RESEARCH LETTER

Characterization of two glutaminases from the filamentous cyanobacterium *Anabaena* sp. PCC 7120

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Abstract

The *Anabaena* genome contains two ORFs that appear to encode glutaminases. The genes were expressed as histidine-tagged fusion proteins in *Escherichia coli*. The purified proteins possessed glutaminase activity using L-glutamine as the substrate, but differed in biochemical properties. All2934 showed an optimal activity at 20 °C and pH 6.0, with a higher affinity for L-glutamine than All4774, which had optimal activity at 37 °C and pH 7.5. Remarkably, the glutaminase activity of All2934 was phosphate dependent, while All4774 was phosphate independent. The expression of *all2934* and *all4774* was analyzed using semi-quantitative reverse transcriptase-PCR. The expression level of *all2934* was much higher than that of *all4774* under normal and nitrogen-depletion conditions, indicating that All2934 may play an important role in metabolizing glutamine in *Anabaena*.

Introduction

Glutamine plays a crucial role in cellular metabolism as a nitrogen donor in the biosynthesis of many metabolic intermediates. Glutaminase (EC 3.5.1.2) is the major enzyme in glutamine degradation, catalyzing the hydrolytic deamidation of L-glutamine to L-glutamate and ammonia (Krebs, 1935; Nandakumar *et al*., 2003). Glutaminase has been intensively investigated in several bacterial genera and in mammals. In bacteria, two forms of glutaminases have been reported in *Escherichia coli*. These were distinguished as glutaminase A (Prusiner, 1973) and B (Prusiner *et al*., 1976), mainly based on their pH optima. In *Rhizobium etli*, two glutaminases were also identified, with different thermostabilities and electrophoretic mobilities (Duran *et al*., 1996). The three-dimensional structures of glutaminases from *Pseudomonas* 7A (Lubkowski *et al*., 1994a) and *Acinetobacter glutaminasificans* have been determined (Ammon *et al*., 1988). Recently, salt-tolerant glutaminases with potential applications in the food industry were isolated from *Aspergillus oryzae* RIB40 and *Stenotrophomonas maltophilia* NYW-81 (Masuo *et al*., 2004; Wakayama *et al*., 2005). In mammals, two isoforms of glutaminase, liver type and kidney type, have been identified, each having distinct kinetic, immunological and genetic characteristics (Kovacevic & McGivan, 1983; Márquez *et al*., 2006). The mammalian glutaminases play a key role in several physiological processes such as renal ammoniagenesis, hepatic ureagenesis and synthesis of the neurotransmitter glutamate in the brain (Curthoys & Watford, 1995; Márquez *et al*., 2006). Although the glutaminase reaction is the same in mammalian and microbial glutaminases, they have different biochemical and functional characteristics. Most remarkably, all mammalian glutaminases have been found to be activated by phosphate (Kvamme *et al*., 2000).

Although glutaminases are well characterized in heterotrophs, little is known regarding the genetic and biochemical properties of the enzyme in autophototrophs. Cyanobacteria are the only prokaryotes that perform plant-like photosynthesis. *Anabaena* sp. PCC 7120 is a filamentous cyanobacterium capable of nitrogen fixation in addition to

photosynthesis. *Anabaena* is therefore used as a valuable model for studying the genetics and physiology of cell differentiation, as well as nitrogen-sensing in cyanobacteria (Zhang *et al.*, 2006). The key enzymes required for ammonium assimilation, glutamine synthetase (GS) and glutamate synthase (GOGAT), have been well documented in cyanobacteria (Muro-Pastor *et al.*, 2005). The roles of the glutaminases in these pathways have not yet been established, however, despite a report on glutaminase activity in cyanobacterium *Anabaena* sp. strain 1F (Chen *et al.*, 1987). In the genome of *Anabaena* sp. PCC 7120, two ORFs, *all2934* and *all4774*, were identified encoding putative glutaminases (http://www.kazusa.or.jp/cyano). To verify whether the gene products are catalytically active, we examined the biochemical properties of these putative glutaminases using recombinant proteins. We also used semi-quantitative reverse transcriptase (RT)-PCR to examine the expression profiles of these two genes in response to nitrogen starvation. Molecular insights into the causes of gene expression should lead to a clearer understanding of their physiological roles in *Anabaena*.

Materials and methods

Reagents

Restriction enzymes BamHI, HindIII and PstI, and the T4 DNA ligase were purchased from New England Biolabs. rTaq DNA polymerase and the corresponding buffer were supplied by Takara Bio Inc. Isopropyl-β-D-thiogalactopyranoside (IPTG), NAD^+, ADP, kanamycin and ampicillin were obtained from Amersham Biosciences. Nickel–nitrilotriacetic acid slurry was purchased from Qiagen, Germany. Bovine liver glutamate dehydrogenase was obtained from Sigma. All other chemicals and reagents used in this study were of analytical grade.

Strains and culture conditions

The *E. coli* strain DH5α was used for cloning the constructed expression vectors and maintaining the plasmids. Strain M15 [pREP4], which was maintained in Luria–Bertani (LB) supplemented with 50 mg L^{-1} kanamycin, was used as the expression host. *Anabaena* sp. PCC 7120 cells were grown photoautrophically on a rotary shaker at 30 °C under 60 μmol m^{-2} s^{-1} of white light in BG-11 medium (Allen, 1968). For nitrogen starvation experiments, *Anabaena* sp. PCC 7120 cells growing exponentially in BG11 medium were collected by centrifugation, washed three times with $BG11_0$ (Rippka *et al.*, 1979) and then resuspended in this medium and incubated further according to Baier (Baier *et al.*, 2004).

Gene cloning and plasmid construction

Genomic DNA was extracted from the mid-log phase growing cultures of *Anabaena* sp. PCC 7120 as described previously (Cai & Wolk, 1990). Full-length *all2934* and *all4774* ORFs (1002 and 924 bp) were amplified from genomic DNA using PCR. The PCR primers contained restriction enzyme sites for the purpose of cloning and had the following sequences: for *all2934*, 5′-GGATCCATGAGCG ATCAAGCCAATCAAG-3′ (forward) with a BamHI restriction site (underlined) and 5′-AAGCTTTCAACACTCACAA TTTCCCCATTC-3′ (reverse) with a HindIII restriction site (underlined); for *all4774*, 5′-GGATCCGTGAAGGGATTTG ATAGACTAG-3′ (forward) with a BamHI restriction site (underlined), and 5′-CTGCAGCTAAAGGTGGGAAAATCT CCC-3′ (reverse) with a PstI restriction site (underlined). Thermocycling conditions were 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min 20 s at 72 °C, and an additional reaction extension for 10 min at 72 °C. The PCR product was cloned directly into T/A cloning vector pBS-T (Tiangen Biotech Co. Ltd, Beijing, China), which was then transformed into competent *E. coli* DH5α cells. Positive clones containing the recombinant plasmids were selected and sequenced to ensure the authenticity of the ORFs. The plasmid containing *all2934* was digested with BamHI and HindIII. The fragment was purified and subcloned into a similarly treated vector pQE-30 (Qiagen) to generate pQE-all2934. For *all4774*, the ORF was subcloned into BamHI/PstI-digested pQE-30, yielding pQE-all4774.

Overexpression and purification of recombinant proteins

The constructed expression plasmids (pQE-all2934 and pQE-all4774) were transformed into *E. coli* M15 [pREP4]. The cells were grown in LB medium at 37 °C containing 100 mg L^{-1} ampicillin and 50 mg L^{-1} kanamycin. When the culture reached an $A_{600\,nm}$ of 0.6–0.8, 300 μM IPTG was added and incubated for an additional 3 h at 30 °C. Following induction, cells were harvested by centrifugation at 7000 ***g*** for 15 min at 4 °C and washed once with 50 mM Tris-HCl and 1 mM EDTA (pH 8.5). The recombinant proteins were purified using Ni^{2+}-affinity chromatography at 4 °C as described previously (Cann *et al.*, 2003; Kenny *et al.*, 2003). The proteins collected from the column were dialyzed overnight against 50 mM Tris-acetate (pH 8.0)/1 mM dithiothreitol buffer. The purified proteins were stored at −20 °C in a dialysis buffer containing 20% glycerol until use. The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The homogeneity of purified proteins was confirmed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Glutaminase activity assay and kinetic analysis

Assays were performed by determining the formation of L-glutamate as described previously (Curthoys & Weiss, 1974; Kenny *et al.*, 2003) with the following modifications. Briefly, an initial reaction mixture for recombinant All2934 contained 20 mM L-glutamine, 60 mM K_3PO_4, 0.2 mM EDTA, 50 mM Bis-Tris (pH 6.0) and the diluted protein in a final volume of 100 μL. The mixture was incubated for 10 min at 20 °C. For All4774 recombinant protein, the initial reaction contained 40 mM L-glutamine, 0.2 mM EDTA, 50 mM HEPES (pH 7.5) and the diluted protein in a final volume of 100 μL. The mixture was incubated for 10 min at 37 °C. The concentration of the recombinant protein in each reaction was adjusted so that the conversion of the substrate was $< 10\%$. The reaction was stopped by adding 0.3 N HCl to inactivate the glutaminases, followed by addition of 1 mL of a second reaction mixture containing 80 mM Tris-HCl (pH 9.4), 0.03% (v/v) hydrogen peroxide, 0.25 mM ADP, 2 mM NAD and 18 U purified bovine liver glutamate dehydrogenase (Sigma). The mixture was then incubated for 40 min at 25 °C. After incubation, the sample was placed on ice. The absorbance at $A_{340\,nm}$ was determined vs. a blank in which the HCl was added before adding the recombinant proteins. One unit of glutaminase was defined as the amount of enzyme that catalyzes the formation of 1 μmol of L-glutamate per minute (Wakayama *et al.*, 2005). To obtain the substrate and activator saturation profiles, we varied the glutamine concentration from 5 to 40 mM (for All2934) and 10 to 130 mM (for All4774), and the phosphate concentration from 0 to 150 mM, respectively. To determine whether the two recombinant glutaminases possess glutaminase–asparaginase activity, the formation of NH_3 was determined using Nessler's reagent (Peterson & Ciegler, 1969) with L-asparagine as the substrate. The kinetic parameters of the recombinant All2934 and All4774 were generated according to the curve fitting and nonlinear regression analysis using GRAPHPAD PRISM software (Motulsky & Brown, 2006).

Total RNA isolation and RT-PCR

Total RNA was isolated from *Anabaena* sp. PCC 7120 cells using Redzol reagent and SiMaxTM membrane spin columns (SBS Genetech, China). Residual DNA in RNA preparations was eliminated by digestion with RNAse-free DNAse (Qiagen). Reverse transcription reactions using random primers were performed with M-MLV RT (Takara). To detect possible DNA contamination, control reactions were performed without RT but with Taq DNA polymerase. Reverse transcription products were amplified by PCR and analyzed by electrophoresis on 1.0% (w/v) agarose gels. The *all2934* transcript was amplified using the forward primer 5′-CTCAATCGGGTACTAGAAATGTT-3′ and the reverse primer 5′-CCATTCTCCATTTCCCACTTTCATA-3′. The *all4774* transcript was amplified using the forward primer 5′-GATGAATCTATGCTGGCTTCTGTC-3′ and the reverse primer 5′-CCACACAGACCTAGAGAACGGGATA-3′. The *rnp B* was used as a positive control (Latifi *et al.*, 2005). The log phase of RT-PCR was determined by measuring the amounts of PCR products at different PCR cycles.

Results

Sequence analysis

Two ORFs were found in the sequence of the *Anabaena* sp. PCC 7120 genome, *all2934* and *al4774*, that appeared to encode glutaminases (http://www.kazusa.or.jp/cyano). Sequence comparison using CLUSTAL W (Thompson *et al.*, 1994) revealed that both All2934 and All4774 exhibited significant similarity to glutaminases from mammals as well as microorganisms (Fig. 1). All2934 was 40.0% identical to glutaminase A from *E. coli* and shared 39.5% identity with that of *R. etli*, 38.0% with *Bacillus subtilis* and 36.0% with *E. coli* glutaminase B, respectively. All4774 shared 38.4% identity with Slr2079, a glutaminase from *Synechocystis* sp. PCC 6803, and 30% and 32% identities with *E. coli* glutaminase A or B, and *R. etli*, respectively (Fig. 1). All2934 and All4774 were only 28.9% identical to each other, however, suggesting the possible diverse properties of the enzymes. Furthermore, both All2934 and All4774 shared $< 23\%$ identity with mammalian glutaminases (Fig. 1), indicating a closer relationship with glutaminases from bacteria than with those from mammals.

Heterologous expression and purification of All2934 and All4774 recombinant proteins

In order to determine the enzymatic function of the recombinant proteins, the coding sequences of *all2934* and *all4774* were cloned into the expression vectors pQE-30 with a His-tag sequence at the N-terminus, yielding pQE-all2934 and pQE-all4774, respectively. Expression of recombinant proteins was under the control of the T5 promoter containing the *lac* operator. The constructs were transformed into *E. coli* M15 [pREP4]. The initial analysis of crude protein extracts from induced cells showed that the bacteria containing plasmids pQE-all2934 or pQE-all4774 produced a substantial amount of the expected recombinant proteins. These proteins were purified from crude extracts to homogeneity with Ni-NTA agarose chromatography. The purified proteins showed a single protein band on SDS-PAGE (Fig. 2) with an estimated molecular mass of 36 kDa for All2934 (Fig. 2a) and 33 kDa for All4774 (Fig. 2b), which correspond to the molecular masses calculated from their coding sequences.

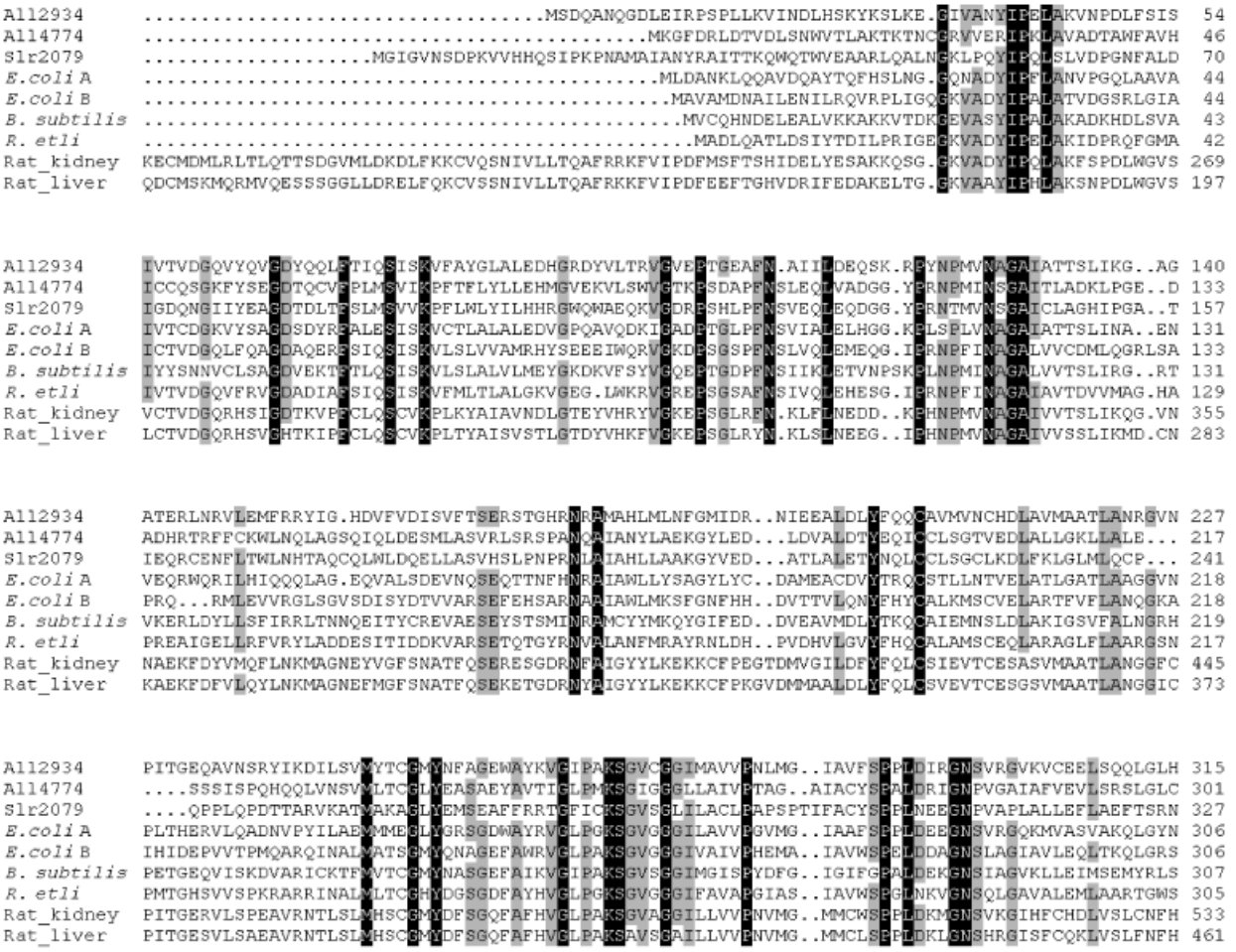

Fig. 1. Sequence alignment of All2934 and All4774 with selected glutaminases. Amino acid sequences are: Slr2079 from *Synechocystis* sp. PCC6803 (accession no. NP441287); *Escherichia coli* glutaminase A (accession no. YP001461674), *E. coli* glutaminase B (accession no. ZP01701382), *Rhizobium etli* glutaminase (accession no. YP469818), *Bacillus subtilis* glutaminase (accession no. NP389366), rat liver glutaminase (accession no. P28492) and rat kidney glutaminase (accession no. P13264). Identical residues are highlighted in black. Residues conserved above 80% are shown in gray. Gaps introduced to optimize sequence alignment are indicated by dots. The sequences were aligned in MEGAV3.1 software using CLUSTAL W.

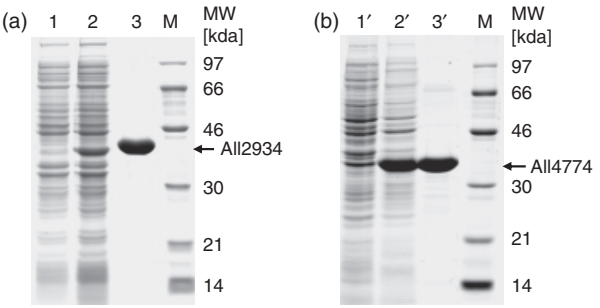

Fig. 2. SDS-PAGE analysis of the purified proteins from *Escherichia coli*. His-tag fused proteins All2934 (a) and All4774 (b) were purified from transformed *E. coli* and analyzed by 12% SDS-PAGE and stained with Coomassie brilliant blue. Lanes 1 and 1′, total protein without IPTG induction (10 μg). Lanes 2 and 2′, total protein with IPTG induction (10 μg). Lane 3 and 3′, recombinant All2934 and All4774 purified using Ni-NTA column (10 μg), respectively. M, protein molecular mass marker.

Functional analysis of All2934 and All4774 recombinant proteins

To determine the substrate specificity of the deduced glutaminases, we measured the enzyme activity using L-glutamine and L-asparagine as substrates. The results showed that both recombinant proteins possessed glutaminase activity on using L-glutamine as the substrate. Neither, however, exhibited glutaminase–asparaginase activity on using L-asparagine as the substrate (data not shown). This indicated that both recombinant proteins were highly specific for glutamine, and catalyzed the hydrolysis of glutamine to glutamic acid (Nandakumar *et al*., 2003). Only L-glutamine was used as the substrate in the subsequent enzyme reactions. To examine the optimum temperature for the enzymatic activity of these proteins, we performed a temperature gradient experiment within the range of

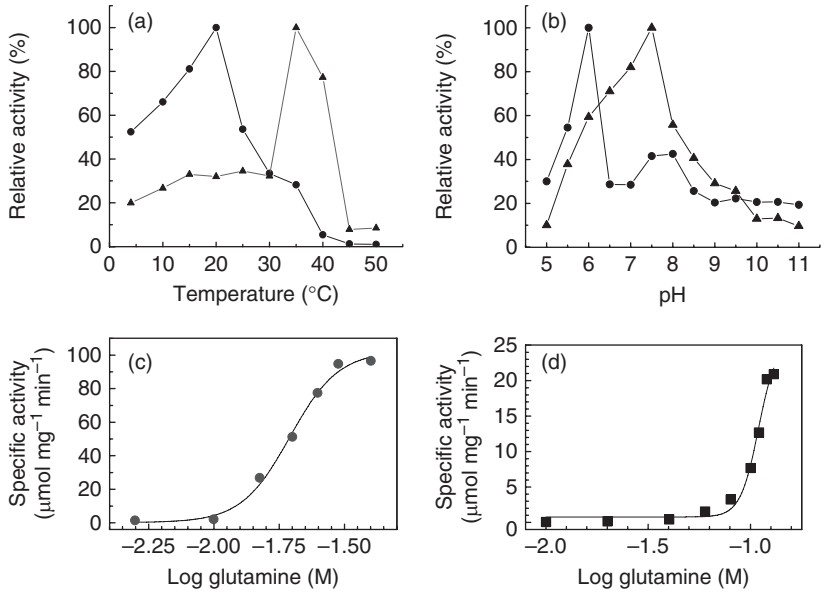

Fig. 3. Analysis of enzymatic properties of recombinant All2934 and All4774. (a) Effects of temperature on glutaminase activity. The optimal temperature of the recombinant All2934 and All4774 was determined using the standard activity assay at various temperatures from 4 to 50 °C. The relative activity of recombinant All2934 (●-●) and All4774 (▲-▲) was normalized to the maximal value obtained at 20 and 37 °C, respectively. (b) Effects of pH on glutaminase activity. The optimal pH of All2934 and All4774 was determined using the standard activity assay from pH 5 to 11. The relative activity of recombinant All2934 (●-●) and All4774 (▲-▲) was normalized to the maximal value obtained at pH 6 and pH 7.5, respectively. (c) and (d) Effect of substrate concentration on glutaminase activity of recombinant All2934 and All4774. Specific activity was calculated as the amount of the substrate converted by the recombinant proteins. All values represent the mean of three independent experiments.

4–50 °C. As shown in Fig. 3, the optimum temperature for the recombinant All2934 was 20 °C. A dramatic decline occurred as the temperature increased (Fig. 3a), with $<$ 6% of activity detected at 40 °C. In contrast, the optimum temperature for the recombinant All4774 was 37 °C. At 30 °C, only about 30% of the activity was retained, whereas as much as 70% of enzyme activity was detected at 40 °C (Fig. 3a). To determine the pH optimum for the enzymatic activity of these proteins, we varied the pH from 5 to 11. The maximum glutaminase activity for the recombinant All2934 was detected at pH 6.0, while the maximum activity for the recombinant All4774 was detected at pH 7.5 (Fig. 3b). To determine the affinity of the two recombinant glutaminases for L-glutamine, kinetics analysis was performed by gradually increasing the substrate concentration from 5 to 40 mM for All2934 (Fig. 3c) and from 10 to 130 mM for All4774 (Fig. 3d). The kinetic parameters of the All2934 and All4774 were generated using the curve-fitting and nonlinear regression analysis tools of the GRAPHPAD PRISM software (Table 1). Neither All2934 nor All4774 showed Michaelis–Menten enzyme kinetics, as their Hill slopes were above 1. The K_m is therefore given as K_m apparent (app), a measure of the affinity for the substrate. As shown in Table 1, the K_m app values of the recombinant All2934 and All4774 for glutamine were 19.5 ± 1.2 and 109.5 ± 10.1 mM, respectively. The V_{max} app of All2934 was 102.3 ± 2.7 μmol mg^{-1} min^{-1}, which was fourfold higher than that of All4774 at 25.71 ± 2.26 μmol mg^{-1} min^{-1}. These observations indicate that the affinity of All2934 for L-glutamine was significantly higher than that of All4774.

Table 1. Kinetic parameters for recombinant All2934 and All4774

Parameter	K_m app* (mmol L^{-1})	V_{max} app (μmol mg^{-1} min^{-1})	Hill slope
All2934	19.5 ± 1.2	102.3 ± 2.7	4.7 ± 0.4
All4774	109.5 ± 10.1	25.71 ± 2.26	9.7 ± 1.5

*app, apparent. All data were obtained from Fig. 3 using the GRAPHPAD PRISM 4.0.

Effect of phosphate on the glutaminase activity

It was reported previously that phosphate is required for the activation of glutaminase isolated from *Bacillus pasteurii* (Klein *et al.*, 2002). Initial studies showed that the glutaminase activity of the recombinant All2934 was phosphate dependent, while All4774 was phosphate independent (data not shown). To further examine the phosphate dependency of All2934, we varied the phosphate concentration in the reaction mixtures. As shown in Fig. 4, no glutaminase activity was detected without addition of phosphate. Activation of glutaminase All2934 by 10 mM phosphate was

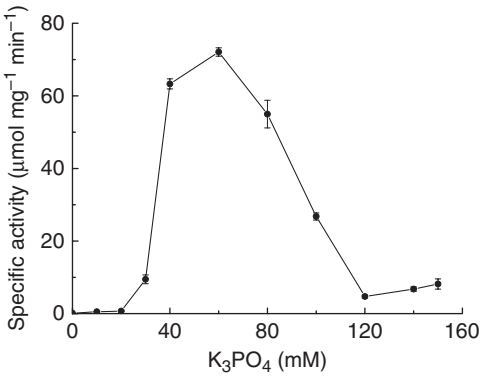

Fig. 4. Effects of phosphate concentration on the glutaminase activity of recombinant All2934. The enzymatic activity of All2934 was measured under optimal conditions with 20 mmol L^{-1} L-glutamine. Values represent the mean of three independent experiments.

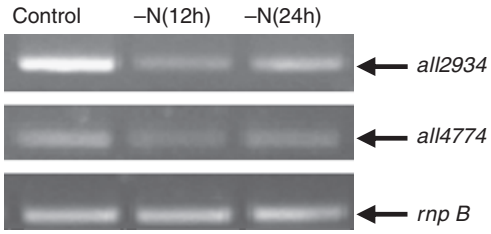

Fig. 5. Semi-quantitative RT-PCR analysis of the expression of *all2934* and *all4774* under normal and nitrogen-deprivation conditions. Expression of *rnpB* was used as a positive control. All RT-PCR experiments were repeated twice, and similar results were obtained.

observed (Fig. 4). The enzyme activity increased rapidly when the phosphate concentration was increased to 30 mM. The maximal glutaminase activity for the recombinant All2934 (72.1 μmol mg^{-1} min^{-1}) was observed at 60 mM. The enzyme activity declined gradually as the phosphate concentration increased further, from 60 to 150 mM (Fig. 4).

Expression of *all2934* and *all4774* in response to nitrogen starvation

To examine the gene expression of the deduced glutaminases, as well as their physiological significance, we carried out a semi-quantitative RT-PCR analysis using total RNA isolated from *Anabaena* cultured under normal and nitrogen-deprivation conditions (Fig. 5). Transcripts of *all2934* and *all4774* were detected under both conditions, indicating that *all2934* and *all4774* are expressed in *Anabaena*. The level of *all4774* transcript was, however, much lower than that of *all2934* (Fig. 5, Control). The low amount of *all4774* transcripts in *Anabaena* may indicate that *all4774* plays a minor role in catalyzing the hydrolysis of glutamine to glutamic acid *in vivo*. Indeed, as shown above, the affinity of All2934 for L-glutamine was in a more physiologically relevant range, and its affinity for L-glutamic acid was significantly higher than that of All4774 (Fig. 3c and d). The expression of *all2934* changed dramatically under nitrogen deprivation (Fig. 5). A dramatic decline of *all2934* transcript levels was observed at 12 h without addition of combined nitrogen. After 24 h of nitrogen starvation, however, the level of *all2934* transcripts appeared to recover to a large extent (Fig. 5). These changes in *all2934* transcript levels indicate that expression of *all2934* in *Anabaena* is regulated by nitrogen availability.

Discussion

As mentioned above, glutaminases play an important role in the nitrogen metabolism of heterotrophic organisms (Halpern, 1988; Márquez *et al.*, 2006). In cyanobacteria, however, little is known about the genetic and biochemical properties of the glutaminases. In this study, two putative cyanobacterial glutaminases were investigated. We searched the *Anabaena* sp. PCC 7120 genome database (http://www.kazusa.or.jp/cyano) and identified *all2934* and *all4774* as genes encoding putative glutaminases. To determine whether the gene products were catalytically active, we examined the functional properties of the putative glutaminases as recombinant proteins. We showed that both recombinant All2934 and All4774 possessed glutaminase activity, but not glutaminase–asparaginase activity. Based on the substrate specificity, both enzymes were placed in the category of glutaminases that catalyze the hydrolysis of glutamine to glutamic acid (Krebs, 1935). Although both recombinant proteins possessed glutaminase activity, the enzymatic properties differed. One difference was their affinity for the substrate L-glutamine (Table 1). The K_m app of recombinant All2934 for L-glutamine was significantly lower than that of All4774, but was in a similar range as *Micrococcus luteus* glutaminases, *E. coli* glutaminase B and kidney-type glutaminase (Prusiner *et al.*, 1976; Kenny *et al.*, 2003; Nandakumar *et al.*, 2003). The high K_m app obtained for the recombinant All4774 protein indicates its low affinity for the substrate L-glutamine. Moreover, only trace amounts of *all4774* transcripts were detected in *Anabaena* cells (Fig. 5). This strongly suggests that All4774 does not play a predominant role in the hydrolytic deamidation of L-glutamine to L-glutamate in *Anabaena*. The presence of All4774 in *Anabaena* could, however, be important for maintaining the balance between glutamine and glutamate in bacteria (Duran *et al.*, 1996).

Another distinctive difference between the two recombinant glutaminases was their response to temperature (Fig. 3a). The recombinant All2934 was found to be most active at 20 °C, which is comparable to glutaminase A from *E. coli*

(Prusiner, 1973), while the recombinant All4774 was active optimally at 37 °C, which is similar to Slr2079, a glutaminase from *Synechocystis* sp. PCC6803 (F. Huang, unpublished data). An additional difference was their response to pH (Fig. 3b). The optimum pH for recombinant All2934 was observed at 6.0, comparable to the glutaminase from *B. subtilis* (Shimizu *et al.*, 1991) but in contrast to glutaminase A from *E. coli*, whose activity was stable at a lower pH (Prusiner, 1973). All4774, whose maximal activity was at pH 7.5, was biochemically similar to *E. coli* glutaminase B (Prusiner *et al.*, 1976) as well as Slr2079 (F. Huang, unpublished data). These results were consistent with the sequence alignment showing that All2934 had the highest similarity to *E. coli* glutaminase A, while All4774 was similar to Slr2079 and *E. coli* glutaminase B (Fig. 1). Taken together, our results indicated that the divergence of the two *Anabaena* glutaminases at the genetic level may represent the diversity of glutaminases among prokaryotes.

Of the functionally characterized glutaminases from microorganisms, only the glutaminase from *B. pasteurii* is reported to have catalytic activity that is activated in the presence of phosphate (Klein *et al.*, 2002). In the present study, we found that All2934, one of the two glutaminases from *Anabaena* sp. PCC 7120, was also phosphate dependent (Fig. 4). To our knowledge, this is the first report on the presence of such a phosphate-activated glutaminase in cyanobacteria. The kinetic analysis of the recombinant All2934 showed that it might not be a Michaelis–Menten enzyme because its Hill slope was 4.7 ± 0.4. Furthermore, because phosphate activation of the native glutaminase resulted from a phosphate-induced association of inactive dimers to form active tetramers (Godfrey *et al.*, 1977; Morehouse & Curthoys, 1981), we postulate that the recombinant All2934 enzyme may act in a similar fashion. The glutaminase All2934 may also be an adaptive strategy of the *Anabaena* cell to cope with different nitrogen states. RT-PCR analysis showed that the transcript level of *all2934* was much higher than that of *all4774* in *Anabaena*, and the expression of *all2934* was repressed in the absence of combined nitrogen for 12 h (Fig. 5). This clearly indicates that the expression of *all2934* is regulated by nitrogen availability. The remarkable repression of *all2934* expression under nitrogen limitation may be correlated to the tight regulation of the GS–GOGAT pathway incorporating ammonium into a carbon skeleton. In nitrogen-fixing cyanobacteria, the activity of GS is increased when the nitrogen source is dinitrogen (N_2) (Flores & Herrero, 1994). This allows enhanced synthesis of glutamine that is required for maintaining the GS–GOGAT cycle. Because GS is the key regulatory point of the GS–GOGAT pathway operating in the cyanobacterial cell (Muro-Pastor *et al.*, 2005), hydrolytic deamidation of glutamine catalyzed by the glutaminase All2934 may be biochemically counteracting or interfering with the process. We postulate that repression of *all2934* expression under nitrogen starvation may serve as an important module in the homeostasis of glutamine under such conditions.

In the filaments of *Anabaena cylindrical*, the main enzyme metabolizing glutamine is GOGAT (Rowell *et al.*, 1977). In the heterocysts, however, the activity of glutamine aminotransferase was detected, but the activity of GOGAT was not (Thomas *et al.*, 1977). Recent data also showed that in heterocysts isolated from *Anabaena* sp. PCC 7120, neither the *glsF* gene transcript nor GOGAT was detected (Martín-Figueroa *et al.*, 2000). This suggests that the main enzyme metabolizing glutamine in the heterocysts is not GOGAT. In the present study, we have verified the expression of putative glutaminases encoded by the *Anabaena* sp. PCC 7120 genome, and demonstrated the glutaminase activity possessed by recombinant proteins encoded by the glutaminase ORFs (Figs 5 and 3). Based on the pattern of gene expression in response to nitrogen starvation as well as their biochemical features, i.e. the lower physiological temperature and pH optima for All2934 and the high K_m app for All4774, we postulate that the glutaminases present in *Anabaena* may play an important role in metabolizing glutamine under N_2-fixation and stress conditions, such as low temperature. Further studies have been initiated to better understand the cyanobacterial glutaminases in the context of their *in vivo* activation, subcellular localization and mechanisms of molecular regulation under adverse conditions.

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Authors' contribution

J.X.Z. and J.Z. contributed equally to this work.

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