and Thr37. These residues are therefore considered to be essential in the discrimination between NADP and NAD molecules. The C-terminal region was estimated to have an extremely flexible conformation and to play an important role in the formation of the supramolecular complex phosphoribulokinase (PRK)–regulatory peptide (CP12)–GAPDH, which regulates enzyme activities.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the reversible interconversion between 1,3-bisphosphoglycerate and D-glyceraldehyde 3-phosphate using either NAD(H) or NADP(H) as a coenzyme. NAD-GAPDH (EC 1.2.1.12) only catalyzes the glycolysis/glyconeogenesis of prokaryotes and eukaryotes in the presence of NAD(H). NADP-GAPDH (EC 1.2.1.13) utilizes both NADP(H) and NAD(H) with a preference for NADP(H) and functions in the second reaction of the photosynthetic carbon reduction (PCR) cycle of higher plants and algae (Wolosiuk & Buchanan, 1976). The activity of NADP-GAPDH is regulated *in vivo* by a system consisting of ferredoxin, thioredoxin *f* and ferredoxin/thioredoxin reductase (Buchanan, 1980; Cséke & Buchanan, 1986). This enzyme is also activated by addition of DTT, NADP(H) or ATP *in vitro* (Wolosiuk & Buchanan, 1976; Wirtz *et al.*, 1982). In higher plants, NADP-GAPDH exists in the form of either a homotetramer A_4 consisting of a subunit *A* or a heterooligomer $(A_2B_2)_n$ consisting of two subunits *A* and *B* in stoichiometric amounts (Cerff, 1978; Shih *et al.*, 1991; Scagliarini *et al.*, 1998). The subunits *A* (36 kDa) and *B* (39 kDa) of chloroplast GAPDHs are homologous and highly conserved, except for the C-terminal extension of the subunit *B*. The extended C-terminal region of subunit *B*, which contains two Cys residues, is thought to be involved in the regulation of GAPDH activity (Sparla *et al.*, 2002, 2005).

The cyanobacterium *Synechococcus* PCC 7942 (*S.* 7942) possesses a unique homotetrameric NADP-GAPDH that is thought to work not only in the PCR cycle but also in glycolysis/gluconeogenesis. The activity of NADP-GAPDH is sixfold higher with NADPH than with NADH. Moreover, it is not greatly activated by light irradiation or by the addition of DTT, NADPH or ATP and is not regulated by a ferredoxin–thioredoxin system, thus making it different from those of higher plants (Tamoi *et al.*, 1996, 1998). *S.* 7942 NADP-GAPDH has an amino-acid sequence that shares about 65–69% identity with subunits *A* and *B* of spinach and tobacco chloroplasts, with the differences in their primary structures mostly being found neigh-

bouring the C-terminus. Of particular significance, *S. 7942* GAPDH lacks the three thiol groups that might be involved in light/dark regulation of the chloroplast enzymes.

Crystal structures have been solved for cytosolic NAD-GAPDHs from a number of organisms including *Escherichia coli* (Duée *et al.*, 1996), *Bacillus stearothermophilus* (Biesecker *et al.*, 1977; Skarzynski *et al.*, 1987), *Trypanosoma cruzi* (Vellieux *et al.*, 1995; Souza *et al.*, 1998), *Leishmania mexicana* (Kim *et al.*, 1995), human muscle (Mercer *et al.*, 1976) and lobster (Moras *et al.*, 1975; Song *et al.*, 1998). Crystal structures of NADP-GAPDHs from *Methanothermobacter ferredoxin* (Charron *et al.*, 2000) and non-regulatory A_4 isoforms from spinach chloroplasts (Fermani *et al.*, 2001; Falini *et al.*, 2003; Sparla *et al.*, 2004) have been reported. These GAPDH structures have contributed to the understanding of phosphate-recognition sites, which are important for the enzyme reaction. GAPDHs have two anion-binding sites, the P_s (substrate phosphate ion site) and the P_i (inorganic phosphate ion site) near the catalytic Cys residue (Moras *et al.*, 1975). A new inorganic phosphate ion-binding site was recently reported in some GAPDH structures (Kim *et al.*, 1995; Korndörfer *et al.*, 1995; Song *et al.*, 1999; Yun *et al.*, 2000; Falini *et al.*, 2003). This site is referred to as the ‘new P_i ’ site to distinguish from ‘classical P_i ’ sites.

The aim of the present study is to clarify the molecular mechanism of the novel NADP-GAPDH from *S. 7942* by analyzing its crystal structure. A detailed study of the three-dimensional structure of the enzyme and comparison with that reported for the A_4 isoform of spinach chloroplasts in complex with NADP should provide information about the recognition mechanism of the two coenzymes NADP and NAD. Furthermore, structure analysis should help determine the activation mechanism produced by light irradiation.

Here, we report the crystal structure of *S. 7942* NADP-GAPDH in complex with NADP at 2.5 Å resolution.

2. Materials and methods

Expression and purification of recombinant NADP-GAPDH from *S. 7942* was performed as reported previously (Tamoi *et al.*, 1996). The initial crystallization condition was found using Crystal Screen 2 from Hampton Research (Nakamura *et al.*, 2001). We slightly optimized the condition. Pure recombinant protein was concentrated to 3.5 mg ml⁻¹ in 50 mM HEPES buffer containing 30 mM NaCl. Crystals of *S. 7942* GAPDH were then grown at 293 K using the hanging-drop vapour-diffusion technique with initial conditions formed by mixing 2 µl protein solution with 1 µl reservoir solution consisting of 44% saturated ammonium sulfate, 0.1 M citrate buffer pH 5.2 and 0.2 M potassium sodium tartrate. Crystals complexed with NADP were produced by soaking the native crystals in solution containing 54% saturated ammonium sulfate, 0.1 M citrate buffer pH 5.2, 0.2 M potassium sodium tartrate and 40 mM NADP⁺.

Diffraction data from the crystals were collected at 100 K on an ADSC CCD detector using synchrotron radiation with a wavelength of 0.97 Å at the BL6A station of KEK-PF, Japan. The crystal-to-detector distance was 220 mm and 180 images were recorded at 1° intervals. For data collection at 100 K, the crystals were loop-mounted in cryoprotectant solution containing 70% saturated ammonium sulfate, 0.1 M citrate buffer pH 5.2, 0.2 M potassium sodium tartrate and 10% (v/v) glycerol. The intensity data were processed with *MOSFLM* (Steller *et al.*, 1997) and scaled using

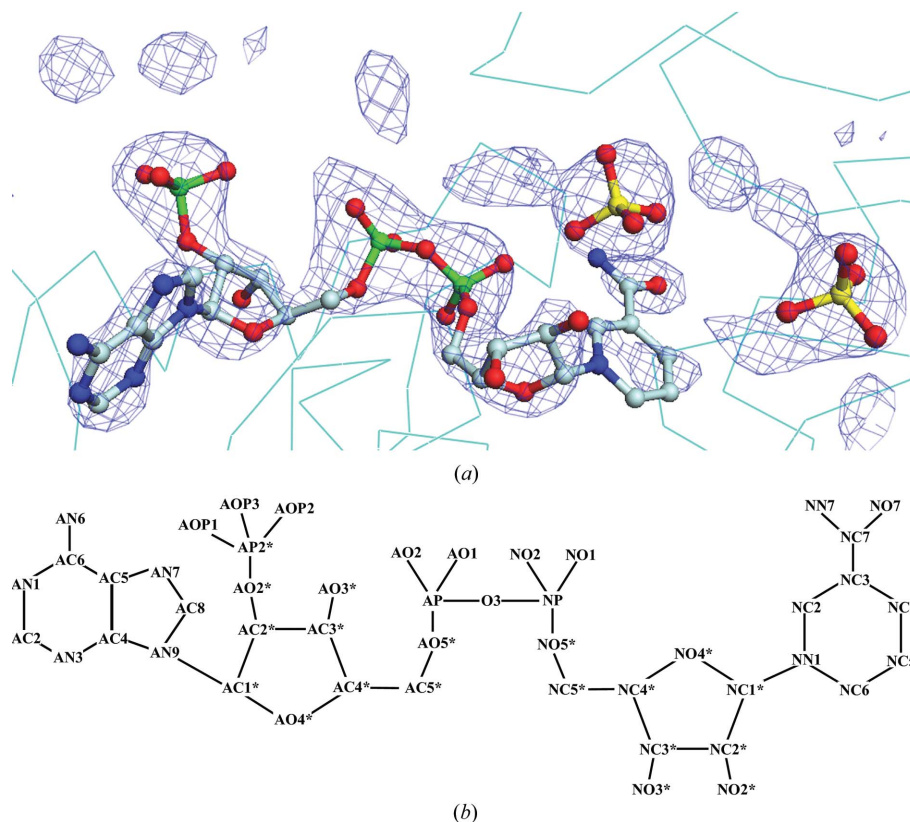


Figure 1
(a) $F_o - F_c$ map of NADP- and sulfate ion-binding sites contoured at 2.5σ . The NADP molecule and sulfate ions are shown as ball-and-stick models. This figure was prepared using *DINO* (<http://www.dino3d.org>). (b) Numbering scheme of the atoms in the NADP molecule using the general nomenclature (Carugo & Argos, 1997).

Table 1

Summary of data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell (2.6–2.5 Å).

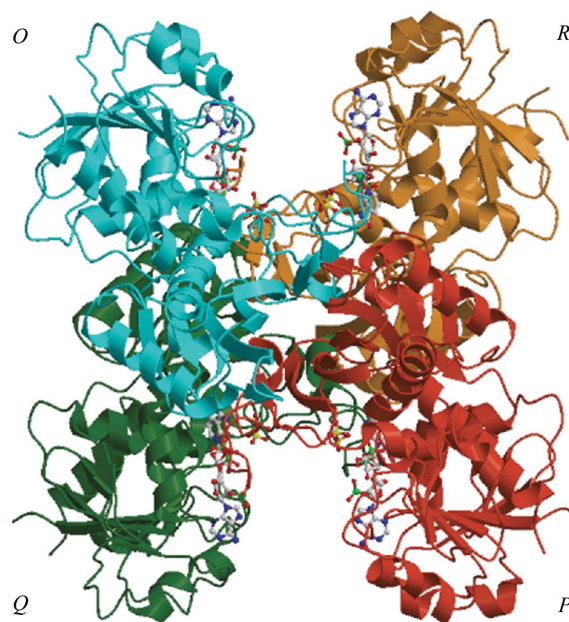
Data-collection statistics	
X-ray source	KEK-PF, BL6A
Wavelength (Å)	0.97
Temperature (K)	100
Resolution (Å)	2.5
Space group	C2
Unit-cell parameters (Å, °)	$a = 150.0, b = 79.6,$ $c = 206.2, \beta = 101.3$
$R_{\text{merge}}^{\dagger}$ (%)	6.7 (26.5)
$I/\sigma(I)$	7.7 (2.8)
No. of observed reflections	701767
No. of unique reflections	71057
Completeness (%)	85.8 (85.8)
Mosaicity (°)	0.86
Model details	
No. of protein residues	2028
No. of sulfate ions	12
No. of NADP molecules	6
No. of water molecules	539
Refinement statistics	
Resolution (Å)	20–2.5
R factor ‡ (%)	19.1 (26.8)
R_{free}^{\S} (%)	24.0 (31.4)
R.m.s.d. in bond distance (Å)	0.013
R.m.s.d. in bond angle (°)	1.7
Average B values	
Overall (Å ²)	33.3
NADP molecules (Å ²)	36.3
Sulfate ions (Å ²)	31.3
Water molecules (Å ²)	29.9
Ramachandran plot	
Most favoured region (%)	87.6
Additionally allowed regions (%)	11.4
Generously allowed regions (%)	0.7
Disallowed regions (%)	0.3

$^{\dagger} R_{\text{merge}} = \sum_{hkl} |I - \langle I \rangle| / \sum_{hkl} I$. $^{\ddagger} R$ factor = $\sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$. $^{\S} R_{\text{free}}$ was calculated using 10% of the data.

SCALA from the CCP4 package (Collaborative Computational Project, Number 4, 1994). Unexpected diffraction overlap arising from high mosaicity of the crystals causes the 85.8% completeness. The crystals belong to the monoclinic space group C2, with unit-cell parameters $a = 150.0, b = 79.6, c = 206.2$ Å, $\beta = 101.3^{\circ}$, and diffracted to 2.5 Å resolution.

The structure of NADP-GAPDH from *S. 7942* was determined by the molecular-replacement method using EP MR (Kissinger *et al.*, 1999). The dimeric structure of NAD-GAPDH from *B. stearothermophilus* (PDB code 1gd1), which exhibits 59.5% identity to *S. 7942* NADP-GAPDH, was used as a search model. The solution revealed that the crystal contains one tetramer and one dimer as the asymmetric unit. Crystallographic refinement was performed by simulated-annealing, positional and individual B -factor refinements with CNS (Brünger *et al.*, 1998), followed by manual rebuilding of the structure with O (Jones *et al.*, 1991). Noncrystallographic symmetry (NCS) restraints were applied during refinement steps. After several rounds of refinement and model building, the R factor and free R factor were reduced to 25.6 and 28.9%, respectively. The $F_o - F_c$ difference map showed clear electron density corresponding to the NADP molecule at the NADP-binding site, although density for the nicotinamide group was somewhat weak (Fig. 1). Two peaks exhibiting an electron density higher than 4σ were found close to the catalytic cavity in the map and were assigned as sulfate ions. After refinement including the NADP molecules and sulfate ions, water molecules were added to the model at locations with $F_o - F_c$ densities higher than 3σ and hydrogen-bonding stereochemistry using the water-picking function of CNS without NCS restraints. The final R factor and free R factor were 19.1 and 24.0%, respectively. The

stereochemistry of the final model was analyzed with PROCHECK (Laskowski *et al.*, 1993). A Ramachandran plot showed 87.6% of the residues in the most favoured regions, with most of the remaining residues in additionally allowed regions. Only Val242 lies in a disallowed region. The electron density for this residue in each subunit clearly confirms its conformation. This structural feature is also

**Figure 2**

Overall view of the NADP-GAPDH tetramer from *S. 7942* in a ribbon representation. NADP and sulfate ions are shown as ball-and-stick models. This figure was prepared using MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt & Murphy, 1994).

**Figure 3**

Overall view of monomer O of NADP-GAPDH from *S. 7942*. NADP and sulfate ions are shown as ball-and-stick models. Helices are represented by crimson cylinders and β -strands are shown in gold. This figure was prepared using MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt & Murphy, 1994).

present in other GAPDH structures (Skarzynski *et al.*, 1987; Kim *et al.*, 1995; Vellieux *et al.*, 1995). The data-collection and final refinement statistics are summarized in Table 1.

3. Results and discussion

The final model comprises six monomers of *S.* 7942 GAPDH complexed with NADP and 539 water molecules in the asymmetric unit. The monomers, *O*, *P*, *Q*, *R*, *O'* and *P'*, were named according to *B. stearothermophilus* GAPDH (Biesecker *et al.*, 1977). Monomers *O*, *P*, *Q* and *R* form a tetramer, *OPQR*, as shown in Fig. 2. Monomers *O'* and *P'* are close to the cell origin and the crystallographic equivalents *Q'* and *R'* are created by a twofold symmetry operation to form the tetramer *O'P'Q'R'*. Although the two tetramers differ crystallographically, the difference in their quaternary structures cannot be regarded as significant.

The structure of monomer *O* is shown in Fig. 3. In all six monomer structures, the 42 amino-acid residues from the C-terminus were disordered and did not show sufficient electron density for assignment, possibly because they are located on the surface of the tetramer. The six monomers present very similar conformations. The root-mean-square deviations (r.m.s.d.) calculated using *LSQKAB* with monomer *O* as a reference for superimposition of the *P*, *Q*, *R*, *O'* and *P'* C α atoms are 0.157, 0.151, 0.165, 0.333 and 0.298 Å, respectively. The values are in the range 0.151–0.333 when calculated using other monomers as a reference. The monomer structures consist of NADP-binding (amino-acid residues 1–153 and 318–338) and catalytic (154–317) domains. The NADP-binding domain has a Rossmann fold (Rossmann *et al.*, 1975) in which six β -strands form a parallel β -sheet and six α -helices lie on both sides of the sheet. The core sheet is completed by two extra β -strands. The catalytic domain is made up of an eight-stranded antiparallel β -sheet, seven α -helices and a loop structure (residues 183–208) called the S-loop (Skarzynski *et al.*, 1987;

Table 2

Short contacts (less than 3.4 Å) of NADP with the protein and sulfate ions.

NADP		Distance (Å)
AOP3	Thr37 OG1	2.6 ± 0.1
AOP3	Ser194 OG†	3.0 ± 0.4
AN6	Arg80 O	3.3 ± 0.2
AO3*	Gly11 N	3.3 ± 0.2
AO2	Arg12 N	2.8 ± 0.1
NO2	Ile13 N	2.9 ± 0.2
NO2*	SO ₄ 340 O4	2.8 ± 0.2
NO7	Asn318 ND2	3.0 ± 0.2
NN7	NADP NO1	3.0 ± 0.1

† Belongs to the S-loop of the neighbouring subunit.

Skarzynski & Wonacott, 1988). Overall, the topology of *S.* 7942 GAPDH is very similar to that of other known GAPDHs.

Each monomer contains one NADP molecule and two sulfate ions, as shown in Fig. 4. The NADP molecule has an extended conformation and is located in the coenzyme-binding site in each monomer, allowing additional interactions with residues of the S-loop in a neighbouring monomer (Table 2); for example, the NADP bound in the coenzyme site of monomer *O* forms additional interactions with the S-loop of monomer *R*. The adenine ring adopts an *anti* conformation, while the nicotinamide ring is in a *syn* conformation stabilized by an internal hydrogen bond between the NN7 atom of the nicotinamide and the NO1 atom of one of the phosphate groups, with an average distance of 2.96 Å between the two. The nicotinamide ring of NADP, which is involved in the redox reaction, is oriented toward the catalytic site. The distance between the NC5 atom of the nicotinamide ring and the S' atom of the catalytic Cys155 is 3.56 Å. One of the 2'-phosphate O atoms of NADP forms hydrogen bonds with the hydroxyl groups of Ser194 belonging to the S-loop and Thr37. The mode of interaction between the NADP molecule and protein is the same as that found in the crystal structure of A₄-GAPDH from spinach chloroplasts (Fermani *et al.*, 2001). Of particular significance, hydrogen bonds between the 2'-phosphate O atoms of NADP and the

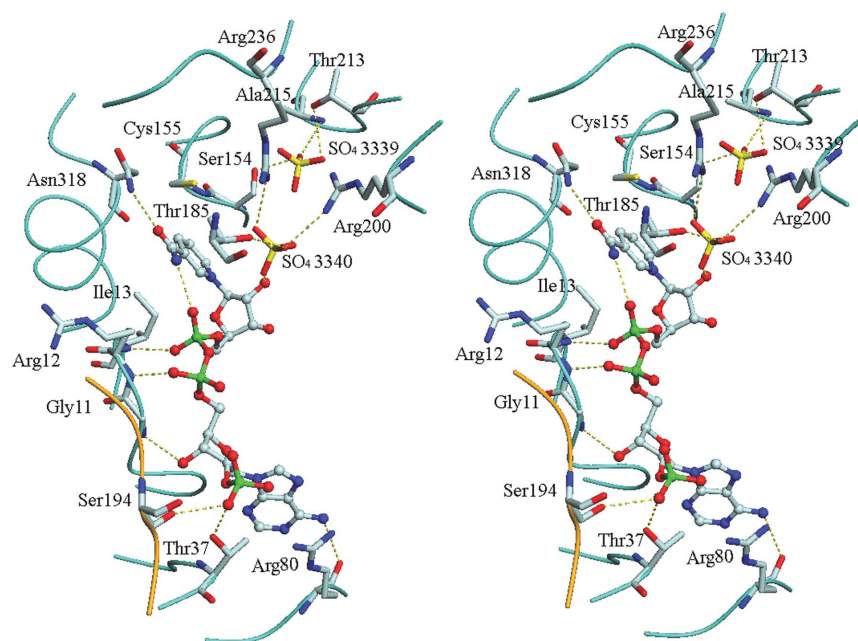


Figure 4

Stereo representation of the NADP- and sulfate-binding sites in monomer *O*. Hydrogen bonds are represented by dotted lines. C atoms are shown in grey, N atoms in blue, O atoms in red, S atoms in yellow and P atoms in green. A C α trace of monomer *O* is shown in cyan and that of subunit *R* is shown in orange. This figure was prepared using *DINO* (<http://www.dino3d.org>).

hydroxyl groups of the residues Ser and Thr were also found in spinach A_4 -GAPDH. Thus, it is suggested that these residues play an essential role in discrimination between NADP and NAD molecules because the two coenzymes only differ structurally in the phosphate group esterified at the 2' position of adenosine ribose. This is supported by evidence that the affinity for NADPH is decreased and $k_{\text{cat}}(\text{NADPH})$ is lowered in mutants of spinach A_4 -GAPDH with the Ser of the S-loop and/or Thr replaced by Ala (Sparla *et al.*, 2004).

Two sulfate ions bind to the active site (Fig. 3). Sulfate ion 3339 occupies a classical P_i site, making hydrogen bonds with Thr213 O^γ, Ser154 O^γ, Ala215 N and one water molecule. Sulfate ion 3340 occupies the P_s site, forming hydrogen bonds with the nicotinamide ribose O2' of NADP, Thr185 O^γ, Arg200 N^{η1} and one water molecule. In our structure, the conformational adjustment of the 220s loop that is necessary for formation of the 'new P_i ' site (Kim *et al.*, 1995) is not observed.

S. 7942 GAPDH is not greatly activated by light irradiation (Tamoi *et al.*, 1996, 1998), although NADP-GAPDH in chloroplast stroma is activated by reduced thioredoxins in light. The regulatory isoform of the chloroplast enzyme is A_2B_2 -GAPDH, containing subunits *A* and *B* in stoichiometric amounts (Scheibe *et al.*, 1996; Scagliarini *et al.*, 1998; Sparla *et al.*, 2002). Two extra Cys residues, Cys349 and Cys358, belonging to the extended C-terminal part of subunit *B*, are considered to be essential for the regulation of GAPDH activity (Sparla *et al.*, 2005). These Cys residues are absent in *S. 7942* GAPDH, although its subunit (41 kDa) has an extended C-terminus. Consequently, it seems likely that the light-activation property of *S. 7942* GAPDH is related to the lack of Cys residues. The 42 amino-acid residues of the C-terminal region did not show sufficient electron density for assignment, indicating that the C-terminal region has an extremely flexible conformation. Recently, it has been reported that oligomerization of the regulatory peptide (CP12) with phosphoribulokinase (PRK) and GAPDH also regulates the activities of both enzymes in the cyanobacteria *Synechococcus* PCC7942 (Tamoi *et al.*, 2005). Therefore, the C-terminal region of *S. 7942* GAPDH might have to be flexible enough for the complicated formation of a supramolecular complex including GAPDH, CP12 and PRK.

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