CphA2 is a novel type of cyanophycin synthetase in \( \text{N}_2 \)-fixing cyanobacteria

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Most cyanobacteria use a single type of cyanophycin synthetase, CphA1, to synthesize the nitrogen-rich polymer cyanophycin. The genomes of many \( \text{N}_2 \)-fixing cyanobacteria contain an additional gene that encodes a second type of cyanophycin synthetase, CphA2. The potential function of this enzyme has been debated due to its reduced size and the lack of one of the two ATP-binding sites that are present in CphA1. Here, we analysed CphA2 from \textit{Anabaena variabilis} ATCC 29413 and \textit{Cyanothece} sp. PCC 7425. We found that CphA2 polymerized the dipeptide \( \beta \)-aspartyl-arginine to form cyanophycin. Thus, CphA2 represents a novel type of cyanophycin synthetase. A \( \text{cphA2} \) disruption mutant of \textit{A. variabilis} was generated. Growth of this mutant was impaired under high-light conditions and nitrogen deprivation, suggesting that CphA2 plays an important role in nitrogen metabolism under \( \text{N}_2 \)-fixing conditions. Electron micrographs revealed that the mutant had fewer cyanophycin granules, but no alteration in the distribution of granules in its cells was observed. Localization of CphA2 by immunogold electron microscopy demonstrated that the enzyme is attached to cyanophycin granules. Expression of CphA1 and CphA2 was examined in \textit{Anabaena} WT and \( \text{cphA} \) mutant cells. Whilst the CphA1 level increased upon nitrogen deprivation, the CphA2 level remained nearly constant.

Received 21 July 2015
Revised 8 January 2016
Accepted 12 January 2016

INTRODUCTION

Cyanophycin [multi-(\( L \)-arginyl-poly-\( L \)-aspartic acid) (Simon, 1971; Simon & Weathers, 1976), also referred to as cyanophycin granule polypeptide, is a branched, nitrogen-rich polymer found in most cyanobacteria and in some heterotrophic bacteria (Füser & Steinbüchel, 2007). The compound consists of a poly-\( \alpha \)-aspartic acid backbone, with arginine residues linked to the \( \beta \)-carboxyl groups of the aspartate units by isopeptide bonds (Simon & Weathers, 1976). Cyanophycin is thought to serve as a dynamic reservoir of nitrogen, carbon and energy in cyanobacteria (Carr, 1988), and has attracted biotechnological attention as a precursor molecule for polyaspartate – a completely biodegradable substitute for polyacrylate (Erickson et al., 2001). The key enzyme in cyanophycin synthesis is cyanophycin synthetase CphA1 (Ziegler et al., 1998). CphA1 synthesizes cyanophycin from its constituent amino acids, aspartate and arginine, in two consecutive, ATP-dependent reactions at its two predicted active sites (Berg, 2003; Berg et al., 2000; Ziegler et al., 1998). The N-terminal active site phosphorylates the \( \alpha \)-carboxyl group at the C terminus of the poly-\( L \)-aspartate backbone, followed by substitution of the phosphoryl group with aspartic acid [cyanophycin :\( L \)-aspartate ligase (ADP-forming; EC 6.3.2.29)]. By the same mechanism, the C-terminal active site connects the \( \beta \)-carboxyl group of the C-terminal aspartic acid residue to the \( \alpha \)-amino group of arginine, thereby forming an isopeptide bond [cyanophycin :\( L \)-arginine ligase (ADP-forming; EC 6.3.2.30)] (Berg, 2003; Berg et al., 2000). In accordance with the proposed mechanism, two ATP per \( \beta \)-aspartyl-arginine, the formal building block of cyanophycin, are converted to ADP and phosphate (Aboulmagd et al., 2001). Genes for CphA1 are present in the genomes of most cyanobacterial species and some heterotrophic bacteria (Füser & Steinbüchel, 2007). Activity for some of these proteins has been shown by heterologous expression in bacteria, yeasts and plants (Frommeyer et al., 2016). In addition, some cyanobacteria possess a second gene named \( \text{cphA2} \) (Picossi et al., 2004). In a CphA2-lacking mutant strain of the filamentous, heterocyst-forming cyanobacterium...
Anabaena sp. PCC 7120, a lower cyanophycin content was observed than in the WT, suggesting a role of CphA2 in cyanophycin synthesis. However, cphA2 encodes what appears to be a shortened version of CphA1. An alignment of the amino acid sequences of CphA1 and CphA2 from the closely related cyanobacterium Anabaena variabilis ATCC 29413 is shown in Fig. 1. CphA2 possesses a region with high sequence similarity to the N-terminal cyanophycin L-aspartate ligase active site of CphA1 with the potential ATP-binding sites in the B- and J-loop (Berg et al., 2000), but is missing the one in the P-loop of the C-terminal cyanophycin L-arginine ligase site. The biochemical function of CphA2 has remained unknown. Here, we report on the catalytic properties of CphA2 from the N2-fixing cyanobacteria A. variabilis and Cyanothece sp. PCC 7425. Our biochemical data show the functionality of CphA2 as a novel type of cyanophycin synthetase that polymerizes β-aspartyl-arginine to cyanophycin. Furthermore, the localization and physiological role of CphA2 in the context of N2 fixation is discussed. A CphA2-lacking mutant of A. variabilis was found to have a reduced viability when deprived of combined nitrogen in high light.

**METHODS**

**Recombinant DNA techniques.** Standard methods (Sambrook et al., 1989) or minor modifications thereof were used. DNA modification enzymes were purchased from New England Biolabs and Fermentas Thermo Scientific. Purification of plasmids, and isolation of DNA fragments after electrophoresis was performed with commercial kits (Macherey-Nagel).

**Construction of cphA2 expression vectors.** Two different expression vectors were generated for the expression of cphA2 (ava_0335) from A. variabilis: pETcphA2, a pET19b (Novagen) derivative containing a gene coding for a CphA2 fusion protein including an N-terminal His_{10}-tag (H_{10}-CphA2), and pGEXava_0335, a pGEX-6-P derivative (GE Healthcare) containing a gene coding for a fusion protein that consisted of an N-terminal GST-tag and CphA2 (GST-CphA2) linked by a PreScission protease recognition and cleavage site. Oligonucleotides used as primers to amplify the genes are listed in Table S1 (available in the online Supplementary Material).

A pET19b-based expression vector containing Cyan7425.4446 was constructed using primer pair D.1/D.2, providing a His_{10}-tagged CphA2 from Cyanothece sp. PCC 7425. The generated expression vectors were verified by restriction analyses and DNA sequencing.

**Production and isolation of H_{10}-CphA2.** An overnight culture of BL21(DE3)[pETcphA2] was used to inoculate lysogeny broth (LB) medium containing 100 μg ampicillin ml⁻¹ to an initial cell density of OD 0.05. The culture was grown in Erlenmeyer flasks at 37 °C under shaking at 200 r.p.m. After inducing expression with 1 mM IPTG at OD_{600} 0.6–0.8, the culture was grown at 18 °C for 18 h. Harvested cells were resuspended in 50 mM Tris/HCl buffer, pH 8.5 containing 300 mM NaCl and 50 mM KCl. This buffer was used for all subsequent purification steps. Cells were treated as described for the purification of H_{10}-CphA2. The GST-CphA2 fusion protein was enriched by affinity chromatography on glutathione agarose in a batch procedure. The supernatant was added to Protino glutathione agarose 4B (Macherey-Nagel) which was equilibrated with the same buffer, and the suspension was inverted at 10 r.p.m. for 30 min at ambient temperature. The sedimented matrix was washed with 30 matrix volumes of buffer and resuspended in 1 matrix volume of buffer. To elute CphA2, 200 U PreScission protease (GE Healthcare Life Science) was added per 1 ml matrix volume. The matrix was inverted for 14 h at 10 r.p.m., and the eluate was collected by centrifugation for 10–30 s at 4 °C and the lowest adjustment. The CphA2-containing supernatant was collected and the purity checked by SDS-PAGE.

**Disruption of cphA2 in A. variabilis.** The cpha2 (ava_0335) gene was interrupted in A. variabilis by insertional mutagenesis. The gene was amplified in two steps. Primer sequences are given in Table S1. One PCR was performed with the primer pair C.1/C.2 and a second PCR with C.3/C.4. A XbaI restriction site was introduced in ava_0335. In a second step, the 5’ and 3’ parts of the gene were ligated by PCR using the PCR products as template and C.1/C.3 as primers. The resulting PCR product was cloned into vector pRL271 (Black et al., 1993) via PstI and SpeI sites (see Physiological role of CphA2). The kanamycin resistance cassette C.K3 from plasmid pRL448 (Elhai & Wolk, 1988a) was inserted into the generated XbaI site. Escherichia coli DH5x cells were transformed by the resulting pRL derivatives, and the transformants checked by PCR and restriction analysis. Two derivatives with resistance cassettes in opposite orientations were isolated, transferred to A. variabilis by conjugation according to published procedures (Elhai & Wolk, 1988b) and double recombinants identified by PCR analysis.

**Preparation of β-aspartyl-arginine.** Cyanophycin was synthesized by heterologous expression of cpha1 from A. variabilis in E. coli and isolated from the cells as described by Ziegler et al. (1998). Cyanophycin was resuspended in 50 mM Tris/HCl, pH 7.5 and enzymically depolymerized to β-aspartyl-arginine using cyanophycinase (CphB) from Thermosynechococcus elongatus BP-1 (Hejazi, 2002). After centrifugation at 10 000 g for 20 min, the enzyme was removed by passing the supernatant through a protein concentrator (Vivaspin 4, cut-off 5 kDa; Sartorius). The flow-through was concentrated by vacuum concentration centrifugation at 80 °C and β-aspartyl-arginine was purified by chromatography on P2 columns (Bio-Rad; 1 × 18 cm, 0.1 M acetic acid, flow rate 0.4 ml min⁻¹). Ninyhydrin-positive fractions were pooled and concentrated at 80 °C as described earlier. The β-aspartyl-arginine was quantified as described by Hejazi et al. (2002). Purity of the substrate was checked by gel filtration on a Superdex Peptide 10/300 GL column (GE Healthcare) with 50 mM Tris/HCl, pH 8.0 as running buffer. The result was verified by MALDI-TOF MS analysis (Oliver Broedel, Technische Hochschule Wildau, Wildau, Germany).
Fig. 1. Comparison of CphA1 and CphA2 amino acid sequences and potential ATP-binding sites. Alignment of the amino acid sequences of CphA1 (Ava_1814) and CphA2 (Ava_0335) from *A. variabilis* ATCC 29413. Strictly conserved residues are shaded in black; similar residues are framed in black. The loop regions predicted to be involved in ATP binding (Berg *et al.*, 2000) are framed in red and are labelled with the corresponding motif name above the sequence.
**In vitro synthesis of cyanophycin and CphA2 activity assay.** The standard reaction mixture for *in vitro* synthesis of cyanophycin contained 50 mM Tris/HCl, pH 8.0, 20 mM MgCl₂, 50 mM KCl, 2 mM DTT, 10 mM ATP (disodium salt), 100 mM β-aspartyl-arginine (substrate) and 0.3 mg H₁₀-CphA2 ml⁻¹. After incubation at 30 °C for defined times, aliquots were withdrawn to determine ATP hydrolysis (phosphate release) and polymer production on SDS-PAGE. Kinetic analysis of CphA2 was performed with (i) 170 mM β-aspartyl-arginine and ATP concentrations ranging from 0.5 to 10 mM, and (ii) 10 mM ATP and β-aspartyl-arginine concentrations ranging from 15 to 210 mM. The kinetic data were analysed with Enzyme Kinetics module 1.3 for SigmaPlot 10 software (Systat). To determine the substrate specificity of CphA2, a range of dipeptides (Bachem) was individually added to the reaction mixture from stock solutions (adjusted to pH 8.0 with 1 M NaOH) to give a final dipeptide concentration of 100 mM. Aliquots of 2.0 or 20 μl were withdrawn at different time points during incubation at 30 °C, and the extent of ATP hydrolysis followed as release of phosphate measured with the malachite green assay (Lanzetta et al., 1979).

**Cyanophycin and protein analysis.** Cyanophycin was isolated from *A. variabilis* WT and cphA2 mutant (Ziegler et al., 1998). The cyanophycin was quantified as aspartate released after total enzymic digestion of cyanophycin (Hejazi, 2002). Protein concentrations were determined using the Lowry assay (Lowery et al., 1951). SDS-PAGE of proteins and of cyanophycin was performed on 15 % polyacrylamide (acylamide/bisacrylamide, 29 : 1, v/v). To analyse the size and purity of *in vitro*-synthesized cyanophycin, 1 μl aliquots of the reaction mixtures were subjected to SDS-PAGE analysis.

**Molecular mass of CphA2.** The molecular mass of both H₁₀-CphA2 and CphA2 was determined by size exclusion chromatography (SEC) on a calibrated column of Superdex 200 10/300 GL (GE Healthcare) using 50 mM Tris/HCl, pH 8.0, 100 mM KCl and 1 mM DTT as running buffer at a flow rate of 0.5 ml min⁻¹. A gel filtration standard from Serva, ranging from 12.4 to 450 kDa, was used for calibration.

**Sequence data analysis.** A BLASTP search (Altschul et al., 1997) was performed against all cyanobacterial sequences available from the Integrated Microbial Genomes database (Markowitz et al., 2012) and GenBank (Benson et al., 2011), and against the recently published sequences of Section V cyanobacteria by Dagan et al. (2013). The amino acid sequences of CphA1 (Ava_1814) and of CphA2 (Ava_0335) from *A. variabilis* were used as queries. Sequence alignment analysis and illustration preparation were performed with CLUSTAL W (Larkin et al., 2007) and ESPript 3.0 (http://espript.ibcp.fr/ESPript/ESPript/; Robert & Gouet, 2014), respectively.

**Strains and culture conditions.** Liquid cultures of *A. variabilis* ATCC 29413 strain FD (Currier & Wolk, 1979) and the cphA2 disruption strain were grown at 30 °C in fourfold-diluted AA medium (Allen & Arnon, 1955) with or without 5 mM KNO₃ in airlift flasks aerated with air enriched with 2 % CO₂. When necessary, the medium was supplemented with 50 μg kanamycin ml⁻¹. Cultures were grown under constant illumination with white light of 50 (low light) or 280 μmol photons m⁻² s⁻¹ (high light), respectively.

**Growth tests.** Growth of *A. variabilis cphA2* and *cphA1* mutants was assayed on solidified AA medium (Allen & Arnon, 1955) supplemented, as indicated, with 5 mM KNO₃ as nitrogen source. Three different dilutions of cells were spotted on agar plates, corresponding to 12.5, 25 and 50 ng chlorophyll *a*. Plates were incubated as stated earlier and photographed after 4 days of constant illumination.

**Ultrastructural and immunocytochemical investigations.** Ultrastructural and immunocytochemical analyses were carried out (Fuhrmann et al., 2009). An anti-CphA2 antiserum against H₁₀-CphA2 and an antiserum against His-tagged CphA1 from *A. variabilis* were generated in rabbits by a commercial supplier (Pineda Antikörper Service). The dilutions of the anti-CphA1 and antiserum were 1 : 100. The secondary antiserum, a gold-coupled anti-rabbit IgG, was used at 1 : 30 dilution. The anti-cyanophycin antiserum was used as described previously (Ziegler et al., 2001).

**Expression analysis of cphA2.** Samples of liquid cultures of *A. variabilis* WT and cphA2 mutant were grown under low-light conditions, and cells were collected at 0, 24 and 48 h after nitrogen step-down. Cell-free extracts were prepared as described previously (Ziegler et al., 1998). The concentration of chlorophyll *a* was determined according to de Marsac & Houmard (1988) for normalization. Cell-free extracts corresponding to 1 or 2 μg chlorophyll *a* were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. The antisera against CphA2 and CphA1 were used in a dilution of 1 : 10 000. Antigen-antibody complexes were visualized with peroxidase-labelled anti-rabbit IgG (Sigma-Aldrich) and an ECL Detection kit (Merck Millipore). After the blot was digitized, the amounts of protein were determined by measuring the signal density using Quantity One software (Bio-Rad).

**RESULTS**

**Distribution of cyanophycin synthetases amongst cyanobacteria.** To gain new insights into the distribution of cyanophycin synthetases amongst cyanobacteria, we performed a BLASTP search (Altschul et al., 1997) against all recently sequenced cyanobacterial genomes (155 genomes). The search included species of the five taxonomic sections defined according to their morphology (Rippka et al., 1979): unicellular forms (Sections I and II), filamentous forms (Section III), filamentous heterocyst-forming forms (Section IV) and filamentous heterocyst-forming branching forms (Section V). The results are summarized in Fig. 2 and Table S2 and S3. A cyanophycin synthetase of type 1 (CphA1) was present in 72.9 % of all sequenced cyanobacteria, whilst only 28.4 % possessed a second cyanophycin synthetase of type 2 (CphA2). CphA2-like proteins were only present when species possess a CphA1. The lowest percentage of CphA1-like proteins was observed for cyanobacteria of Section I at only 50.6 %, whereas almost all of the other cyanobacteria possessed a CphA1-like protein. In filamentous, *N₂*-fixing cyanobacteria of Sections IV and V the percentage of CphA2-like proteins was high, at 71.4 and 81.8 %, respectively. In comparison, only 8.4 % of Section I cyanobacteria possessed an additional CphA2-like protein, including three *Cyanothece* and one *Gloeocapsa* species that grow diazotrophically (Bandypadhyay et al., 2013; Millineaux et al., 1981). CphA-coding ORFs of the sequenced species are summarized in Table S3. In Section II, half of the sequenced cyanobacteria possessed a CphA2-like protein; all these species contained *nifH* genes encoding a subunit of the nitrogenase – a marker gene widely used to study the ecology and evolution of *N₂*-fixing bacteria (Raymond et al., 2004). The presence of CphA2-like proteins in species of Section III was relatively low at 29.4 %, but some of these species have been reported to fix nitrogen (Stal & Heyer, 1987).
CphA2 forms cyanophycin from β-aspartyl-arginine

Preparation and SEC of CphA2. The His_{10}-CphA2 fusion protein from *A. variabilis* (H_{10}-CphA2) was purified from crude extracts of recombinant *E. coli*. The protein was visible as a single band on SDS-PAGE gels with an apparent molecular mass of ~75 kDa (Fig. 3a, lane 2). The size corresponded well to the predicted monomeric mass of 75 kDa calculated for H_{10}-CphA2 using the ProtParam tool (Gasteiger *et al.*, 2005). The molecular mass of the native enzyme was determined by SEC. The His_{10}-CphA2 was eluted as a symmetrical peak corresponding to a molecular mass of 273 ± 2.4 kDa (*n* = 2). Additionally, a recombinant GST-CphA2 variant from *A. variabilis* was purified and cleaved by PreScission protease. SEC was performed with the resulting CphA2. We obtained a mass of 265 ± 0.6 kDa for the untagged complex (*n* = 3). The molecular mass determined for the tagged and untagged CphA2 corresponded to 3.6 monomeric masses (75 and 73 kDa, respectively), suggesting that the native protein was either a trimer or a tetramer.

In vitro activity of CphA2. We used H_{10}-CphA2 from *A. variabilis* to identify potential substrates in vitro. Enzymic activity was measured as ATP consumption (phosphate release) (mg protein)^{-1}. A range of dipeptides was tested as potential substrates in the presence of Mg-ATP (Table 1). The only tested dipeptide found to cause a substantial release of phosphate was β-aspartyl-arginine. The low activity found with α-aspartyl-arginine might have been due to minor β-aspartyl-arginine impurities. In contrast to CphA1, the isolated enzyme did not synthesize cyanophycin from the substrates aspartate, arginine and Mg-ATP (Table 1). SDS-PAGE analyses of the synthesized product over time revealed that CphA2 formed a product with a molecular mass of ~20–30 kDa in a single reaction (Fig. 3b, lanes 1–8). Specific digestion with cyanophycinase CphB (Richter *et al.*, 1999) was used to identify the product as cyanophycin (Fig. 3c, lanes 8 and 9). The amount of cyanophycin was determined based on the amount of aspartate released after treatment of cyanophycin with CphB and isopeptidase, as described previously (Hejazi, 2002; Neumann *et al.*, 2005). The ATP consumption required to form the peptide bond between β-aspartyl-arginine units was determined by comparing the amount of released phosphate to the yield of cyanophycin. To incorporate 1 mol β-aspartyl-arginine into cyanophycin, the hydrolysis of ~1 mol ATP was required (1.15 ± 0.10 mol ATP per 1 mol β-aspartyl-arginine; *n* = 5).

The presence of potassium ions was essential for enzymic activity, as their replacement with sodium ions abrogated the observed phosphate release. Reactions run without β-aspartyl-arginine or ATP also failed to release phosphate, as did reactions in which ATP was replaced with polyphosphate (polyP_{45}; sodium phosphate glass type 45; Sigma-Aldrich). Notably, CphA1 from *A. variabilis* was not able to use β-aspartyl-arginine as substrate, failing to release phosphate or form cyanophycin under our experimental conditions in vitro. As a side note, CphA2 activity was enhanced in vitro by adding water-soluble cyanophycin from *Desulfotobacterium hafniense* (Otterbach, 2010; Ziegler *et al.*, 2002) as an artificial primer (Fig. S1).
Kinetics. The phosphate release kinetics were determined for both β-aspartyl-arginine and ATP substrates. As shown for β-aspartyl-arginine as an example (Fig. S2), complex sigmoid kinetics were recorded for cyanophycin synthesis. The $K_{0.5}$ for β-aspartyl-arginine was 76 ± 10.69 mM ($n = 6$). The $K_{0.5}$ for ATP was 3.5 ± 0.45 mM ($n = 6$). The in vitro turnover number for one monomer of CphA2 was 1.1 Hz at substrate saturation. Hill coefficients of 3.1 and 2 were determined for β-aspartyl-arginine and ATP, respectively, indicating positive cooperativity.

CphA2 from Cyanothece sp. PCC 7425. To confirm our BLASTP results and show the enzymic activity of CphA2 in diazotrophic cyanobacteria, cphA2 of the unicellular cyanobacterium Cyanothece sp. PCC 7425 (Section I) was heterologously expressed in E. coli, purified and characterized. Cyanophycin formation from the dipeptide β-aspartyl-arginine was observed in vitro (Fig. S3). Samples of the reaction batch were taken at different time points and analysed by SDS-PAGE (Fig. S3). The formed cyanophycin was identified by specific CphB degradation (not shown). CphA2 from Cyanothece sp. PCC 7425 showed a lower activity than CphA2 from A. variabilis under in vitro conditions. A ~25 kDa band representing the final mass of cyanophycin appeared later (Fig. S3). A 17 kDa band representing a soluble cyanophycin fraction was found at the initial stages of cyanophycin synthesis (Fig. S3).

Physiological role of CphA2

To further examine the physiological role of CphA2, we generated a cphA2 disruption mutant of A. variabilis by...
insertional mutagenesis (Fig. S4). Cyanophycin isolated from WT and cphA2 mutant cells ranged from 30 to 130 kDa (data not shown). We observed 10–15% less cyanophycin per chlorophyll a in the mutant during its stationary growth phase under low-light conditions than in the WT. Electron micrographs of cells in which cyanophycin granules were immunocytochemically identified with an anti-cyanophycin antiserum confirmed a similar localization of cyanophycin in the WT and mutant cells. Whilst cyanophycin was localized to polar nodules in the neck region of the cell in heterocysts (Fig. 4), cyanophycin granules were visible in various positions of vegetative cells (Fig. S5 and S6). To further ascertain the distribution of CphA2, we analysed its localization in A. variabilis WT cells by immunogold labelling with an anti-CphA2 antiserum. In WT filaments, CphA2, like cyanophycin, was located at the polar nodules of heterocysts and at the cyanophycin granules of vegetative cells. We found no evidence of cell-specific distribution of CphA2 (Figs 4 and S6). CphA2 mutant cells did not show any specific labelling of cyanophycin granules (Fig. 4). Our CphA2 antiserum showed no cross-reaction with CphA1, which is also associated with cyanophycin (Fig. 5). To further examine CphA1 and CphA2 expression at the protein level, samples from A. variabilis WT and mutant cells were taken at different time points after nitrogen step-down. Crude extracts were separated by SDS-PAGE and subjected to Western blot analysis using antisera raised against CphA1 and CphA2. The results were quantified by densitometric analysis. Mutants lacking CphA1 (Ziegler et al., 2001) and CphA2 were used as controls. As shown in Fig. 5 (lane 1), the expression level of CphA2 in WT cells was relatively consistent for 48 h after nitrogen step-down (100 ± 16%). In contrast, the expression level of CphA1 in WT cells increased to 148 ± 27 (Fig. 5, lane 2) and to 304 ± 64% in cphA2 mutant cells following nitrogen step-down (Fig. 5, lane 3). Growth profiles of WT, cphA1 and cphA2 mutants of A. variabilis were examined under different light and nitrogen conditions (Fig. 6). Under low-light conditions (Fig. 6a), no significant difference in growth was observed in the presence and absence of nitrate amongst WT, cphA1 and cphA2 mutant cells. In contrast, the cphA2 mutant was impaired in growth under high-light conditions (Fig. 6b). In the absence of combined nitrogen, the mutant showed pronounced bleaching after 4 days of high-light exposure. The altered phenotype under high-light conditions and nitrogen deprivation was confirmed by growth experiments in liquid medium. Whilst the doubling time for the WT and cphA2 mutant was similar in the presence of nitrate (~20 h), it was significantly reduced under nitrogen deprivation (53 versus 28 h for WT).

**DISCUSSION**

**CphA2 presence strongly corresponds to N₂ fixation**

The distribution analysis of CphA1 and CphA2 proteins amongst cyanobacteria revealed that the appearance of CphA2 is strictly dependent on the presence of CphA1. CphA2 is predominantly found in filamentous growing species of Section IV and V that are able to fix N₂ in heterocysts (Fig. 2, Tables S2 and S3). CphA2 genes were also found in unicellular cyanobacteria of Section I that are known to fix N₂ in diurnal rhythms in the absence of photosynthesis. The function of CphA2 in one of these strains, i.e. *Cyanothece* sp. PCC 7425, was verified *in vitro*. The presence of CphA2 in species of Section II and III correlates with the presence of both CphA1 and nitrogenase, indicating their capacities for N₂ fixation. We conclude that the presence of CphA2 strongly corresponds to the ability of a cyanobacterium to fix N₂.

![Fig. 4. Localization of CphA2. Electron micrographs of A. variabilis WT and cphA2 mutant cells grown under N₂-fixing conditions. Ultrastructural investigations were performed using electron microscopy following the immunocytochemical visualization of CphA2 with antisera raised against CphA2 and a gold-coupled anti-rabbit IgG antibody in *Anabaena* cultures.](image-url)
CphA2 – a second type of cyanophycin synthetase

CphA2 acts as cyanophycin synthetase

Although previous studies based on cphA1 deletion mutants in *A. variabilis* and *Anabaena* sp. PCC 7120 suggested that CphA2 cannot synthesize cyanophycin *de novo* (Picossi et al., 2004; Ziegler et al., 2001), the catalytic activity of CphA2 has remained unknown. Here, we demonstrated substrate consumption as well as product synthesis of CphA2 *in vitro*. The isolated enzyme did not show the canonical activity of CphA1, but was found to be specific for the dipeptide β-aspartyl-arginine and ATP as substrates. Potassium ions were essential for the *in vitro* reaction. SDS-PAGE analyses and specific CphB digestion of the synthesized product revealed that cyanophycin was formed. The ATP:β-aspartyl-arginine ratio of ~1 to incorporate 1 mol β-aspartyl-arginine into cyanophycin suggests that the reaction mechanism involved may be similar to that described for CphA1, which forms cyanophycin via acyl-phosphate intermediates (Ziegler et al., 1998).

The $K_{0.5}$ values obtained *in vitro* are quite high, at 76 ± 10.69 mM for β-aspartyl-arginine and 3.5 ± 0.45 mM for ATP, and probably do not reflect the *in vivo* situation where CphA2 is attached to the insoluble cyanophycin (Fig. 4a). We expect further cofactors, not yet identified, as well as ambient conditions to influence the *in vivo* activity of CphA2. However, these results collectively show that CphA2 is an active cyanophycin synthetase that ligates two β-aspartyl-arginine units by catalysing the formation of a peptide bond between their aspartate residues. As CphA1 was not able to use β-aspartyl-arginine as substrate, we assume that the two enzymes may play different roles in the nitrogen metabolism of *A. variabilis* – whilst CphA1 forms cyanophycin by ligating aspartate and arginine, CphA2 rebuilds cyanophycin from β-aspartyl-arginine. Reactions that affect the cyanophycin/β-aspartyl-arginine pool are presented in Fig. 7. Considering that β-aspartyl-arginine is transferred as the main nitrogen carrier between heterocysts and vegetative cells in *Anabaena* sp. PCC 7120 (Burnat et al., 2014), CphA2 provides the option to form cyanophycin as a storage compound when degradation to the single amino acids is not required. This would be beneficial for the cell due to the lower cost of ATP.

CphA2 and its function

The lack of CphA2 did not affect the molecular mass of cyanophycin in *A. variabilis*, as cyanophycin isolated from CphA2-lacking cells ranged from 30 to 130 kDa, as previously reported for *A. variabilis* WT (Ziegler et al., 2001). However, the mutant produced only 85–90 % of cyanophycin per chlorophyll a in comparison with the WT level during its stationary growth phase under low-light conditions. This is consistent with the results of Picossi et al. (2004), who found that the cyanophycin content of a ΔcphA2 mutant from *Anabaena* sp. PCC 7120 was ~20 % lower than that of the WT. The role of CphA2 remained unclear at that time. Based on our findings, we conclude that cells lacking CphA2 possess a lower overall cyanophycin synthesis capacity and need to consume more ATP per cyanophycin unit. The reduced cyanophycin amount observed in cphA2 mutant cells compared with WT cells might reflect in part the lack of CphA2 activity. We further suggest that nitrogen storage and mobilization in *Anabaena* species are highly dynamic processes that involve cyanophycin/β-aspartyl-arginine pools as reservoirs. In WT and cphA2 mutant cells, cyanophycin was localized to polar nodules in the neck regions of heterocysts and in the granules of vegetative cells. In WT filaments, CphA2 and cyanophycin were co-located at the polar nodules of heterocysts and in the cyanophycin granules of vegetative cells. We did not observe any evidence of cell-specific distribution along the filaments. These results are supported by recently published transcriptional data (Park et al., 2013) showing no cell-specific cphA2 expression. The spatial proximity of CphA1 and CphA2 signals with cyanophycin prompted us to speculate that CphA1 and CphA2 co-localize, and are both predominantly attached to cyanophycin granules. Although no indications for a CphA1/CphA2 assembly were observed *in vitro* (Nürnberg, 2008), we would not exclude a direct interaction of CphA1 and CphA2. Both cyanophycin

Fig. 5. Expression of cphA1 and cphA2 in *A. variabilis* WT and its cphA2 disruption mutant. Immunoblot analysis of *A. variabilis* cell-free extracts is shown. Samples were taken at 0, 24 and 48 h after a nitrogen step-down (*N*). Cell-free extracts corresponding to 1 μg chlorophyll a were loaded per lane. The enzymes were detected with antisera raised against CphA2 and CphA1 (extracts and antisera are indicated on the left). As negative controls, cell-free extracts were used. 1, cphA2 mutant; 2, cphA1 mutant; 3, cphA1 mutant.
synthetases might support each other in vivo. The nitrogen-dependent expression of cphA1 and cphA2 in Anabaena sp. PCC 7120 as mono- and polycistronic transcripts was reported previously (Picossi et al., 2004). Here, we analysed the CphA1 and CphA2 protein levels in A. variabilis mutants at different time points after nitrogen step-down. As shown in Fig. 5, the CphA2 level remained nearly unchanged when the culture was transferred from the presence of nitrate to the absence of nitrate. This result was consistent with that reported previously at the transcript level in Anabaena PCC 7120 subjected to nitrogen step-down (Picossi et al., 2004). Therefore, the expression of CphA2 does not appear to be affected by the supplied nitrogen source. The increased CphA1 level in WT cells following nitrogen step-down is consistent with a previous report that CphA1 mRNA expression was increased under combined nitrogen starvation in Anabaena PCC 7120 (Flaherty et al., 2011; Picossi et al., 2004). In the mutant lacking CphA2, the CphA1 level increased at a higher rate than in WT cells following nitrogen step-down (Fig. 5, lane 3). The enhanced increase of CphA1 in the cphA2 disruption mutant compared with the WT suggests that CphA1 might substitute for CphA2 in the ‘rebuilding’ of cyanophycin (Fig. 7). Although the cphA2 mutant was found to express cphA1 at a higher level than that seen in the WT, this did not fully restore the WT level of cyanophycin.

In the absence of combined nitrogen, the CphA2-lacking mutant strain showed pronounced bleaching after 4 days of high-light exposure (Fig. 6b). Thus, although CphA2 activity was not generally required for growth, it appeared to be essential for the survival of cells in the absence of combined nitrogen under high-light conditions. A similar effect was observed in a CphA1-lacking mutant of A. variabilis (a cyanophycin-free mutant) (Ziegler et al., 2001). Whether other stress conditions, such as phosphate and sulphate limitations, that increase the level of cyanophycin (Allen et al., 1980) lead to a similar phenotype remains to be investigated.

In Anabaena sp. PCC 7120 and A. variabilis, the expression levels of the cyanophycin-degrading enzymes isoaspartyl dipeptidase and cyanophycinase were increased under N2-fixing conditions (Flaherty et al., 2011; Park et al., 2013; Picossi et al., 2004). A basal, nitrogen-independent CphA2 activity might therefore act as an additional control element at the protein level that is involved in the fine tuning of aspartate and arginine release from cyanophycin. The additional β-aspartyl-arginine in CphA2-lacking mutants might lead to an increase of arginine and aspartate in the cell, and a subsequent imbalance in nitrogen metabolism.
CphA2 – a second type of cyanophycin synthetase

Fig. 7. Storage and mobilization of aspartate and arginine. Illustration of cyanophycin (CP) synthesis by CphA1 and CphA2 (upper panel) and degradation by CphB and isoaspartyl dipeptidase (lower panel) in N₂-fixing cyanobacteria.

CONCLUSIONS

Here, we show that CphA2 is a novel type of cyanophycin synthetase using ATP and β-aspartyl-arginine as substrates to form cyanophycin. As recently published by Burnat et al. (2014), β-aspartyl-arginine seems to be the main transport unit for combined nitrogen between heterocysts and vegetative cells in Anabaena sp. PCC 7120. In our opinion, CphA2 contributes to a continuous relocation process that shifts combined nitrogen within the filaments of cyanobacteria. However, given that CphA2-like proteins are also found in diazotrophically growing unicellular cyanobacteria such as Cyanothecae sp. PCC 7425, the CphA2 activity should be considered in the context of N₂ fixation and nitrogen mobilization in addition to nitrogen transfer.

ACKNOWLEDGEMENTS

We dedicate this work to our colleague Karl Ziegler, who died during the preparation of this paper. We would like to thank Oliver Brödel (Technische Hochschule Wildau, Wildau, Germany) for performing the MS measurements. We are also grateful to Sabine Nicklisch for excellent technical support.

REFERENCES


Edited by: E. Flores