Purification and Characterization of 6-Phosphogluconate Dehydrogenase from *Phormidium* sp.

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A procedure for the purification of 6-phosphogluconate dehydrogenase from the cyanobacterium *Phormidium* sp. is described. The molecular weight of the native enzyme was 104,000 by gel filtration, and SDS-polyacrylamide gel electrophoresis showed that the enzyme consisted of two subunits with an identical molecular weight of 52,000. The optimum pH of the reaction was 8.0. The K_m values for 6-phosphogluconate and NADP were 3.6×10^{-5} M and 1.3×10^{-5} M, respectively. The enzyme showed no Mg^{2+} requirement for the activity, but was activated by Mn^{2+} and Ca^{2+} . The enzyme was inhibited by sulfhydryl reagents, indicating that a sulfhydryl group may be involved in the active site of the enzyme. The enzyme was also inhibited by NADPH₂, ATP, and the intermediates formed during photosynthesis. The substrate 6-phosphogluconate and cofactor NADP partially protected the enzyme from inactivation. The enzyme had enzymological and physicochemical properties similar to enzymes isolated from other sources.

The enzyme 6-phosphogluconate dehydrogenase [6PGDH; EC 1.1.1.44, 6-phospho-D-gluconate: NADP 2-oxidoreductase(decarboxylating)] has been extensively studied and isolated from a number of sources because of its key role in the pentose phosphate pathway.^{1~7)}

In cyanobacteria sugar breakdown occurs exclusively *via* the oxidative pentose phosphate pathway, predominantly consisting of the same enzymes operating in the Calvin cycle, supplemented with two NADP-specific dehydrogenases, glucose 6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) and 6PGDH.⁸⁾ The metabolic links between photosynthetic and respiratory metabolism in cyanobacteria are therefore close. The formed NADPH₂ can be used for ATP formation during the dark phase.⁸⁾ For the oxidative pentose phosphate pathway, G6PDH is thought to be the key enzyme of regulation. Inhibition of cyanobacterial G6PDH by NADPH₂, ATP, glutamine, and ribulose 1,5-bisphosphate (RUBP),

as well as some enzymatic properties have been reported.^{9~11)} However, little is known of the cyanobacterial 6PGDH.

We describe here a purification procedure and the characterization of the enzymatic, physical, and chemical properties of 6PGDH from *Phormidium* sp. These studies permit elucidation of the mechanisms that regulate 6PGDH activity *in vivo*.

MATERIALS AND METHODS

Biological material and culture methods. The filamentous cyanobacterium, *Phormidium* sp., isolated from Matsue hot springs, was cultured photoautotrophically at 47°C as described previously.¹²⁾ Cells were harvested in the late logarithmic phase by filtration and washed with distilled water.

Enzyme assays. The 6PGDH activity was measured at 340 nm in a Shimadzu model UV 240 spectrophotometer with a thermostated cuvette holder maintained at 43°C. The standard assay mixture contained the following components in a final volume of 1.0 ml: 100 mM Tris-HCl buffer, pH 8.2; 0.5 mM sodium 6-phosphogluconate (6PG);

Abbreviation: PCMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethylmaleimide; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid).

and 0.5 mM NADP. The reaction was initiated by the addition of 6PG and followed by the rate of NADPH₂ formation. The *K_m* for the substrate was measured under saturating conditions of the co-enzymes (0.5 mM). The *K_m* for the co-enzyme was measured under saturating conditions of the substrate (0.5 mM). Activities were calculated from the initial slopes and the unit of activity was defined as the amount of enzyme catalyzing the reduction of 1 μ mol of NADP/min. Specific activity was expressed as enzyme units per mg of protein. Protein was estimated by the method of Lowry *et al.* with bovine serum albumin as a standard.¹³⁾

Preparation of blue-Sepharose 4B. Blue-Sepharose was prepared by the method of Henys and DeMoor.¹⁴⁾ Sepharose 4B agarose gel (150 ml) was washed twice with distilled water and filtered on a Buchner funnel. After the last filtration, 2 g of Na₂CO₃ in 50 ml of water were combined with 1 g of Cibacron F3GA dye in 50 ml of water to give 100 ml of solution; to this was added the washed Sepharose 4B with mild agitation. The mixture was left at 43°C for 48 hr with occasional agitation. The gel was then washed extensively with water and stored in distilled water at 4°C until use.

Electrophoresis. Analytical polyacrylamide gel electrophoresis was done by the method of Davis¹⁵⁾ in a column of 7.5% polyacrylamide gel with pH 9 buffer system, with a current of 4 mA per column. Gels were stained with Amido Black 10B. To find bands with 6PGDH activity, gels were also stained with the following mixture: 0.5 mM 6PG, 0.5 mM NADP, 65 μ M phenazine methosulfate, 35 μ M nitroblue tetrazolium, and 50 mM Tris-HCl buffer, pH 8.2. Gels were immersed in this solution and incubated at 45°C in the dark. SDS polyacrylamide gel electrophoresis was done by the method of Laemmli.¹⁶⁾ Gels were stained with Coomassie Brilliant Blue. Cytochrome *c* monomer (12,400), dimer (24,800), trimer (37,200), tetramer (49,600), and hexamer (74,400) were used as standards.

Measurement of molecular weight by gel filtration. The molecular weight of 6PGDH was estimated by descending chromatography using a Sephadex G-150 column (1.8 $\times$ 114 cm), equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, at a flow rate of 4.5 ml per hr column was calibrated with the following proteins of known molecular weights: cytochrome *c* (12,500), chymotrypsinogen A (25,000), egg albumin (45,000), bovine serum albumin (67,000), and alcohol dehydrogenase (148,000).

Chemicals. 6-Phosphogluconate, NADP, glucose 6-phosphate, ATP, ADP, AMP, alcohol dehydrogenase, and molecular weight standards for SDS gel electrophoresis were purchased from Oriental Yeast Co. RUBP, ribulose 5-phosphate, cytochrome *c*, and chymotrypsinogen A were obtained from the Sigma Chemical Co. DEAE-cellulose was obtained from Serva. Hydroxy-

apatite was purchased from Seikagaku Kogyo Co. DEAE-Toyopearl was a product of Toyo Soda Co. All other chemicals were reagent grade.

RESULTS

Enzyme purification

All steps of the purification procedure were done at 4°C. Unless otherwise specified, Tris-HCl buffer was used. All the buffer solutions contained 1 mM EDTA and 0.05% 2-mercaptoethanol, and were adjusted to pH 7.5.

Step I

Extraction. About 80 g of wet algal cells were suspended in 5 volumes of 0.1 M buffer and ground in a mortar with 40 g of aluminum oxide powder. Extraction was completed by ultrasonic treatment (in a Tomy Seiko UR-200P for 5 min at full power). After centrifugation at 15,900 *g* 15 min, solid ammonium sulfate was added to the supernatant solution and the precipitates formed at 0.70 saturation were collected by centrifugation, dissolved in a minimal volume (45 ml) of 0.1 M buffer, and dialyzed against 10 mM buffer overnight.

Step II

DEAE-cellulose column chromatography. The dialyzed solution was then put on a DEAE-cellulose column (6 $\times$ 15 cm) equilibrated with 10 mM buffer containing 80 mM NaCl. The column was then washed with the same buffer until the absorbance at 280 nm was less than 0.1. The enzyme was eluted with 10 mM buffer containing 160 mM NaCl. Active fractions (1830 ml) were collected, and dialyzed against 50 mM buffer overnight.

Step III

Blue-Sepharose column chromatography. The dialyzed solution was then put on a Blue-Sepharose column (3 $\times$ 14 cm) equilibrated with 50 mM buffer. The column was then washed with the same buffer until the blue color derived from phycobilin-protein disappeared. The enzyme was eluted with 50 mM buffer containing 1 mM NADP. Active fractions (225 ml) were pooled and then promptly con-

TABLE I. PURIFICATION STEPS OF *Phormidium* 6-PHOSPHOGLUCONATE DEHYDROGENASE

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purity	Yield (%)
Crude	2534.0	115.5	0.046	1	100
DEAE-cellulose	1406.6	98.9	0.070	1.52	85.6
Blue gel	146.8	64.4	0.438	9.52	55.8
DEAE-Toyopearl	18.8	45.8	2.45	53.3	39.7
Hydroxyapatite	1.78	12.9	7.22	156.9	11.2
Sephadex G-150	0.13	5.1	38.0	826.9	4.4

centrated in a Toyo ultrafiltration cell (model UHP-62) equipped with a UK-10 membrane.

Step IV

DEAE-Toyopearl column chromatography.

The enzyme solution (25 ml) from step III was put on a DEAE-Toyopearl column (3×14 cm) equilibrated with 50 mM buffer containing 80 mM NaCl. The enzyme was eluted with 50 mM buffer containing 160 mM NaCl. Active fractions (280 ml) were collected and then concentrated as above. The enzyme solution was then dialyzed against 5 mM potassium phosphate buffer (K-phosphate), pH 7.5 overnight.

Step V

Hydroxyapatite column chromatography.

The dialyzed solution was put on a hydroxyapatite column (1.6×20 cm) equilibrated with 5 mM K-phosphate. The 6PGDH activity passed through the column unadsorbed. Active fractions were collected and then concentrated in Toyo ultrafiltration cell.

Step VI

Sephadex G-150 column chromatography.

The enzyme solution from step V was put on a Sephadex G-150 column (1.8×114 cm) equilibrated with 50 mM buffer. The active fraction (70 ml) was used as the purified enzyme preparation.

The results of a typical purification scheme are summarized in Table I. By this procedure, the enzyme was obtained in 4.4% yield with an overall purification of about 830-fold and a specific activity of 38.

FIG. 1. Polyacrylamide Gel Electrophoresis of the Purified 6-Phosphogluconate Dehydrogenase.

Electrophoresis was done using a 7.5% gel as described in MATERIALS AND METHODS. Protein (30 μ g) was stained with Amido Black.

FIG. 2. Molecular Weight Measurement by Gel Filtration.

A, alcohol dehydrogenase (148,000); B, bovine serum albumin (67,000); C, egg albumin (45,000); D, chymotrypsinogen A (25,000); E, cytochrome c (12,500).

Purified 6PGDH was homogeneous by polyacrylamide gel electrophoresis as shown in Fig. 1. The activity staining also showed a

FIG. 3. SDS-Polyacrylamide gel Electrophoresis and Molecular Weight Measurement of the Purified 6-Phosphogluconate Dehydrogenase.

Samples ($10 \mu\text{g}$) were put on a 12.5% acrylamide gel and electrophoresed as described under MATERIALS AND METHODS.

A, cytochrome *c* hexamer (74,400); B, cytochrome *c* tetramer (49,600); C, cytochrome *c* trimer (37,200); D, cytochrome *c* dimer (24,800); E, cytochrome *c* monomer (12,400).

single purple band at the same position as that of the protein band.

Molecular weight

The molecular weight of the enzyme was estimated as 104,000 by Sephadex G-150 gel filtration as shown in Fig. 2. SDS polyacrylamide gel electrophoresis gave a single protein band (Fig. 3A) corresponding to a molecular weight of approximately 52,000 as shown in Fig. 3B.

FIG. 4. Effects of pH on Activity and Stability of the Enzyme.

A: Effects of pH on the activity. The enzyme reaction was done at various pHs (7~9.6). Buffer used was 0.2 M Tris-HCl.

B: Effects of pH on the stability. The enzyme solution was kept at 50°C for 5 min in various buffers with pH ranging from 4 to 9.6, and the remaining activity was assayed. Buffers used were: acetate (pH 4~6), Tris-maleate (pH 5.6~7.8) and Tris-HCl (pH 7.8~9.6).

TABLE II. EFFECTS OF METAL IONS ON THE ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

The mixture of 0.05 ml of 10 mM NADP, 0.4 ml of 0.25 M Tris-HCl buffer (pH 8.2), 0.05 ml of the enzyme solution (15.3 m units), 0.35 ml of distilled water, and 0.1 ml of 10 mM salts was preincubated at 43°C for 10 min, and then to the mixture was added 0.05 ml of 10 mM 6-phosphogluconate. The reaction was assayed under the standard conditions.

Compounds	Conc. (mM)	Relative activity
CaCl_2	1.0	146.6
MnCl_2	1.0	142.1
MgCl_2	1.0	102.6
None	—	100.0
NH_4Cl	1.0	98.7
NiCl_2	1.0	68.1
BaCl_2	1.0	61.7
CoCl_2	1.0	47.7
CdCl_2	1.0	13.0
ZnCl_2	1.0	9.1
CuCl_2	1.0	6.0

Effects of pH on the enzyme activity and stability

The effects of pH on the enzyme activity are shown in Fig. 4. The enzyme showed a sharp pH optimum of 8 and was stable between pH 6 and 9.

TABLE III. EFFECTS OF SULFHYDRYL REAGENTS ON THE ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

Compounds	Conc. (mM)	Relative activity
None	—	100.0
PCMB	0.2	60.1
	0.1	83.5
HgCl ₂	0.5	0.0
	0.1	95.0
NEM	0.5	16.8
	0.1	98.2
ICH ₂ COOH	0.5	97.7
	0.1	100.1
DTNB	0.1	87.2

TABLE IV. EFFECTS OF METABOLITES ON THE ACTIVITY OF 6-PHOSPHOGLUCONATE DEHYDROGENASE

Compounds	Conc. (mM)	Relative activity
None		100.0
Glucose 6-P	1	100.0
Fructose 6-P	1	95.0
Fructose 1,6-P	1	93.6
Ribulose 5-P	1	69.1
Ribulose 1,5-P	1	85.6
ATP	1	92.6
ADP	1	93.4
AMP	1	105.0
NADPH ₂	0.1	78.2
	0.2	67.0

Effects of metal ions

The effects of metal ions (1.0 mM) on the enzyme activity were investigated. As shown in Table II, the enzyme was activated by Ca²⁺ and Mn²⁺ up to about 140~150%, but Zn²⁺, Cu²⁺, and Cd²⁺ strongly inhibited the activity.

K_m values

The K_m values for 6-phosphogluconate and NADP of the 6PGDH were 1.3×10^{-5} M and 3.6×10^{-5} M, respectively. No activity was observed when NAD was substituted for NADP.

Effects of sulfhydryl reagents

The effects of some reagents on the enzyme

FIG. 5. Effects of Temperature on the Enzyme Activity.

The enzyme sample, 0.05 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05% 2-mercaptoethanol was incubated at various temperatures for 10 min.

●—●, no addition; ○—○, +0.3 mM NADP; △—△, +0.3 mM 6-phosphogluconate.

activity are listed in Table III. The enzyme activity was inhibited by sulfhydryl reagents such as PCMB, NEM, DTNB, Hg²⁺ when these reagents were added in the reaction mixture, and the enzyme was protected from this inhibition by 2-mercaptoethanol.

Effects of metabolites

As shown in Table IV, the enzyme was inhibited by RUBP, ribulose 5-P, and NADPH₂, and also weakly inhibited by fructose 6-P, fructose 1,6-P, ATP, and ADP.

Thermal properties

When the enzyme was incubated at various temperatures for 10 min at pH 7.5, it was stable below 50°C and about 50% of the original activity was retained at 57°C (Fig.5). 6-Phosphogluconate and NADP gave only slight protection. On the other hand, the enzyme activity increased between 35°C to 50°C and the maximal activation (140%) was at 40°C. The enzyme activity was also increased up to 135% between 30°C to 50°C by the addition of 0.3 mM NADP.

Effects of urea

Figure 6 shows that the enzyme was stable in

FIG. 6. Influence of Urea on the Inactivation of the Enzyme.

The enzyme sample, 0.05 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05% 2-mercaptoethanol was incubated at 25°C in the presence of various concentration of urea and samples were removed at the indicated times for measurement of residual activity.

○—○, 1.0 M; □—□, 2.0 M; △—△, 4.0 M.

FIG. 7. Kinetics of Photoinactivation of the Enzyme.

The enzyme sample, 0.05 mg/ml in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05% 2-mercaptoethanol and 1 μ M Rose Bengal, was stirred and illuminated at 25°C with a 400-W lamp placed 15 cm above the surface of the solution (55,000 lux).

●—●, no addition; ○—○, 0.3 mM NADP; ▲—▲, 0.3 mM 6-phosphogluconate.

1 M urea, being inactivated at higher molarities with a half-denaturation time of 5 min in 4 M urea. The inactivation by urea was a first-order process.

Photooxidation

Exposure of the enzyme to low concentrations of Rose Bengal and light led to an irreversible loss of activity. As shown in Fig. 7, the addition of 6PG or NADP slightly protected the enzyme from this photo-inactivation and in all cases inactivation occurred as a

biphasic process.

DISCUSSION

6-PGDH of *Phormidium* sp. was purified about 830-fold. This is the first report of purification of the cyanobacterial 6PGDH to homogeneity. The molecular weight of the cyanobacterial 6PGDH was estimated to be 104,000 by gel filtration. The result of SDS polyacrylamide gel electrophoresis suggested that the enzyme consisted of two apparently identical subunits with a molecular weight of 52,000. The molecular weight of cyanobacterial enzyme is similar to those reported for the enzymes extracted from *Candida utilis* (101,000),¹⁾ *Streptococcus faecalis* (108,000),⁵⁾ *Neurospora crassa* (110,000)³⁾ and rat liver (112,000),²⁾ but somewhat different from that of the enzyme from sheep liver (94,000).⁴⁾ The enzymes, regardless of their sources, are dimers with subunits of equal molecular weight.

Cyanobacterial 6PGDH showed a sharp pH profile around pH 8, distinct from the enzymes from *Bacillus*⁷⁾ and *Streptococcus*⁵⁾ which have broad pH profiles around pH 7.5 to 8. 6PGDH from *Candida*¹⁾ and *Bacillus*⁷⁾ required Mg^{2+} ion for its full activity, while the cyanobacterial 6PGDH showed no Mg^{2+} requirement but was activated by Mn^{2+} and Ca^{2+} .

The apparent K_m values of cyanobacterial 6PGDH for NADP and 6-PG were of the same order of magnitude reported for the enzymes from other sources. Recently, Lubberding *et al.*¹⁷⁾ reported the results of a comparative study on the K_m for substrates and co-enzymes of the soluble enzymes G6PDH and 6PGDH in two closely related cyanobacteria, a mesophilic strain and a thermophilic strain. In the mesophilic strain the apparent K_m values of 6PGDH are 9.0×10^{-5} M (substrate) and 2.5×10^{-5} M (NADP) and in the thermophilic strain they are 4.0×10^{-5} M (substrate) and 1.0×10^{-5} M (NADP). They suggested that, in general, thermophilic enzymes possess lower affinities for their substrates and co-enzymes than do mesophilic

enzymes. Since the cyanobacterium *Phormidium* sp., which we used, shows an optimum growth temperature near 47°C, the result that the apparent *K_m* values of *Phormidium* 6PGDH for 6-phosphogluconate and NADP are 3.6×10^{-5} M and 1.3×10^{-5} M, respectively, supports the above view.

Phormidium 6PGDH was markedly sensitive to sulfhydryl reagents, suggesting that a sulfhydryl groups may be involved in the active site of the enzyme. The enzyme was also protected from heat inactivation and photo-oxidation in the presence of the substrate and co-substrate. Similar results were reported for the enzymes from *Bacillus*,⁷⁾ *E. coli*,⁶⁾ and *Neurospora*³⁾ but not for that from *Candida*.¹⁾

The inhibition of cyanobacterial G6PDH by NADPH₂ and ATP, and by RUBP, an intermediates of the Calvin cycle has been reported.^{9~11)} *Phormidium* 6PGDH was also inhibited by RUBP, ribulose 5-P, NADPH₂, and ATP, but in less degree than that of G6PDH.¹¹⁾ Similar inhibition of 6PGDH by the intermediates of the Calvin cycle was reported by Lubberding *et al.*¹⁷⁾ Although these intermediates can affect the activity of the G6PDH and the 6PGDH, and can individually regulate these enzymes to some extent, they cannot be responsible for the total deactivation of the enzyme activities observed in the light. It is likely that a substantial regulation of the oxidative pentose phosphate pathway during photosynthesis would require the concerted action of several regulating factors.

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