P$_H$-Regulated Arginine Synthesis Controls Accumulation of Cyanophycin in Synechocystis sp. Strain PCC 6803

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Cyanophycin (multi-L-arginyl-poly-L-aspartic acid) is a nitrogen storage polymer found in most cyanobacteria and some heterotrophic bacteria. The cyanobacterium Synechocystis sp. strain PCC 6803 accumulates cyanophycin following a transition from nitrogen-limited to nitrogen-excess conditions. Here we show that the accumulation of cyanophycin depends on the activation of the key enzyme of arginine biosynthesis, N-acetyl-L-glutamate kinase, by signal transduction protein P$_H$.

Cyanophycin (multi-L-arginyl-poly-L-aspartic acid) is a nitrogen-rich reserve polymer present in most cyanobacteria (reviewed in references 4, 5, 34, and 43) as well as in some heterotrophic bacteria (27, 49). It consists of a poly-$\alpha$-aspartic acid backbone, with arginine linked to the $\beta$-carboxyl group of almost every aspartyl residue via isopeptide bonds (44). Cyanophycin is synthesized by a single enzyme, cyanophycin synthetase, from aspartate and arginine in an ATP-dependent reaction using a still-unidentified primer (1, 2, 8, 17, 42, 48). The amount of cyanophycin in cyanobacteria varies considerably with growth conditions. Its content is usually less than 1% of dry weight in rapidly growing cultures but is high (up to 18%) in stationary-phase cultures and under conditions of unbalanced growth such as sulfate or phosphate limitation (6, 30, 40, 45). When nitrogen-starved cyanobacterial cultures were provided with combined nitrogen sources, a rapid but transient accumulation of cyanophycin occurred (3). The cyanophycin contents of Anabaena cylindrica and Synechocystis sp. strain PCC 6803 increased severalfold when translation was inhibited by chloramphenicol (6, 41), indicating that rapid synthesis of the polymer did not depend on de novo synthesis of cyanophycin synthetase and that consumption of amino acids by protein synthesis may compete with the accumulation of cyanophycin. Furthermore, no correlation was found between the extractable activity of cyanophycin synthetase and the rate of polymer accumulation (31). These and several similar studies could not, so far, elucidate the mechanism(s) by which cyanophycin accumulation is regulated. Recently, it was shown that the genes for cyanophycin metabolism are under nitrogen control in the diazotrophic strain Anabaena sp. strain PCC 7120 (35). Furthermore, an involvement of the signal transduction protein P$_H$ in the control of cyanophycin synthesis was suggested (19, 29) (see below).

The cyanobacterial P$_H$ protein is a member of the large family of P$_H$ signal transduction proteins, which play pervasive roles in nitrogen control in bacteria, plants, and some archaea.
The transformable wild-type Synechocystis sp. strain PCC 6803 (15) and the isogenic Synechocystis mutants MPphA (PphA deficient; pphA::kan [23]) and ΔPII (PII deficient; glnB::spc [22]) were routinely grown in liquid BG11 medium (38) supplemented with 5 mM NaHCO₃. The MPphA strain was maintained with kanamycin (50 μg ml⁻¹) selection and the ΔPII strain with spectinomycin selection (35 μg ml⁻¹). In the first set of experiments, wild-type cells of Synechocystis sp. strain PCC 6803 and the mutants ΔPII and MPphA were shifted from nitrogen-poor to nitrogen-excess conditions. Nitrogen-limited cultures were prepared by harvesting cells from 2 ml of nitrate-replete stock culture and resuspending them in 100 ml of modified BG11 medium (low-N BG11) containing 1 mM of nitrate. These cultures were grown in triple-baffled 100 ml flasks with shaking at 30°C, under continuous illumination of 50 μmol photons m⁻² s⁻¹ from white fluorescent lamps. When an optical density at 750 nm of 0.8 to 1.0 was reached (after approximately 4 days for the wild-type and MPphA strains and 5 days for the ΔPII strain), cells started to get slightly bleached due to consumption of nitrate. After the time zero aliquots were taken ammonium chloride was added to a final concentration of 5 mM (nitrogen excess), and aliquots of the culture were harvested in the course of time. From these samples, the accumulation of cyanophycin, activity of NAGK, and phosphorylation status of PII were analyzed. Furthermore, at selected time points, the amount of cellular arginine was determined. Cyanophycin was extracted (40) from 10-ml samples and enzymatically hydrolyzed by recombinant cyanophycinase (37) and recombinant isoaspartyl dipeptidase from Synechocystis sp. strain PCC 6803 (20) to arginine and aspartic acid. The mass of the polymer was calculated from the liberated aspartic acid, quantified enzymatically (33). Values were reproducible within ±5%. For the determination of NAGK activity and the phosphorylation status of PII, cell extracts of the samples were prepared using a Ribolyser (Hybaid) as described previously (19) and protein concentration was estimated using the Brad- ford assay (9). One hundred microliters of extract protein was used for a NAGK assay, and 5 μg of protein was used for PII phosphorylation state analysis. To measure the intracellular arginine level, cells from 4 ml of culture were harvested, suspended in 1 ml of 80% ethanol, and incubated for 3 h at 65°C. Following centrifugation, the supernatant was dried and the arginine content was determined by high-pressure liquid chromatography according to reference 16.

As shown in Fig. 1A, wild-type cells rapidly accumulated cyanophycin following ammonium treatment. By contrast, cyanophycin accumulation was completely absent in the PII-deficient mutant. The PphA-deficient strain showed an intermediate phenotype, having a delayed accumulation of cyanophycin compared to the wild type. Determination of NAGK activity revealed that, following ammonium upshift, wild-type cells rapidly increased the activity of this enzyme. By contrast, the PII-deficient mutant was unable to increase NAGK activity, the same result observed previously in PII-deficient cells of Synechococcus elongatus sp. strain PCC 7942 (19). The PphA-deficient cells showed a delayed increase of NAGK activity, compared to the wild type. Quantification of intracellular arginine following ammonium upshift revealed that the arginine level in wild-type treatments. In the PII-deficient mutant was unable to increase NAGK activity, and cyanophycin accumulation following nitrogen-excess treatments.

The activation state of NAGK, cyanophycin accumulation, and intracellular arginine concentration strongly correlated with the phosphorylation state of PII (Fig. 1C). Previously, we demonstrated that, in Synechococcus sp. strain PCC 7942, the nonphosphorylated form of PII strongly activates NAGK activity (19). Similarly, in the Synechocystis sp. strain PCC 6803 wild-type cells, dephosphorylation of PII correlates with an increase in NAGK activity and cyanophycin accumulation. The PII-deficient mutant showed a delayed increase of NAGK activity, compared to the wild type. The activation state of NAGK, cyanophycin accumulation, and intracellular arginine concentration strongly correlated with the phosphorylation state of PII (Fig. 1C). Previously, we demonstrated that, in Synechococcus sp. strain PCC 7942, the nonphosphorylated form of PII strongly activates NAGK activity (19). Similarly, in the Synechocystis sp. strain PCC 6803 wild-type cells, dephosphorylation of PII correlates with an increase in NAGK activity and cyanophycin accumulation. The PII-deficient mutant showed a delayed increase of NAGK activity, compared to the wild type.
increase in NAGK activity. The increased NAGK activity is accompanied by increased intracellular arginine and cyanophycin concentrations. In contrast, the delay in $P_\text{II}$ dephosphorylation in the MPphA strain correlated with delayed NAGK activation and cyanophycin and arginine accumulation. Immunoblot analysis using NAGK-specific antibodies revealed that the amount of NAGK protein did not significantly change during the time course of the experiment (data not shown).

The above results strongly suggested that $P_\text{II}$-mediated NAGK activation is responsible for increased arginine synthesis, which then leads to cyanophycin accumulation. To verify independently that impaired cyanophycin synthesis in $P_\text{II}$-deficient cells is indeed due to limiting arginine levels and not caused by impaired cyanophycin synthetase activity (26), ammonium upshift experiments in the presence of 5 mM arginine were carried out with wild-type and $P_\text{II}$-deficient cells (Fig. 3).

*Synechocystis* has a highly active arginine transport system (28), resulting in a rapid uptake of externally added arginine. As shown in Fig. 3A, the $P_\text{II}$-deficient mutant, despite low NAGK activity, was now able to accumulate cyanophycin, although to a lesser extent than the wild type (Fig. 3B). The difference between wild type and mutant may be due to the lack of intracellularly synthesized arginine in the $\Delta P_\text{II}$ strain or may indicate an additional requirement for $P_\text{II}$ in cyanophycin synthesis. The recently discovered $P_\text{II}$ receptor PamA in *Synechocystis* may be considered in this context (47). In any case, cyanophycin accumulation can be restored in the $P_\text{II}$-deficient mutant by bypassing the impaired NAGK activity through external addition of arginine, implying that cyanophycin synthesis in the $\Delta P_\text{II}$ strain was limited by the availability of arginine.

Arginine has a dual role in cyanobacteria, first as an amino acid for protein synthesis and second as a nitrogen buffer, storing excess nitrogen in the form of cyanophycin and making it easily available through efficient arginine metabolism (21, 36). $P_\text{II}$ controls the committed step in arginine synthesis as it activates NAGK activity by complex formation. In addition to increasing NAGK catalytic activity, complex formation with $P_\text{II}$ also causes a dramatic reduction in arginine feedback inhibition. Whereas free NAGK was almost completely inhibited by arginine concentrations above 50 $\mu$M, the $P_\text{II}$-complexed NAGK was barely inhibited (32). Therefore, under physiological conditions of $P_\text{II}$-NAGK complex formation, efficient arginine synthesis occurs in the presence of appreciably higher levels of arginine compared to conditions favoring $P_\text{II}$-NAGK complex dissociation. Complex formation occurs with non-phosphorylated $P_\text{II}$ at low levels of 2-oxoglutarate, corresponding to nitrogen-rich conditions. Under nitrogen-poor conditions, however, complex formation is impaired, since $P_\text{II}$ is phosphorylated and the 2-oxoglutarate concentrations are high (19, 32). Cyanophycin synthetase in *Synechocystis* sp. strain PCC 6803 has a $K_m$ for arginine of 49 $\mu$M (2), a concentration which is already inhibitory for free NAGK but not for NAGK in complex with $P_\text{II}$. Therefore, under nitrogen-poor conditions, cyanophycin cannot be formed. Instead, arginine levels should be just sufficient to meet the requirement for protein synthesis, since the $K_m$ values of aminoacyl-tRNA synthetases for their cognate amino acids are usually in the micromolar range. Under conditions of nitrogen excess, however, the NAGK-$P_\text{II}$ complex is formed and arginine synthesis is stimulated, allowing cyanophycin synthesis to occur. The other substrates of cyanophycin synthetase, aspartate and ATP (exhibiting $K_m$ values for Asp of 0.45 mM and for ATP of 0.2 mM [2]), do not seem to limit the reaction in the $P_\text{II}$-deficient mutant, since arginine addition alone was sufficient to restore cyanophycin synthesis. The regulation of NAGK activity by $P_\text{II}$ in response to the nitrogen status thus provides the mechanistic
basis for the dual role of arginine: in the nonactivated state, NAGK activity is sufficient to provide arginine for the purpose of protein synthesis; in the PII-activated state, excess nitrogen can be stored in the form of cyanophycin.

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