Characterization of three putative xylulose 5-phosphate/fructose 6-phosphate phosphoketolases in the cyanobacterium*Anabaena* sp. PCC 7120

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Xylulose 5-phosphate/fructose 6-phosphate phosphoketolase (Xfp) is a key enzyme in the central carbohydrate metabolism in heterofermentative bacteria, in which enzymatic property of Xfps is well characterized. This is not the case in other microbes. The cyanobacterium*Anabaena* sp. PCC 7120 possesses three putative genes encoding Xfp, *all1483*, *all2567*, and *alr1850*. We purified three putative Xfps as recombinant proteins. The results of gel filtration indicated that these proteins form homomultimer complex. *All1483* and *All2567* showed phosphoketolase activity, whereas *Alr1850* did not show the activity. Kinetic analyses demonstrated that substrates, fructose 6-phosphate and inorganic phosphate, are cooperatively bound to enzymes positively and negatively, respectively.

**Key words:** cooperative binding; cyanobacteria; phosphoketolase; Xfp

Xylulose 5-phosphate/fructose 6-phosphate (Xu5P/F6P) phosphoketolases (Xfps) are glycolysis-related enzymes, which catalyze the conversion of F6P and inorganic phosphate (Pi) to erythrose 4-phosphate (E4P), acetyl phosphate (AcP), and H₂O (EC 4.1.2.9). There are two types of enzymes with different preferences for substrates: namely, those having a preference for Xu5P/F6P and those having a comparable preference for both Xu5P and F6P. Xfps are key enzyme of bifid shunt of bifidobacteria natively lacking phosphofructokinase, which catalyzes F6P to fructose 1,6-bisphosphate (F1,6BP), and that of phosphoketolase pathway of heterofermentative and facultative hom fermentative lactic acid bacteria and xylose fermenting yeast.

Xfps require thiamine pyrophosphate (TPP) and divalent metal ions as cofactors in their activity, and the N-terminal part of the enzyme is responsible for TPP-binding. Recently, crystal structures of Xfps were reported in* Bifidobacterium breve* and *Bifidobacterium longum*. The authors indicated key amino acid residues for catalysis and substrate binding and also demonstrated that the minimum functional unit of Xfp is a homodimer. By gel filtration chromatography, homohexamer formation of Xfp was indicated in* Bifidobacterium lactis* and* B. breve*. Enzymatic kinetics of Xfp was determined in* Bifidobacterium* and* Lactobacillus*, and the *Kₘ* value for F6P is 5–26 mM, and for Xu5P is 4–45 mM, and for Pi is 3–8 mM. By detailed kinetic analyses, it was reported that the reaction mechanism of Xfp is a ping-pong bi–bi mechanism, in which Xu5P/F6P is the first substrate and E4P is released, and Pi is the second substrate and AcP is released.

Recently, comprehensive phylogenetic analysis of Xfp was performed by Sánchez et al., indicating that Xfps are mainly divided into three groups. Group 1 encompasses Xfps of fungi and bacteria including* Bifidobacterium*, *Lactobacillus*, and cyanobacteria. Groups 2 and 3 contain Xfps of proteobacteria and cyanobacteria, respectively. The authors also showed that the cyanobacterium* Anabaena* sp. PCC 7120 encodes three Xfp homologs: two Group 1 Xfps (*All1483* and *All2567*) and one Group 3 Xfp (*Alr1850*). However, enzymatic study of cyanobacterial Xfp proteins has not been reported.

In this study, we first purified three putative Xfps of* Anabaena* as recombinant proteins expressed in* Escherichia coli* and showed that two Group 1 Xfps have F6P phosphoketolase (F6PPK) and Xu5P phosphoketolase (Xu5PPK) activities. Dependency (for TPP and divalent metal ion) and kinetics (for F6P and phosphate) were examined in the two Xfps.

**Materials and methods**

Growth of the organism. *Anabaena* sp. PCC 7120 was grown photoautotrophically in the BG-11 medium buffered with 5 mM HEPES-NaOH (pH 7.5) at 32 °C under continuous illumination provided by fluorescent tubes at a fluence rate of 50 μmol m⁻² s⁻¹, with aeration by air containing 1% CO₂.

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Phylogenetic analysis. Sequence data of phospho- 
ketolase proteins were obtained from the CyanoClust 
database (cluster 940, version 2010-04, data-set Cyano- 
clust4; http://cyanoclust.c.u-tokyo.ac.jp/). Amino 
acid alignments were prepared by the MUSCLE soft-
ware version 3.6. Manipulation of sequence data was 
performed by the SISEQ software version 1.59. The 
alignments were used to infer phylogeny by the maxi-
mum-likelihood method using the TreeFinder software 
version March 2011 with the WAG model with the 
“Empirical” option.

Overexpression and purification of Anabaena Xfp 
proteins. A full-length sequence of three xfp homologs 
was amplified by PCR from the Anabaena sp. PCC 7120 
genome using the following primer pairs; for all1483,
InF_all1483-F (5′-CCTCTAGAGTCGACCTGCAG 
TA-CTATTTCTCCTTCTAAAGCC-3′) and InF_all1483-R 
(5′-ACCCTGTAAGTTAATGCAAGTGAAGGCCC- 
ACTTCCAGT-3′); for all2567, InF_all2567-F (5′-CCTCTAGAGTCGACCTGCAG 
CAGTACTGCACATCACAACCAGGC-3′) and InF_ 
all2567-R (5′-ACCCTGTAAGTTAATGCAAGTGAAG 
GCCCCTTCCAC-3′); for alr1850; InF_alr1850-F (5′-CCTCTAGAGTCGACCTGCAG 
TACTGCAATCACACCCAAGGC-3′) and InF_ 
alr1850-R (5′-ACCCTGTAAGTTAATGCAAGTGAAG 
GCCCCTTCCAC-3′). The underlined sequences 
correspond to the ends of PstI-digested pThioHisA vec-
tor (Invitrogen, Carlsbad, CA, USA) and are required 
for the in-fusion cloning reaction (Clontech Laboratories, 
Mountain View, CA, USA). The PCR product was 
cloned into the linearized pThioHisA vector digested 
with PstI by in-fusion reaction. These constructs were

![Fig. 1. Phylogenetic trees of Xfps in cyanobacteria.](image)

Notes: Xfps are classified into three groups by Sánchez et al. Cyanobacteria contain Group 1 and/or 3 Xfp homologs. Bold shows Xfp homolo-
gs of Anabaena sp. PCC 7120. Sequences were obtained from Cluster 940, which contains both Groups 1 and 3 Xfp homologs, of the data-set 
Cyanoclust4 in the CyanoClust database. Groups 1 and 3 Xfp were individually calculated. The numbers on each branch indicate bootstrap value 
obtained by NJ analysis, and values are shown in percentile.
transformed into *E. coli* TOP10 cells (Invitrogen), and the cells were grown in 500 mL of LB medium containing 100 μg mL⁻¹ ampicillin at 37 °C. When the OD₅₆₀ of the cultures became 0.5, isopropyl-β-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and the cells were cultured overnight at 22 °C. The cells were harvested by centrifugation (2600 × g for 10 min) and resuspended in 20 mL of binding buffer (20 mM Na₂HPO₄, pH 7.4, 500 mM NaCl, 20 mM imidazole, 2 mg mL⁻¹ lysozyme). For purification of Alr1850, binding buffer without imidazole was used. After disruption of the cells by sonication, the cleared lysate was adsorbed onto a HisTrap HP column (a Ni²⁺-chelating resin; GE Healthcare Bio-Science, Piscataway, NJ, USA). Thioredoxin-fusion proteins were eluted with a linear gradient of 20–500 mM imidazole. The eluted proteins were desalted by PD MiniTrap G-25 column (GE Healthcare Bio-Science) equilibrated in XfP buffer (20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol). Glycerol was added to the protein solution to a final concentration of 50%, which was then stored at −80 °C until use.

**Gel filtration chromatography.** Purified protein solution was applied to Superdex 200HR 10/30 column (GE Healthcare Bio-Science) equilibrated in gel filtration buffer (50 mM HEPES [pH 7.5], 200 mM NaCl) at a flow rate of 0.4 mL min⁻¹. A standard of molecular size, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa), which were purchased from GE Healthcare Bio-Science, were used.

**Measurement of phosphoketolase activity.** Phosphoketolase reaction was measured spectrophotometrically as ferric acetyl hydroxamate produced from the enzymatically generated AcP by the procedure of Racker and Meile et al., which are predicted to encode polypeptides of 810, 793, and 737 amino acids (aa), corresponding to molecular masses of 91, 90, and 79 kDa, respectively. According to phylogenetic analysis by Sánchez et al., All1483 and All2567 belong to Group 1 Xfp and Alr1850 belongs to Group 3. The grouping was confirmed by our phylogenetic analysis using sequence data obtained from Cyanoblast database (Fig. 1). Groups 1 and 3 Xfps are conserved in many cyanobacteria, but not in the all species of *Prochlorococcus marinus* and half of *Synecochoccus* species (Table S1). Based on results of crystal structure analysis in Group 1 Xfp proteins, key amino acid residues for catalysis and substrate binding were suggested. Nearly, all key residues are conserved in *Anabaena* Group 1 Xfp, All1483, and All2567 (Fig. S1). Group 3 Xfp Alr1850 has low sequence similarity with Group 1 protein. However, some of the key residues are conserved also in Alr1850. To characterize enzymatic property, three Xfp proteins were expressed as

**Fig. 2.** Purification of recombinant Xfps. Full length of All1483, All2567, and Alr1850 expressed as thioredoxin (Trx) fusion protein. Notes: Trx was fused to N-terminus of each protein. The fusion proteins were purified by Ni²⁺-chelating column. The SDS–PAGE was performed using a 4–20% polyacrylamide gradient gel and stained with Coomassie Brilliant Blue. Aliquots (5 μg each) of purified protein were electrophoresed. An arrowhead indicates Trx-Xfp proteins.
recombinant proteins fused with thioredoxin (Trx) in _E. coli_ cells and purified with a Ni$^{2+}$ chelating column (Fig. 2). Nominal molecular masses of Trx-All1483, All25674, and Alr1850 are calculated to be 103, 102, and 91 kDa, which were consistent with the results of SDS–PAGE.

**Gel filtration chromatography of three recombinant Xfp homolog proteins**

The molecular mass of native form of recombinant Xfp proteins was estimated by gel filtration chromatography (Fig. 3(A), (C) and (E)), and peak fractions were identified by SDS–PAGE (Fig. 3(B), (D) and (F)). A

![Gel filtration chromatogram](image)

Fig. 3. Gel filtration chromatography of three Xfps.

Notes: Purified protein solution was applied to Superdex 200HR 10/30 column (GE Healthcare Bio-Science) at a flow rate of 0.4 mL min$^{-1}$. The results of gel filtration chromatography and SDS–PAGE staining with Coomassie Brilliant Blue of All1483 (A, B), All2567 (C, D), and Alr1850 (E, F) were shown. Arrows in A, C, and E show detection time and protein size in parentheses that were calculated from the result of standard proteins shown in Fig. S2. Arrowheads in (B, D and F) show Trx-Xfp proteins.
plot of elution time of standard proteins is shown in Fig. S2. The Trx-All1483 protein (103 kDa) was eluted as a 1740 kDa complex. Since it was reported that the minimum functional unit of Xfp is a homodimer from the results of crystal structure analysis,8,9) Trx-All1483 appears to form 16- or 18-mer. In the assay of Trx-All1483, peaks of a 146 and a 316 kDa proteins were also detected (Fig. 3(A)). Because these fractions also contained an 80 kDa protein (Fig. 3(B)), which might be contaminants from E. coli proteins or a partial protein of Trx-All1483, it was unclear that Trx-All1483 forms a monomer (146 kDa) and a dimer (316 kDa). The Trx-All2567 protein (102 kDa) was eluted as a 115 and a 838 kDa proteins, corresponding to the estimated size of a monomer and an octamer, respectively. The Trx-Alr1850 protein (91 kDa) was eluted as a 216 kDa protein, corresponding to the estimated size of a dimer.

Measurement of phosphoketolase activity
Since no study on cyanobacterial Xfp and Group 3 Xfp was reported, whether three purified proteins harbor Xu5PPK and/or F6PPK activity was examined by spectrophotometric assay. In this assay, one of the product AcP is converted to ferric acetyl hydroxamate tinged with red–purple after the phosphoketolase reaction, and absorbance at 505 nm was measured by a spectrophotometer. Fig. 4 shows the results of measurement of F6PPK activity in the three recombinant proteins. All1483 and All2567 showed the activity, namely, increase of amount of reaction product AcP was dependent on protein amount and reaction time. Alr1850 did not show such activity, even if large amount of protein was added to the reaction mixture.

Xu5PPK activity was also examined. Because commercial availability of the substrate Xu5P was extremely limited, the reaction was performed in a small scale (5 μL) at high concentration of Xu5P (25 mM). In this volume, we could not measure the absorbance of 505 nm by a spectrophotometer, and images of chromogenic reaction are shown in Fig. S3. The reaction solutions of All1483 and All2567 turned to red–purple in the mixture reacted for 120 min, whereas that of Alr1850 did not show changes in color. These results indicated that All1483 and All2567 can use both Xu5P and F6P as the substrate, and Alr1850 has neither activity of F6PPK nor Xu5PPK. In the following analysis, enzyme activity of All1483 and All2567 was examined using F6P as a substrate.

Effect of TPP and divalent metal ions
Xfp proteins are known as a TPP-dependent enzyme. By the BLAST search, the three cyanobacterial Xfp homologs were found to contain TPP-binding domain at the N-terminus. The effect of TPP was evaluated (Table 1). Comparing to the activity without TPP, in a reaction in 1 mM TPP, F6PPK activity was increased to 168 and 644% in All1483 and All2567, respectively. Because the activity could be detected even in the absence of added TPP, purified proteins are likely to contain TPP originated from E. coli cells. It is also known that TPP-dependent enzymes including Xfp proteins depend on the presence of a divalent metal ion.7) Effects of divalent metal ions, Mg2+, Mn2+, and Ca2+, were examined (Table 2). The F6PPK activity of All1483 was slightly increased by Mg2+ and Ca2+ and remained unchanged by Mn2+. In All2567, no dependency was observed to any divalent metal ions, and the activity was slightly inhibited by the addition of Mn2+ or Ca2+.
Kinetics for F6P and inorganic phosphate in the phosphoketolase activity of All1483 and All2567

Fig. 5 shows Michaelis–Menten plots for F6P (Fig. 5(A)) and Pi (Fig. 5(B)) in All1483 and All2567. Reaction buffers of kinetics analysis for F6P and Pi contain 25 mM Pi and 25 mM F6P, respectively. The kinetic plot for F6P was sigmoidal in both proteins. Curve fitting was performed assuming the Hill equation. 

$K_m$ values for F6P were $7.8 \pm 0.1$ mM in All1483 and $14.8 \pm 1.2$ mM in All2567. Hill coefficient ($n$) values were $3.1 \pm 0.1$ and $1.9 \pm 0.2$ in All1483 and All2567, respectively. Namely, F6P showed positive cooperativity in the binding with both Xfps.

In the kinetics for Pi, curve fitting was also performed with the Hill equation. Although the plots for Pi seem to obey the Michaelis–Menten equation (Fig. 5(B)), coefficient of determination ($R^2$) with Hill equation ($R^2 = 0.999$ and 0.994 in All1483 and All2567, respectively) was higher than that with Michaelis–Menten equation ($R^2 = 0.986$ and 0.963 in All1483 and All2567, respectively). $K_m$ values for Pi were $10.9 \pm 1.6$ mM in All1483 and $2.6 \pm 0.9$ mM in All2567. $n$ values were $0.7 \pm 0.0$ and $0.6 \pm 0.0$ in All1483 and All2567, respectively, suggesting that Pi was bound with two Xfps with a negative cooperativity.

Discussion

Here, we demonstrated that at least two cyanobacterial Xfp homologs had phosphoketolase activity. All previous studies on Xfp were performed in Group 1 Xfps, and there was no report of enzyme activity of Group 3 Xfps. In this study, enzymes showing Xu5PPK and F6PPK activities were Group 1 enzymes, and we did not find evidence for activity of Group 3 Xfp (Alr1850). In the data base for searching homolog proteins, CyanobClust, Groups 1 and 3 proteins were in the same cluster. However, these groups were clearly divided into different clades in reported phylogenetic analysis. In accordance with the phylogenetic analysis, alignment of Xfp proteins belonging to Group 1 and 3 showed low similarity (Fig. S1) and showed that a part of key amino acids for TPP or substrate binding are not conserved in Group 3 proteins, which were indicated by crystal structure analysis of Group 1 Xfps. In the BLAST search, Alr1850, as well as All1483 and All2567, contains a TPP-binding domain at the $N$-terminus. These findings suggest that Alr1850 appears to be TPP-dependent enzyme and might catalyze some reaction with metabolic intermediates other than Xu5P and F6P. As another possibility, we could not purify the Alr1850 protein with in a native state, although the protein was purified as a soluble protein.

Reported $K_m$ values of Xfps are 5–26 mM for F6P and 3–8 mM for Pi in *Bifidobacterium* and *Lactobacillus*. The values of two cyanobacterial Xfps are consistent with the previous values. However, in All1483 and All2567, kinetic plots for F6P and Pi showed a sigmoidal curve with positive and negative cooperativities, respectively. Recently, similar curves were also reported in Xfp2 protein of a fungus *Cryptococcus neoformans*, in which the Hill coefficient values were $1.4$ and $0.6$ for F6P and Pi, respectively. Cooperative binding to substrate has not been reported in other bacterial Xfps.
The filamentous cyanobacterium *Anabaena* sp. PCC 7120 can fix dinitrogen. Under nitrogen-depleted conditions, vegetative cells of the filament differentiate into nitrogen-fixing cells called heterocysts with a spacing of about 10 cells,21 where O₂-evolving photosystem II is inactivated and respiration rate is increased. Fig. S4 shows the microarray data adopted from the data by Kushige et al.20) The cells of *Anabaena* sp. PCC 7120 were cultured in continuous light for circadian analysis after light/dark cycles. The transcript level of all2567 oscillated with circadian rhythm; namely, the expression was decreased during the subjective light, but increased during the subjective dark, especially under the condition of nitrogen depletion. On the other hand, the levels of all1483 and allr1850 remained unchanged during the experiment. The expression level of all2567 was considerably higher than that of all1483 and allr1850 in the peaks, suggesting that All2567 protein could account for the most part of Xfp activity in *Anabaena* sp. PCC 7120. Mechanism of regulation was reported for the expression of all2567 under nitrogen-depleted conditions.21,22) all2567 is expressed as part of the xfp operon (xfp [all2567]-gap1-pyk1-talB) under the control of the transcription factor NrnA, which also drives the expression of *glpP*1, leading to activation of glycolytic catabolism. In *Cyanothecae* spp. ATCC 51142, which encodes two Group 1 Xfps and one Group 3 Xfp homologs (Fig. 1; Table S1), under the condition of nitrogen depletion, it is estimated that 90% of intracellular acetyl-CoA is produced through phosphoketolase pathway.22,23) From these reports, it is considered that Xfp functions in efficient supply of energy in the nitrogen fixation under the non-photosynthetic condition. AcP is one of the product of phosphoketolase reaction, and ATP can instantly be produced from AcP by the reaction of acetate kinase (All2561), which catalyzes AcP and ADP into acetate and ATP. Because Xfp catalyzes the conversion of Xu5P/F6P into GAP/E4P, all of which are intermediates in glycolysis including pentose phosphate pathway and Calvin–Benson cycle, the existence of Xfp might increase plasticity and complexity in these metabolic pathways in photosynthetic organisms. Although expression and metabolome analyses suggest some function of Xfp in cyanobacteria, actual role of Xfp in vivo remains unclear. Mutant analysis on xfp genes will provide clues for the problem.

**Supplemental material**

The supplemental material for this paper is available at [http://dx.doi.org/10.1080/09168451.2014.993357](http://dx.doi.org/10.1080/09168451.2014.993357).

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