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Cyanophycin: A Nitrogen-Rich Reserve Polymer

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Abstract

Cyanophycin is a nitrogen/carbon reserve polymer present in most cyanobacteria as well as in a few heterotrophic bacteria. It is a non-ribosomally synthesized polyamide consisting of aspartate and arginine (multi-L-arginyl-poly-L-aspartic acid). The following chapter provides an overview of the characteristics and occurrence of cyanophycin in cyanobacteria. Information about the enzymes involved in cyanophycin metabolism and the regulation of cyanophycin accumulation is also summarized. Herein, we focus on the main regulator, the P_{II} signal transduction protein and its regulation of arginine biosynthesis. Since cyanophycin could be used in various medical or industrial applications, it is of high biotechnological interest. In the last few years, many studies were published aiming at the large-scale production of cyanophycin in different heterotrophic bacteria, yeasts and plants. Recently, a cyanobacterial production strain has been reported, which shows the highest so ever reported cyanophycin yield. The potential and possibilities of biotechnological cyanophycin production will be reviewed in this chapter.

Keywords: cyanophycin, cyanophycin synthetase, cyanophycinase, nitrogen reserve, polyamide, L-arginine, L-aspartate, P_{II} protein

1. Introduction

Cyanophycin, abbreviated CGP (cyanophycin granule peptide), is next to poly- γ -glutamic acid and poly- ϵ -lysine, the third polyamino acid known to occur in nature [1]. It serves as a nitrogen/carbon reserve polymer in many cyanobacterial strains as well as in a few heterotrophic bacteria. CGP consists of the two amino acids, aspartate and arginine, forming a poly-L-aspartic acid backbone with arginine side chains. The arginine residues are linked to the β -carboxyl group of every aspartyl moiety via isopeptide bond [2].

CGP was discovered in 1887 by the botanist Antonio Borzi during microscopic studies of filamentous cyanobacteria [3]. He observed opaque and light scattering inclusions by using light microscopy and created the name *cianoficina*. Early electron microscopic studies showed a strong structure variation of the CGP granules, depending on the fixatives and poststains used during electron microscopic examinations [4, 5]. This led to a controversy about the ultrastructure of these inclusions until the 1970s. Later, electron microscopic studies described CGP granules as membrane less, electron dense and highly structured cytoplasmic inclusions [6, 7].

With a C/N ratio of 2:1, CGP is extremely rich in nitrogen and consequently an excellent nitrogen storage compound. During the degradation of CGP and subsequent degradation of arginine, a function as energy source was also proposed [8].

2. CGP occurrence

Most cyanobacteria, including unicellular and filamentous, as well as diazotrophic and non-diazotrophic groups are able to accumulate CGP (**Figure 1**).

In non-diazotrophic cyanobacteria, the amount of CGP is usually less than 1% of the cell dry mass during exponential growth. CGP accumulates conspicuously under unbalanced growth conditions including stationary phase, light stress or nutrient limitation (sulfate, phosphate or potassium starvation) that do not involve nitrogen starvation [9, 10]. Under such unbalanced conditions, the amount of CGP may increase up to 18% of the cell dry mass [10]. During the recovery from nitrogen starvation by the addition of a usable nitrogen source, CGP is transiently accumulated [11, 12].

In the unicellular diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, nitrogen fixation and photosynthesis can coexist in the same cell, but temporarily separated. The nitrogen-fixing enzyme, nitrogenase, is highly sensitive to oxygen. Nitrogen fixation occurs in dark periods and the fixed nitrogen is stored in CGP. In the light period, when photosynthesis is performed, the CGP is degraded to mobilize the fixed nitrogen [13]. Transient CGP accumulation during dark periods was also reported in the filamentous cyanobacterium *Trichodesmium* sp., which has a high abundance in tropical and subtropical seas and is an important contributor to global N and C cycling [14].

Furthermore, in heterocysts of diazotrophic cyanobacteria of the order *Nostocales*, polar nodules consisting of CGP are deposited at the contact site to adjacent vegetative cells [15] (**Figure 1**). The heterocystous CGP seems to be involved in transport of fixed nitrogen to the adjacent photosynthetically active vegetative cell. CGP catabolic enzymes are present at significantly higher levels in vegetative cells than in heterocysts. Moreover, CGP could serve as a sink for fixed nitrogen in the heterocyst to avoid feedback inhibition from soluble products of nitrogen fixation [16, 17]. In *Anabaena* sp. PCC 7120 and *Anabaena variabilis*, mutational studies have shown that strains lacking CGP synthetic genes are little affected in diazotrophic growth under standard laboratory conditions [15, 18]. However, a growth defect was observed under high light conditions [15]. Moreover, diazotrophic growth is significantly decreased in strains that are unable to degrade CGP [16, 18].

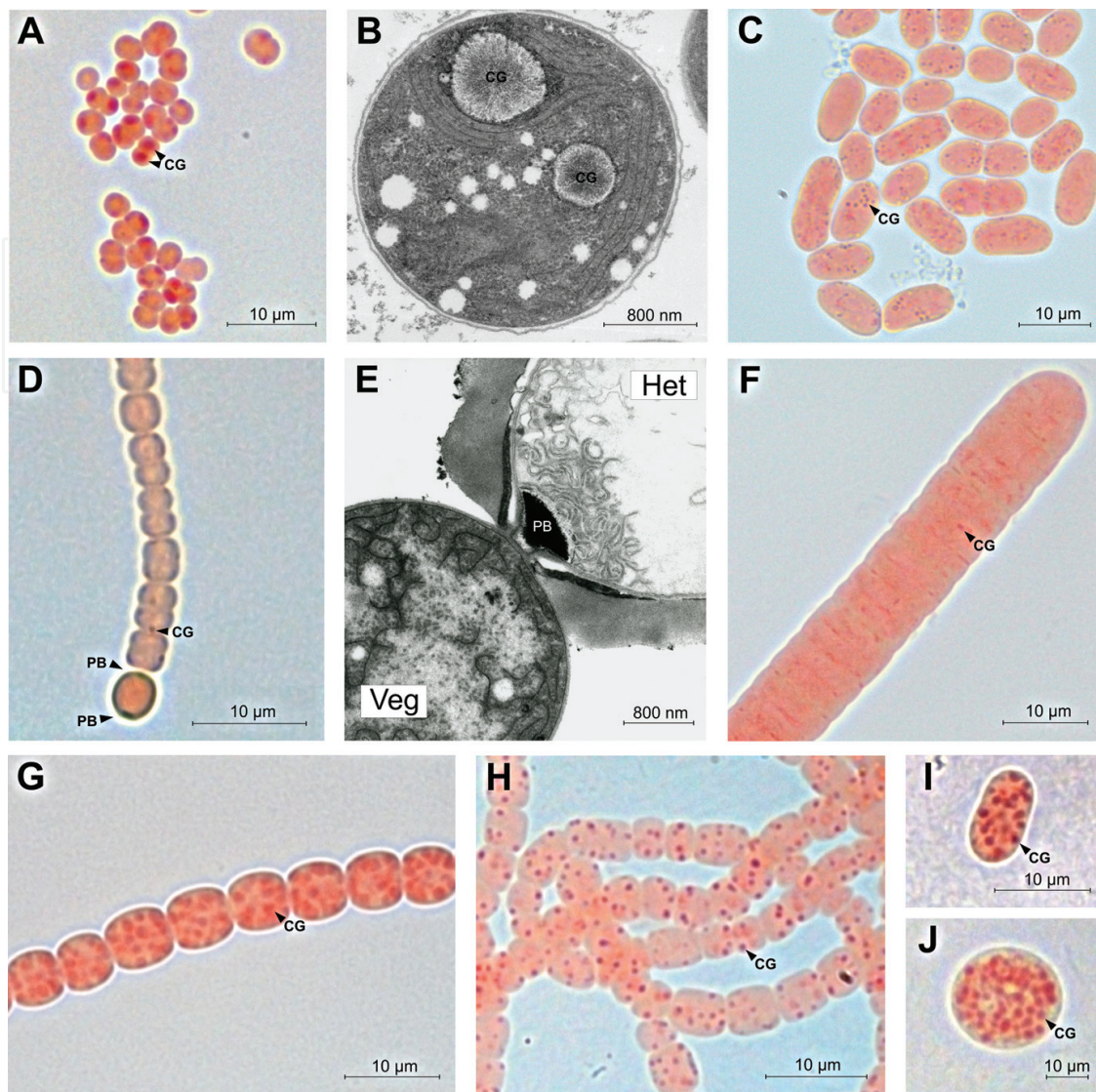


Figure 1. Light and electron microscopic pictures of CGP accumulating cyanobacteria. In light microscopic pictures, CGP was stained using the Sakaguchi reaction [10]. The intensity of the red color indicates the amount of arginine. Dark red to purple dots are CGP granules [CG]. (A) and (B) Phosphate starved *Synechocystis* sp. PCC 6803 in light and transmission electron microscopy, respectively. (C) *Cyanothece* sp. PCC 7424 cultivated in presence of nitrate and continuous light. (D) Filament of diazotrophic growing *Anabaena* sp. PCC 7120 with terminal heterocyst containing polar bodies [PB]. (E) Transmission electron micrographs of a heterocyst and adjacent vegetative cell from *Anabaena* sp. PCC 7120, showing a GCP consisting polar body [PB]. (F) *Oscillatoria* sp. cultivated with nitrate supplementation, showing small CGP granules. (G) Phosphate starved *Anabaena variabilis* ATCC 29413 under nitrate supplemented growth. (H) *Nostoc punctiforme* ATCC 29133 under phosphate starvation and nitrate supplementation. (I) and (J) Mature akinetes of *Anabaena variabilis* ATCC 29413 and *Nostoc punctiforme* ATCC 29133, respectively.

Akinetes are resting spore-like cells of a subgroup of heterocyst-forming cyanobacteria for surviving long periods of unfavorable conditions. During akinete development, the cells transiently accumulate storage compounds, namely glycogen, lipid droplets and CGP [19, 20] (**Figure 1**). CGP granules also appear during germination of dormant akinetes [21]. *Anabaena variabilis* akinetes lacking CGP granules were also able to germinate. This behavior agrees with early observations that CGP is not the direct nitrogen source for protein biosynthesis and therefore not essential for akinete germination [21, 22].

CGP was formally thought to be unique in cyanobacteria. In 2002, Krehenbrink et al. and Ziegler et al. discovered through evaluation of obligate heterotrophic bacteria genomes that many heterotrophic bacteria possess CGP synthetase genes [23, 24]. Genes of CGP metabolism occur in a wide range of different phylogenetic taxa and not closely related to cyanobacteria [25].

3. CGP characteristics

In 1971, Robert Simon isolated CGP granules for the first time by using differential centrifugation. Along with this study, CGP has shown its special and unique solubility behavior [26]. CGP is insoluble at physiological ionic strength and at neutral pH, but soluble in solutions which are acidic, basic or highly ionic. In non-ionic detergents such as Triton X-100, CGP is insoluble; however, in ionic detergents like SDS, it is soluble [6]. Present-day CGP extraction methods are based on its solubility at low pH and insolubility at neutral pH [27].

The chemical structure of CGP was proposed in 1976 by Simon and Weathers [2]. According to this model, CGP has a polymer backbone consisting of α -linked aspartic acid residues. The α -amino group of arginine is linked via isopeptide bonds to the β -carboxylic group of every aspartyl moiety. Because every aspartate residue is linked to an arginine residue, CGP contains equimolar amounts of aspartate and arginine [2]. This structure has been confirmed via enzymatic degradation studies. CGP-degrading enzymes (see below) release β -Asp-Arg dipeptides [28]. CD spectroscopy data suggest that the acid-soluble and neutral insoluble forms of CGP have similar conformations. Both forms contain substantial fractions of β -pleated sheet structure [29].

Cyanobacterial CGP has a molecular weight and polydispersity ranging from 25 to 100 kDa [26]. In contrast, the native CGP producer *Acinetobacter* sp. ADP1 synthesizes CGP with a lower molecular weight ranging from 21 to 28 kDa [30]. Recombinant bacteria or genetically engineered yeast harboring heterologous expression of cyanobacterial CGP synthesis genes also show a lower molecular weight of 25–45 kDa [27, 31]. Transgenic plant-produced CGP also shows a reduced polydispersity between 20 and 35 kDa [32]. A possible explanation would be that cyanophycin synthesis in the native cyanobacterial background involves additional factors contributing the polymer length. These additional factors should also be absent in *Acinetobacter* sp. ADP1.

Native CGP is exclusively composed of aspartate and arginine. By contrast, in CGP isolated from recombinant *E. coli* expressing cyanophycin synthetase (see below) from *Synechocystis* sp. PCC 6803, besides aspartate and arginine, lysine has been found [33]. The amount of incorporated lysine in CGP influences its solubility behavior. Recombinant CGP with a high lysine amount (higher than 31 mol%) is soluble at neutral pH [34].

4. CGP metabolism

4.1. Cyanophycin synthetase

CGP is non-ribosomally synthesized from aspartate and arginine by cyanophycin synthetase (CphA1) (**Figure 2**). In 1976, CphA1 was enriched and characterized for the first time by

Simion [35]. The enzyme incorporates aspartate and arginine in an elongation reaction, which requires ATP, KCl, MgCl_2 and a sulfhydryl reagent (β -mercaptoethanol or DTT). For its activity, CphA1 needs a so far unknown CGP primer, as a starting point of the elongation reaction [35]. By using synthetically primers, Berg et al. could show that a single building block of CGP (β -Asp-Arg) does not serve as an efficient primer for CphA1 elongation reaction in vitro. The primers need to consist of at least three Asp-Arg building blocks (β -Asp-Arg) $_3$ to detect CphA1 activity [36]. Other peptides, like cell wall or other cellular components, have been suggested to serve as an alternative priming substance for the CphA1 reaction [37]. This could be an explanation for the functionality of CGP synthesis in recombinant bacteria, without the ability to produce native CGP primers [38]. Interestingly, the CphA1 of *Thermosynechococcus elongatus* strain BP-1 shows primer-independent CGP synthesis [39].

Today, CphA1 enzymes from several bacteria, including cyanobacteria and heterotrophic bacteria, have been purified and characterized [33, 39–42]. The molecular mass of the characterized CphA1 enzymes ranges from 90 to 130 kDa. The active form of CphA1s from *Synechocystis* sp. PCC6308 and *Anabaena variabilis* PCC7937 is most likely homodimeric [33, 41], while the primer-independent CphA1 from *Thermosynechococcus elongatus* strain BP-1 forms a homotetramer [39]. The primary structure of cyanobacterial CphA1 can be divided into two regions [33]. The C-terminal region shows sequence similarities to peptide ligases that include murein ligases and folyl poly- γ -glutamate ligase. The N-terminal part of CphA1 shows sequence similarities with another superfamily of ATP-dependent ligases that include carboxylate-thiol

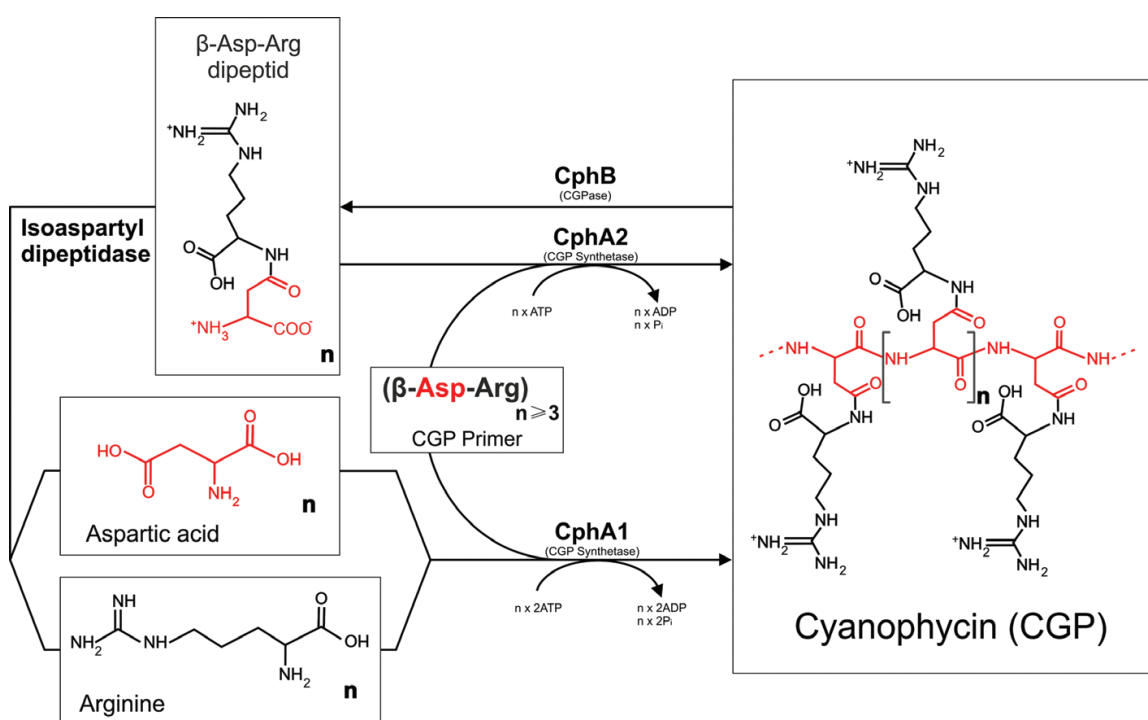


Figure 2. Schematic illustration of CGP metabolism in cyanobacteria. CGP is synthesized from aspartate and arginine by CGP synthetase (CphA1) in an ATP-depending elongation reaction using CGP primers, containing of at least three Asp-Arg building blocks. Intracellular CGP degradation is catalyzed by the CGPase (CphB). The β -Asp-Arg dipeptides resulting from cleavage of CGP are further hydrolyzed by isoaspartyl dipeptidase, releasing aspartate and arginine. In many nitrogen-fixing cyanobacteria, an additional CGP synthetase is present, termed CphA2. CphA2 can use β -aspartyl-arginine dipeptides to resynthesize CGP.

and carboxylate-amine ligase. Since the C- and N-terminal parts show similarity to different superfamilies of ATP-dependent ligases, two ATP-binding sites and two different active sites have been predicted [36]. In vitro experiments revealed that arginine is probably bound in the C-terminal and aspartate in the N-terminal active site [43].

The mechanism of CGP synthesis by CphA1 has been suggested by Berg et al. in 2000, by measuring the step-wise incorporation of amino acids to the C-terminus of the CGP primer. The putative CGP elongation cycle starts at the C-terminal end of the poly-aspartate backbone. First, the carboxylic acid group of the poly-aspartate backbone is activated by transfer of the γ -phosphoryl group of ATP. In the second step, one aspartate is bound at the C-terminus of the growing polymer by its amino group, forming a peptide bound. Subsequently, the intermediate (β -Asp-Arg)_n-Asp is transferred to the second active site of CphA1 and phosphorylated at the β -carboxyl group of the aspartate. Finally, the α -group of arginine is linked to the β -carboxyl group of aspartate, forming an isopeptide bound [36].

Various CphA1 enzymes have been characterized with respect to their substrate affinity and specificity. For CphA1 of *Synechocystis* sp. PCC 6308, apparent K_m values were determined to be 450 μ M for aspartate, 49 μ M for arginine, 200 μ M for ATP and 35 μ g/ml CGP as priming substance. The lower K_m of arginine compared to aspartate indicates a higher affinity of CphA1 towards arginine. During the in vitro reaction, CphA1 converts per mol incorporated amino acid 1.3 ± 0.1 mol ATP to ADP. The optimal reaction conditions of this enzyme were at pH 8.2 and 50°C [41].

CphA homologs are widely distributed in eubacteria. In silico analysis proposes 10 different groups of cyanophycin synthetases [25]. In cyanobacteria, cyanophycin synthetases of group I–III (CphA, CphA2 and CphA2') can be found.

Recently, the function of a cyanophycin synthetase of group II (CphA2) has been characterized. Most non-diazotrophic cyanobacteria use a single type of cyanophycin synthetase (CphA1). However, in many nitrogen-fixing cyanobacteria, an additional version of CphA is present, termed CphA2. In 2016, Klemke et al. resolved the function of CphA2 [44]. Compared to CphA1, CphA2 has a reduced size and just one ATP-binding site. CphA2 uses the product of CGP hydrolysis, β -aspartyl-arginine dipeptide as substrate to resynthesize cyanophycin, consuming one molecule of ATP per elongation. A mutant lacking CphA2 shows only a minor decrease in the overall CGP content. However, a CphA2-deficient mutant displays similar defects under diazotrophic and high light conditions than a CphA1 mutant [15, 44]. This observation suggests that the apparent “futile cycle” of CGP hydrolysis and immediate re-polymerization is probably of physiological significance in the context of nitrogen fixation [17].

4.2. Cyanophycinase

Since 1976, it is known that CGP is resistant against hydrolytic cleavage by several proteases or arginase [2, 45]. This resistance is probably due to the branched structure of CGP [38]. Therefore, the presence of a highly specified peptidase for CGP hydrolysis was suggested.

In 1999, Richter et al. reported a CGP hydrolyzing enzyme from the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, called CphB [28] (**Figure 2**). During this study, CphB was purified

and studied in detail. CphB is a 29.4 kDa C-terminal exopeptidase, catalyzing the hydrolyzation of CGP to β -Asp-Arg dipeptides [28]. Based on sequence analysis and inhibitor sensitivity to serine protease inhibitors, CphB appears to be a serine-type exopeptidase related to dipeptidase E (PepE) [28]. According to its sequence, CphB contains a serine residue within a lipase box motive (Gly-Xaa-Ser-Xaa-Gly). The serine residue together with a glutamic acid residue and a histidine residue forms the catalytic triad, which is typical for serine-type peptidases [28]. In 2009, the crystal structure has been solved at a resolution of 1.5 Å, showing that CphB forms a dimer. Site-directed mutagenesis confirms that CphB is a serine-type peptidase, consisting of a conserved pocket with the catalytic Ser at position 132 [46]. Structure modeling indicates that the cleavage specificity occurs due to an extended conformation in the active site pocket. The unique conformation of the active site pocket requires β -linked aspartyl peptides for binding and catalysis, preventing CphB from non-specific cleavage of other polypeptides next to CGP [46].

In addition to CphB, which catalyzes the intracellular cleavage of CGP, other versions of cyanophycinase exist, catalyzing the extracellular hydrolysis of CGP. In 2002, Obst et al. isolated several Gram-negative bacteria from different habitats, which were able to utilize CGP as a source of carbon and energy [47, 48]. One isolate was affiliated as *Pseudomonas anguilliseptica* strain BI. In the supernatant of a *Pseudomonas anguilliseptica* culture, a cyanophycinase was found and purified, called CphE [47]. CphE exhibits a high specificity for CGP; however, proteins were not or only marginally hydrolyzed. Degradation products of CphE are β -Asp-Arg dipeptides. Inhibitor sensitivity studies indicated that the catalytic mechanism of CphE is related to serine-type proteases. CphE from *Pseudomonas anguilliseptica* strain BI exhibits an amino acid sequence identity 27–28% to intracellular CphB enzymes of cyanobacteria [47]. Today, extracellular CGPases has been found in a high variety of bacteria including Gram-positive, Gram-negative, aerobic and anaerobic strains. This indicates that the extracellular cleavage and utilization of CGP as carbon, nitrogen and energy source is a common principle in nature [47–53].

In 2007, in silico analysis showed that CphB homologs are widely distributed in eubacteria, proposing eight different groups including intracellular and extracellular CGPases. CGPases from cyanobacteria belong to group I, II and partially group III (CphB₁₋₃). Groups IV–VIII, including CphE, are present in a large variety of non-photosynthetic bacteria [25].

4.3. Aspartyl-arginine dipeptidase

The last step in catabolism of CGP is the cleavage of β -Asp-Arg dipeptides to monomeric amino acids, arginine and aspartate (**Figure 2**). In 1999, Richter et al. found β -Asp-Arg dipeptides hydrolyzing activity in extracts of *Synechocystis* sp. PCC 6803 [28]. In *Synechocystis* sp. PCC 6803, the ORF sll0422 as well as ORF all3922 from *Anabaena* sp. PCC 7120 is annotated as “plant-type asparaginase,” because of sequence similarities to the first cloned asparaginase from plants [54]. During characterization of plant-type asparaginase in general, including Sll0422 and All3922, Hejazi et al. were able to show that these enzymes are able to hydrolyze a wide range of isoaspartyl dipeptides [55]. Isoaspartyl peptides arise from two biological pathways: First, proteolytic degradation of modified proteins containing isoaspartyl residues and second, as primary degradation product of CGP cleavage from CGPases. Thus, the plant-type

asparaginases, Slr0422 and All3922, have not only a function in asparagine catabolism but also in the final step of CGP and protein degradation [55].

The mature isoaspartyl dipeptidases of *Synechocystis* sp. PCC 6803 and *Anabaena* sp. PCC 7120 consist of two protein subunits that are generated by autocleavage of the primary translation product between Gly-172 and Thr-173 (numbering according to *Synechocystis* sp. PCC 6803) within the conserved consensus sequence GT(I/V)G [55]. The native molecular weight of approximately 70kD of this enzyme suggests that it has a subunit structure of $\alpha_2\beta_2$ (α derived from the N-terminal part and β from the C-terminal part of the precursor) [55].

In *Anabaena* sp. PCC 7120, all genes involved in CGP metabolism as well as the isoaspartyl dipeptidases All3922 are expressed in vegetative cells and heterocysts but in different expression levels. Both, CGP synthetases and CGPases are much higher expressed in heterocysts than in vegetative cells [56]. However, asparaginase All3922 is present in significantly lower levels in heterocysts than in vegetative cells [57]. A deletion of All3922 in *Anabaena* sp. PCC 7120 causes an increased accumulation of CGP and β -Asp-Arg dipeptides. Furthermore, a deletion mutant shows an impaired diazotrophic growth similar to the phenotype known from CphB deletion mutants in *Anabaena* sp. PCC 7120 [18, 57]. This observation implies that the first step of CGP catabolism, the cleavage catalyzed by CphB, takes place in the heterocyst. The released β -Asp-Arg dipeptides are transported to the adjacent vegetative cells. Isoaspartyl dipeptidase All3922, present in the vegetative cells, cleaves the β -Asp-Arg dipeptides and releases monomeric aspartate and arginine [57]. When CGP synthesis is not possible, due to a deletion of CphA, arginine and aspartate might be transferred directly from heterocysts. This explains the minor effects on diazotrophic growth in a CphA deletion mutant [15]. These results identified β -Asp-Arg dipeptides as nitrogen vehicle in diazotrophic heterocyst-forming cyanobacteria, next to glutamine and arginine alone or with aspartate [57–59]. A benefit of β -Asp-Arg dipeptides as nitrogen transport substance is avoiding the release of free arginine and aspartate in the heterocyst. This indicates that CGP metabolism has evolved in multicellular heterocyst-forming cyanobacteria to increase the efficiency of nitrogen fixation [57].

5. CGP regulation

5.1. Genetic organization of CphA and CphB

Usually, genes involved in CGP metabolism are clustered. The organization of these clusters can be different, depending on the respective organism [25]. In *Synechocystis* sp. PCC 6803, *cphA* and *cphB* are adjacent; however, they are expressed independently [60]. A hypothetical protein named slr2003 is located downstream of *cphA* and is transcribed in a polycistronic unit with *cphA* [60]. However, the function of Slr2003 is unknown. In the gene of CphB (slr2001), a small antisense RNA was detected (transcriptional unit 1486) [60].

In *Anabaena* sp. PCC 7120, two clusters containing CphA and CphB were identified [18]. In the *cph1* cluster, *cphB1* and *cphA1* were expressed under ammonia and nitrate supplemented growth, but the expression of both genes was higher in the absence of combined nitrogen in

heterocysts and vegetative cells. In the *cph1* operon, *cphB1* and *cphA1* were cotranscribed. In addition, *cphA1* can be expressed from independent promoters, of which one is constitutive and the other regulated by the global nitrogen control transcriptional factor NtcA [18].

In cluster *cph2*, the *cphB2* and *cphA2* genes were found in opposite orientation and both genes were expressed monocistronically. The genes were expressed under conditions of ammonia, nitrate or N₂ supplementation, but the expression was higher in the absence of ammonia. Generally, the expression of the *cph2* is lower compared to *cph1* [18].

In addition to these two gene clusters, a third set of ORFs containing putative *cphA* and *cphB* genes was found in *Nostoc punctiforme* PCC 73102 and *Anabaena variabilis* ATCC 29413 [25].

5.2. Dependence of CGP metabolism on arginine biosynthesis

Generally, CGP accumulation is triggered by cell growth arresting stress conditions, such as entry into stationary phase, light or temperature stress, limitation of macronutrients (with the exception of nitrogen starvation) or inhibition of translation by adding antibiotics like chloramphenicol [9, 10, 61]. All of these CGP triggering conditions result in a reduced or arrested growth. In exponential growth phase the amino acids arginine and aspartate are mostly used for protein biosynthesis with the consequence of a low intracellular level of free amino acids. Under growth-limiting conditions, protein biosynthesis is slowed down, which yields an excess of monomeric amino acids in the cytoplasm, triggering the CGP biosynthesis [10].

CGP accumulation also requires an excess of nitrogen. For the filamentous cyanobacterium *Calothrix* sp. strain PCC 7601, it was shown that CGP accumulation occurs preferably in the presence of ammonia [62]. The addition of amino acids to the media further increased CGP formation [63]. During process optimization studies for heterotrophic CGP production in the strain *Acinetobacter calcoaceticus* ADP1, it was shown that addition of arginine to the medium as sole carbon source increased CGP accumulation drastically. When, in *A. calcoaceticus* strain ADP1, CGP synthesis is induced by phosphate starvation, it accounts to 3.5% (w/w) of the cell dry matter (CDM) with ammonia as nitrogen source. Additional supply of the medium with arginine increases the CGP amount to 41.4% (w/w) (CDM). Notably, a combined supply of arginine and aspartate has a much lower stimulating effect to CGP accumulation than arginine alone [30].

A potential link between regulation of arginine biosynthesis and GCP metabolism was suggested in many previous studies. In a transposon mutagenesis study in the filamentous cyanobacterium *Nostoc ellipsosporum*, an arginine biosynthesis gene, *argL*, was interrupted by a transposon. This mutation partially impairs arginine biosynthesis but does not strictly result in L-arginine auxotrophy. Without arginine supplementation, heterocysts failed to fix nitrogen, akinetes were unable to germinate and CGP granules did not appear. However, when both nitrate and arginine are present in the media, the impaired arginine biosynthesis is bypassed. Under this condition, the mutant could form CGP and was able to differentiate functional akinetes, which contained CGP granules [64].

In metabolic engineering studies of the CGP production strain *Acinetobacter calcoaceticus* ADP1, several genes related to the arginine biosyntheses or its regulation were modified to yield higher amounts of arginine. As a consequence, significant higher CGP production was observed [65].

Bacteria produce arginine from glutamate in eight steps. The first five steps involving N-acetylated intermediates lead to ornithine. The conversion of ornithine to arginine requires three additional steps [66]. The second enzyme of ornithine biosynthesis is the N-acetylglutamate kinase (NAGK), which catalyzes the phosphorylation of N-acetyl glutamate to N-acetylglutamyl-phosphate. NAGK catalyzes the controlling step in arginine biosynthesis [67]. NAGK activity is subjected to allosteric feedback inhibition by arginine and is, moreover, positively controlled by the P_{II} signal transduction protein (see below) [67, 68]. Maheswaran et al. showed that arginine production and the following CGP accumulation depend on the catalytic activation of NAGK by the signal transduction protein P_{II} [69]. In a P_{II} -deficient mutant of *Synechocystis* sp. PCC 6803, NAGK remained in a low activity state, which caused impaired CGP accumulation [69].

The nitrogen-regulated response regulator NrrA also has influence on arginine and CGP biosynthesis. An NrrA-deficient mutant in *Synechocystis* sp. PCC 6803 shows reduced intracellular arginine levels and, consequently, reduced CGP amount [70].

All these results and observations point towards arginine as main bottleneck of CGP biosynthesis, while aspartate plays a minor role. CGP accumulation occurs as a result of arginine enrichment in the cytoplasm. Reasons for increased arginine content in the cell are lowered protein biosynthesis as a result of various growth limiting conditions. Furthermore, an excess of nitrogen and energy sensed by P_{II} leads to NAGK activation and thereby increased arginine biosynthesis.

5.3. P_{II} regulation of arginine metabolism

The P_{II} signal transduction proteins are widely distributed in prokaryotes and chloroplasts, where they play a coordinating role in the regulation of nitrogen assimilatory processes [71–73]. For this purpose, P_{II} senses the energy status of the cell by binding ATP or ADP in a competitive way [74]. Binding of ATP and synergistic binding of 2-oxoglutarate (2-OG) allows P_{II} to sense the current carbon/nitrogen status of the cell [75]. 2-OG is the carbon skeleton for the GS/GOGAT reactions and thereby links the carbon and nitrogen metabolism in all domains of life [76, 77]. The pool size of 2-OG reacts quickly to changes in nitrogen availability, wherefore 2-OG is an indicator of the carbon/nitrogen balance [78, 79]. Depending on the nitrogen supply, P_{II} may be phosphorylated at the apex of the T-loop at position Ser49 [80, 81]. Binding of the effector molecules ATP, ADP and 2-OG as well as phosphorylation leads to conformational rearrangements of the large surface-exposed T-loop, P_{II} 's major protein-interaction structure [82]. These conformational states direct the interaction of P_{II} with its various interaction partners and thereby regulate the cellular C/N balance [83].

In cyanobacteria, P_{II} regulates the global nitrogen control transcriptional factor NtcA, through binding to the NtcA co-activator PipX [84]. In common with other bacteria, cyanobacterial P_{II} proteins can interact with the biotin carboxyl carrier protein (BCCP) of acetyl-CoA carboxylase (ACC) and thereby control the acetyl-CoA levels [85]. Furthermore, P_{II} controls arginine biosynthesis via regulation of NAGK [68, 69, 86].

P_{II} proteins form a cylindrical-shaped homotrimer with 12–13 kDa per subunits. The T-loop, a large and surface-exposed loop, protrudes from each subunit. The effector binding sites are positioned in the three inter-subunit clefts [87, 88]. If sufficient energy and nitrogen are available,

indicated by a high ATP and low 2-OG level, non-phosphorylated P_{II} forms an activating complex with NAGK.

The crystal structure of the P_{II} -NAGK complex from *Synechococcus elongatus* strain PCC 7942 revealed two P_{II} trimers sandwiching a NAGK homohexamer (trimer of dimers) [88]. Each P_{II} subunit contacts one NAGK subunit [88]. Two parts of P_{II} are involved in interaction with NAGK. The first structure, called B-loop, is located on the P_{II} body and interacts with the C-domain of NAGK subunit, involving residue Glu85. The interaction of the B-loop is the first step in complex formation. Second, the T-loop must adopt a bent conformation and insert into the interdomain cleft of NAGK [89]. This enhances the catalytic efficiency of NAGK, with the V_{max} increasing fourfold and the K_m for N-acetylglutamate decreasing by a factor of 10 [86]. Furthermore, feedback inhibition of NAGK by arginine is strongly decreased in the presence of P_{II} [86].

During P_{II} mutagenesis, a P_{II} variant was identified that binds constitutively NAGK in vitro. This P_{II} variant exhibits a single amino acid replacement, Ile86 to Asn86, hereafter referred as P_{II} (I86N) [89]. The crystal structure of P_{II} (I86N) has been solved, showing an almost identical backbone than wild-type P_{II} . However, the T-loop adopts a compact conformation, which is a structural mimic of P_{II} in the NAGK complex [89, 90]. Addition of 2-OG in the presence of ATP normally leads to a dissociation of the P_{II} -NAGK complex, however P_{II} (I86N) no longer responds to 2-OG [90].

The P_{II} (I86N) variant enables a novel approach of metabolic pathway engineering by using custom-tailored P_{II} signaling proteins. By replacing the wild-type P_{II} with a P_{II} carrying the mutation for I86N in *Synechocystis* sp. PCC 6803, it was possible to engineer the first cyanobacterial CGP overproducer strain. Strain BW86, containing the P_{II} (I86N) version, shows an increase of NAGK activity, which causes a more than 10-fold higher arginine content than the wild-type [10]. Under balanced growth conditions with nitrate as nitrogen source, strain BW86 accumulates up to $15.6 \pm 5.4\%$ CGP relative to the CDM, i.e., on average almost sixfold more than the wild type. Appropriate starvation conditions can further increase the CGP content of strain BW86 up to $47.4 \pm 2.3\%$ per CDM under phosphate starvation and $57.3 \pm 11.1\%$ per CDM under potassium starvation, without addition of arginine to the medium [10]. Furthermore, the CGP, which is produced by strain BW86, shows a high polydispersity ranging from 25 to 100 kDa, similar to the polydispersity of cyanobacterial wild-type CGP, which contrasts CGP from recombinant producer strains using heterologous expression systems with heterotrophic bacteria, yeasts or plants [10]. CGP isolated from those strains have a size ranging of 25–45 kDa [27, 31, 32].

6. Industrial applications

Industrial applications for CGP have previously mainly focused on chemical derivatives. CGP can be converted via hydrolytic β -cleavage to poly(α -L-aspartic acid) (PAA) and free arginine. PAA is biodegradable and has a high number of negatively charged carboxylic groups, making PAA a possible substituent for polyacrylates [48, 50, 91]. PAA can be employed as anti-scalant or dispersing ingredient in many fields of applications, including washing detergents or suntan lotions. Furthermore, PAA has potential application areas as an additive in paper, paint, building or oil industry [48, 50].

CGP can also serve as a source for dipeptides and amino acids in food, feed and pharmaceutical industry. The amino acids arginine (semi-essential), aspartate (non-essential) and lysine (essential) derived from CGP have a broad spectrum of nutritional or therapeutic applications. Large-scale production of these amino acids, as mixtures or dipeptides, is established in industry, with various commercial products already available on the market (reviewed by Sallam and Steinbuechel [92]).

Potential applications of non-modified CGP have been discussed but remain so far largely unexplored. This can partially be explained by the lack of research being conducted on the material properties of CGP. Recently in 2017, the first study regarding CGP material properties has been published. In this study, Khlystov et al. focused on the structural, thermal, mechanical and solution properties of CGP produced by recombinant *E. coli*, giving new insights in the nature of this polymer as bulk chemical [91]. They describe CGP as an amorphous, glassy polyzwitterion with high thermostability. The dry material is stiff and brittle. According to these properties, CGP could be used to synthesize zwitterionomeric copolymers or as reinforcing fillers [91].

7. Biotechnological production

Previous ventures to produce CGP in high amounts were mainly focused on heterotrophic bacteria, yeasts and plants as production host. These recombinant production hosts heterologously express CGP synthetase genes, mostly from cyanobacteria. In this way, heterotrophic bacteria, which are established in biotechnological industry including *E. coli*, *Corynebacterium glutamicum*, *Cupriavidus necator* (formally known as *Ralstonia eutropha*) and *Pseudomonas putida*, were used for heterologous production of CGP [93].

Strain *E. coli* DH1, containing *cphA* from *Synechocystis* sp. PCC6803, was used for large-scale production of CGP in a culture volume of up to 500 liter, allowing the isolation of CGP in a kilogram scale. During process optimization, the highest observed CGP content was 24% (w/w) per CDM. However, the synthesis of CGP was strongly dependent on the presence of complex components in the medium (terrific broth complex medium). In mineral salt medium, CGP accumulation only occurs in the presence of casamino acids [27]. An engineered version of *CphA* from *Nostoc ellipsosporum*, transformed in *E. coli*, shows a further increase in CGP production, up to 34.5% (w/w) of CDM. However, this production strain also requires expensive complex growth media to yield such a high amount of CGP [94].

Cupriavidus necator and *Pseudomonas putida* are known as model organisms for the industrial scale production of polyhydroxyalkanoates (PHA). Therefore, they have been considered as candidates for large scale CGP production [93, 95]. Metabolic engineering and process optimization studies of *Cupriavidus necator* and *Pseudomonas putida* harboring *cphA* from *Synechocystis* sp. PCC 6803 or *Anabaena* sp. PCC 7120 were performed. In these organisms, the accumulation of CGP is mainly depending on the origin of the *cphA* gene, the accumulation of other storage compounds like PHA as well as the addition of precursor components like arginine to the medium [96]. PHA-deficient mutants of *Cupriavidus necator* and *Pseudomonas putida* accumulate in general more CGP compared to the PHA containing strains [96]. During genetic modification of *cphA* expression in *Cupriavidus necator*, CGP accumulation turned out to be

strongly affected by the expression system. A stabilized multi-copy *cphA* expression system, using the KDPG-aldolase gene (*eda*)-dependent addiction system, allows cultivation without antibiotic selection. The multi-copy *cphA* expression results in a CGP yield between 26.9% and 40.0% (w/w) of CDM. The maximum amount of 40.0% (w/w) of CDM was observed in a 30- and 500-l pilot plant. In the absence of the amino acids arginine and aspartic acid in the medium, the CGP amount was still between 26.9% and 27.7% (w/w) of CDM [97].

The industrially established host *Saccharomyces cerevisiae* has also been used for CGP production, by expression of *cphA* from *Synechocystis* sp. PCC 6803. *S. cerevisiae* harboring *cphA* accumulated up to 6.9% (w/w) of CDM. Two CGP species were observed in this strain: water-soluble and the typical water-insoluble CGP. Furthermore, the isolated polymer from this transgenic yeast contained 2 mol% lysine, which can be increased up to 10 mol% when cultivation occurs with lysine in the medium [31]. During metabolic engineering studies, several arginine biosynthesis mutants have been analyzed concerning their CGP accumulation abilities. Surprisingly, strains with defects in arginine degradation accumulated only 4% CGP (w/w) of CDM; however, arginine auxotrophic strains were able to accumulate up to 15.3%. Depending on the cultivation conditions, between 30 and 90% of the extracted CGP was soluble at neutral pH. In addition to arginine, aspartate and lysine, further amino acids, such as citrulline and ornithine, have been detected in isolated CGP from different arginine biosynthesis mutants [98]. Furthermore, it was also possible to produce CGP and CGP derivatives in *Pseudomonas putida* and the yeast *Pichia pastoris* [99, 100].

CGP and CGP derivatives are important sources for β -dipeptides for several applications. A large-scale method was developed to convert CGP into its constituting β -dipeptides by using CphE from *Pseudomonas alcaligenes*. This allows the large-scale production of customized β -dipeptides, depending on the composition of the CGP derivatives [92, 101].

Production of CGP has also been attempted in several transgenic plants. Here, ectopic expression of the primer-independent CphA from *Thermosynechococcus elongatus* BP-1 leads to an accumulation of CGP up to 6.8% (w/w) in tobacco leaves and to 7.5% (w/w) of CDM in potato tubers [102, 103]. CGP production and extraction in plants can be coupled with the production of other plant products like starch [103]. The peculiarities and challenges of plant-produced CGP have been reviewed by Nausch et al. [32].

Compared to bacteria that are used so far in biotechnological industry, cyanobacteria are unique as they use sunlight and CO₂ as energy and carbon source. Cyanobacteria have been identified as rich source of various biologically active compounds, biofertilizers, bioplastics, energy, food and feed [104]. Obviously, the importance of environmentally friendly production processes increases more and more. Hence, Cyanobacteria are expected to play a major role in future industry. *Synechocystis* sp. PCC 6803 strain BW86 is the first reported bulk chemical producing cyanobacterial strain in the literature. CGP production in *Synechocystis* BW86 does not require organic carbon or CGP precursor substances. Growth limiting conditions like phosphate and potassium starvation can further increase the CGP production up to $47.4 \pm 2.3\%$ and $57.3 \pm 11.1\%$ per CDM, respectively. The studies of Trautmann et al. showed that strain BW86 can be cultivated in flat plate photobioreactors (Midiplate reactor system [105]). During this optimization study, the optimal light intensity as well as the phosphate concentration was determined to maximize CGP synthesis. Under optimal production conditions, highest amount of CGP was around 40% of CDM with a total yield of 340 mg CGP per liter in 9 days [106].

The main bottleneck of CGP production in Cyanobacteria is the relatively slow growth rate, which is much lower than in biotechnologically established bacteria. Conventional cultivation methods of cyanobacteria reach a biomass of roughly 1 g dry mass per liter [107]. To overcome this limitation, a new cultivation method was developed, using a two-tier vessel with membrane-mediated CO₂ supply. By using this cultivation setup, it was possible to enable rapid growth of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 up to 30 g CDM per liter [108]. *Synechocystis* sp. PCC 6803 strain BW86 was also used in this high-density cultivation setup. During this study, CGP amounts up to 1 g per liter were reached in 96 h. This is approximately four times higher compared to the maximum CGP yield observed during conventional cultivation after 12 days [106, 109].

In comparison, the recombinant *E. coli* strain DH1 harboring *cphA* from *Synechocystis* sp. PCC 6803 produces between 6.7 and 8.3 g CDM per liter culture in 16 h. CGP amounts during this fed-batch fermentations were between 21 and 24% of the CDM [27], resulting in a CGP production rate of 87.9 to 124.5 mg/l and hour. Although this exceeds the production rate in *Synechocystis* sp. PCC 6803 strain BW86 by a factor of 10, the recombinant *E. coli* requires terrific broth complex medium, while *Synechocystis* sp. PCC 6803 strain BW86 is cultivated in simple mineral medium and additionally sequesters hazardous greenhouse gas CO₂. Considering these super ordinate factors, production of biopolymers with cyanobacteria may in fact become an alternative to heterotrophic bacteria.

8. Conclusions

CGP is well researched and its occurrence in cyanobacteria is known for more than 100 years. However, many questions are still open. Most obviously, the cell biology of the CGP granules remains largely unknown. In the last decades, research on CGP mainly focused on biotechnological purposes, like strain or process optimization. Most work has been carried out with short-chain CGP from recombinant producer strains; however the biophysical properties of the long-chain native CGP remain largely unexplored. So far, heterotrophic bacteria were mainly used to produce industrial biocompounds including CGP. In this chapter, we discussed the possibility of a cyanobacterial CGP production strain. The main disadvantages of cyanobacteria, their slower growth and the low abundance of product can be compensated using genetic engineering together with appropriate production processes. Future industry has to cope with the manifold challenges to counteract environmental pollution and climate change. The use of cyanobacteria in CGP production and, more generally, in biotechnological applications for bioproduct synthesis provides an environmentally friendly alternative to conventional biotechnological approaches.

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Conflict of interest

The authors declare that they have no competing interests.

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