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Inorganic Carbon Assimilation in Cyanobacteria: Mechanisms, Regulation, and Engineering

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1.1 Introduction – The Need for a Carbon-Concentrating Mechanism

Cyanobacteria are oxygenic phototrophs that occur in almost all photic habitats on the Earth. During their long-lasting evolution, they adapted to a wide range of abiotic conditions. Among them, the availability of the photosynthetic substrate inorganic carbon (C_i, including CO_2 and dissolved inorganic carbon, mostly bicarbonate – HCO_3^-) can fluctuate on different timescales. It is generally accepted that oxygenic photosynthesis evolved in cyanobacteria at least 2.7 billion years ago, a time when C_i was at much higher levels in the Earth atmosphere (6000 ppm) and the oceans [1–3]. Because of the activity of photosynthetic organisms, particularly after the rise of eukaryotic algae and plants, the cyanobacterial habitats became strongly depleted of C_i, accompanied by an increase in molecular oxygen (O_2). Over the long term, cyanobacteria adapted from a C_i-rich, O_2-free atmosphere to a C_i-poor, O_2-rich atmosphere. This change had drastic consequences for C_i assimilation, in particular for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the carboxylating enzyme of the Calvin–Benson–Bassham cycle (CBBC). RubisCO is not a *bona fide* carboxylase; it evolved from an enolase in the methionine salvage pathway [4]. In CBBC, RubisCO catalyzes the activation of the acceptor molecule ribulose 1,5-bisphosphate (RuBP) by changing its enol into the reactive keto conformation; this activation allows CO_2 binding to RuBP and its carboxylation. Then, the non-stable carboxylation product is hydrolytically split into two stable molecules of 3-phosphoglycerate (3PGA). Generally, RubisCO is characterized by a rather low speed and low affinity toward CO_2. Cyanobacterial RubisCO proteins, belonging to the "type 1" RubisCOs, have a particularly high K_m for CO_2 of approximately 300 μM, which is about 10-fold higher than the concentration of dissolved CO_2 in water [5]. In addition, RuBP activated by RubisCO can also react with O_2 in the oxygenase reaction, which leads to the formation of one-molecule 3PGA and one-molecule 2-phosphoglycolate (2PG). The latter product has inhibitory effects

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on the enzymes of CBBC in plants, such as triosephosphate isomerase and sedoheptulose 1,7-bisphosphate phosphatase [6, 7]. Hence, 2PG concentrations need to be kept low; this is maintained by the 2PG salvage pathway called photorespiration (see Section 1.5). In conclusion, the long-term change in the atmospheric composition introduced problems for RubisCO, which were solved by the evolution of the carbon-concentrating mechanism (CCM, see Section 1.2) in cyanobacteria, as well as in many other photosynthetic organisms [8].

C_i availability not only changes over long timescales but can also fluctuate on short timescales. The solubility of CO_2 in water is mainly influenced by pH, temperature, and total ion content. In general, high temperature decreases the solubility of gases, including CO_2. It can also be dissolved in water, leading to the formation of bicarbonate and carbonate, whereby the occurrence of the different C_i species depends on pH. For example, in the slightly alkaline water of oceans, less than 1% of C_i is in the form of CO_2 and most of the dissolved C_i is present as bicarbonate (87.5%). Carbonate dominates in alkaline lakes, while acidic waters preferentially contain only CO_2. The pH-dependent interconversion of the C_i species is rather slow and can be highly accelerated in biological systems by carbonic anhydrases (CA). Such enzymes, which belong to different enzymatic classes, are present in all cyanobacteria and many other photosynthetic organisms [9]. In addition to abiotic factors, the C_i availability is also strongly influenced by biotic factors, especially in eutrophic waters. In dense phytoplankton population, for example, in cyanobacterial surface scums that appear during the so-called bloom situations, the high photosynthetic activity will strongly deplete C_i and O_2 will accumulate to high levels [10]. Heterotrophic organisms, for example, bacteria specifically associated with cyanobacteria, may not only provide vitamins and other growth-stimulating molecules but can also locally enrich water with CO_2 released via respiration. Hence, cyanobacteria also have to acclimate to short-term variations in C_i; hence, the response to varying C_i needs to be tightly regulated at different levels.

1.2 The Carbon-Concentrating Mechanism (CCM) Among Cyanobacteria

The above-mentioned problems of RubisCO due to the long-term depletion of the atmospheric CO_2 and the enrichment of O_2 initiated diverse adaptation mechanisms. For example, RubisCO proteins of type 1 in C3 plants increased the CO_2 affinity to respond to low atmospheric CO_2 partial pressure [11]. Cyanobacteria used another strategy; they did not invest in the improvement of RubisCO but instead evolved a CCM. CCM is defined as the measures that result in higher C_i affinity of the intact photosynthetic organism/cell compared to the CO_2 affinity of its RubisCO. Hence, the CCM works to increase the CO_2 partial pressure in the vicinity of RubisCO, which allows the enzyme to efficiently perform the carboxylation reaction and at the same time suppresses the oxygenase reaction to a large extent. The cyanobacterial CCM is a so-called biophysical CCM because it mainly depends on transport/uptake of C_i by different mechanisms, leading to high intracellular

accumulation of the charged bicarbonate, which in contrast to CO_2 cannot easily diffuse from the cell through biological membranes. RubisCO is confined to the carboxysome, a bacterial micro-compartment with a protein shell containing in addition CA that efficiently converts bicarbonate into CO_2 (Figure 1.1). Hence, RubisCO operates in a highly CO_2 enriched environment inside the carboxysome, which is also believed to exclude O_2, thereby minimizing the competing oxygenase reaction. When the cyanobacterial CCM evolved among cyanobacteria is still a matter of discussion. One common assumption dates it to the low CO_2, high O_2 period about 400 million years ago [12], while much earlier dates for its appearance have also been reported [13]. Because of the notion that the CCM is functionally and structurally well conserved in the majority of cyanobacterial clades, including the basal and most distant forms, it is more parsimonious to assume that the CCM evolved much earlier than 400 million years ago in the cyanobacterial radiation [14].

1.2.1 C_i Uptake Proteins/Mechanisms

Over the past 30 years, five different C_i uptake systems have been identified in different model cyanobacteria (Figure 1.1). Their activity allows a high accumulation of bicarbonate inside the cells, which can exceed the external amount of 100–1000-fold [15].

First, the bicarbonate transporter BCT1 has been characterized [16]. BCT1 belongs to the group of primary active ATP-binding cassette (ABC) transporters, which energize the uptake of substrates via ATP hydrolysis. In cyanobacteria, BCT1 is usually encoded by the *cmp* operon, which comprises the genes for the ATP-binding subunit, the bicarbonate pore protein(s), and a periplasmic bicarbonate-binding protein that determines the high bicarbonate affinity of the system. The *cmp* operon expression is strongly stimulated by the activator protein CmpR under low C_i conditions (LC, usually ambient air), which is often encoded upstream of the structural *cmp* operon [17].

Second, the sodium-dependent bicarbonate transporter (SbtA) has been identified in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which is a high-affinity bicarbonate transporter presumably executing a bicarbonate/Na^+ symport [18]. The Na^+ dependency of SbtA has been concluded from the high stimulation of the transporter by increasing Na^+ amounts and its dysfunction upon mutation of Na^+ transport protein J (NtpJ), a protein assumed to be involved in sodium export [19]. However, it turned out that NtpJ is not involved in Na^+ export but instead is part of the Ktr-type K^+ transport system [20, 21]. Hence, whether or not Na^+ and bicarbonate transport are indeed directly coupled in SbtA still needs to be verified experimentally on isolated SbtA reconstituted into liposomes. Like BCT1, *sbtA* expression is highly induced under LC conditions.

Third, BicA, another widespread bicarbonate transporter, was identified in *Synechococcus* sp. PCC 7002, which belongs to the SulP family and is also believed to couple bicarbonate uptake to Na^+ symport [22]. In contrast to BCT1 and SbtA, the BicA transporter exhibits a more constitutive, low-affinity, but highly active bicarbonate uptake.

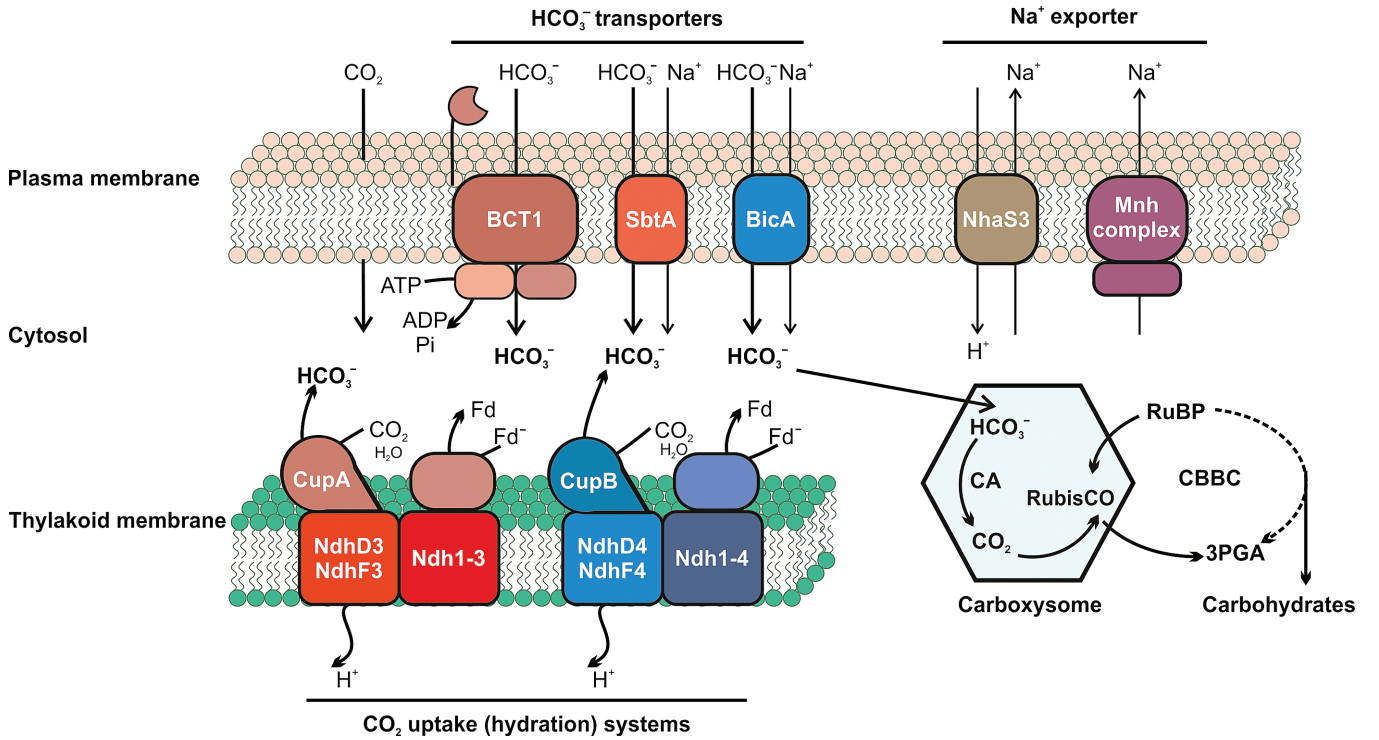

Figure 1.1 Schematic presentation of cyanobacterial carbon-concentrating mechanism (CCM). The cyanobacterial CCM utilizes three bicarbonate transporters, BCT1, SbtA, and BicA, located in the plasma membrane. The two thylakoid-embedded CO_2 uptake (hydration) systems convert cytoplasmic CO_2 to bicarbonate: Ndh1-3 and Ndh1-4 comprising the cyanobacteria-specific small subunits NdhD3/4, NdhF3/4, and CupA/B. Among them, the expression of transporters BCT1, SbtA, and Ndh1-3 complex (in warm color) are strongly induced under LC conditions, while BicA and Ndh1-4 complexes (in cold color) are constitutively expressed. The Na^+ gradients are kept by at least two Na^+ export systems: NhaS3 (brown) and Mnh complex (purple). The accumulated bicarbonate penetrates into carboxysomes, where bicarbonate is dehydrated back to CO_2 by carbonic anhydrase (CA) in proximity to RubisCO and fixed into carbohydrates through CBBC.

In addition to the three bicarbonate transporters, two CO_2 uptake (hydration) systems have been identified. CO_2 is a gas and no direct transport protein has been characterized to date. Hence, CO_2 passively diffuses inside the cell driven by its partial pressure gradient, whereby the diffusion can be accelerated by specific aquaporin-like channels [23]. Inside the cell, CO_2 is efficiently converted into bicarbonate by two specialized NDH1-like complexes located in the thylakoid membrane [19, 24–26]. The conversion of CO_2 into bicarbonate fulfills two tasks; it maintains the partial pressure gradient for CO_2, promoting inward diffusion, and reduces the diffusion of CO_2 that is not fixed by RubisCO in the carboxysome from the cell. The latter function has been demonstrated by the observation that cyanobacterial cells release CO_2 during the first minutes after illumination of darkened cells, a time span when RubisCO is still inactive because of missing activation via carbamylation, but carboxysome-located CA is already working [27].

The cyanobacterial NDH1-like complex is involved in different functions. In addition to CO_2 hydration into bicarbonate, it participates in respiration and cyclic electron transport around photosystem 1 [24, 26]. Proteomic and mutant studies revealed that these multiple tasks are fulfilled by structurally different complexes, which are associated with a different set of auxiliary subunits, leading to different complex sizes (see e.g. [25]). Among them, the two small complexes Ndh1-MS and Ndh1-MS′ are specifically involved in the CO_2 uptake [26]. In *Synechocystis*, one has been characterized as a constitutive system with low CO_2 affinity named as Ndh1-4 (NDH1-MS′), while the other represents a high-affinity system named as Ndh1-3 (NDH-MS) that is highly induced under LC conditions [19, 25]. Cyanobacteria-specific small NDH1 subunits have been identified, which also play an essential role in the CO_2 to bicarbonate conversion [26, 28, 29]. Among them, the CupA or CupB subunits, which reach into the cytoplasm on the distal membrane part of the NDH-MS complexes, are specifically involved in the CO_2 uptake [19, 30–32].

Despite the impressive progress in functional and structural studies of NDH1 complexes, some questions remained open for a long time [26]. For example, the nature of the electron donor for the cyanobacterial NDH1 has been discussed because the NAD(P)H_2-oxidizing subunits were never identified in isolated NDH1 complexes nor harbor cyanobacterial genome homologs for these proteins. Recently, the structures of the large NDH1 complex from *Thermosynechococcus elongatus* have been solved, which unequivocally showed that reduced ferredoxin donates electrons to the complex. Hence, the NDH1 complex among cyanobacteria is not a *bona fide* NAD(P)H_2 dehydrogenase complex [33]. Another open question is related to the mechanism, which permits the CO_2 conversion by the specialized NDH1-MS complexes. It is assumed that because of light-driven trans-thylakoid proton transport via the NdhD subunits [32], an alkaline region is created at the cytoplasmic site of the NDH1-MS complexes, which forms the predicted “alkaline pocket” driving the CO_2 conversion [34]. Recently, the CA EcaB was found at the thylakoid membrane, where it seems to be specifically linked to the NDH1-3 and NDH1-4 (NDH1-MS) complexes involved in CO_2 uptake in *Synechocystis* [35]. The authors concluded that EcaB might be involved in the proton extraction by the

NDH1-3 and NDH1-4 complexes to allow for continuous CO_2 to bicarbonate conversion. Moreover, the EcaB protein also seems to regulate the CO_2 uptake activity of these specialized NDH1 complexes [35]. The structure of the NDH-MS complex has recently been solved by cryo-electron microscopy, which permitted to gain a mechanistic understanding of the CO_2-hydrating reaction performed by CupA [36].

The multiple systems involved in C_i uptake among cyanobacteria seem to cooperate in the accumulation of bicarbonate inside the cell. This redundancy explains why the mutation of single bicarbonate transporters or CO_2 uptake systems causes usually no severe phenotype, i.e. these mutants can still grow at ambient air conditions (LC). Only under specific conditions do growth phenotypes of single mutants become visible, such as variation of the external pH to make CO_2 or bicarbonate the predominantly available C_i form [32]. However, the combined inactivation of several transporters, including a *Synechocystis* mutant with all five C_i uptake systems inactivated [37], resulted in the characteristic high CO_2-requiring (HCR) phenotype. The HCR phenotype of these mutants directly supports the notion that despite the existence of an intact carboxysome, the accumulation of high cellular bicarbonate concentrations is an essential part of the cyanobacterial CCM. This has also been shown in experiments expressing a highly active recombinant CA in the cytoplasm of *Synechococcus elongatus*. The activity of this human CA collapsed the internal accumulation of bicarbonate, resulting in the HCR phenotype [38].

It should be noted that not all cyanobacteria possess all the five described C_i uptake systems [39]. For example, it has been shown that *Microcystis* spp. ecotypes are characterized by variable numbers of C_i transporters, especially the SbtA protein is often absent while BicA is retained [40]. Competition experiments showed that strains having both SbtA and BicA outcompete *Microcystis* spp. with only BicA under LC conditions. Consistently, *sbtA* and *bicA* genotypes of *Microcystis* strains were found predominantly during cyanobacterial bloom development, leading to C_i-limiting conditions, while *bicA*-only genotypes were successful in seasons with enriched C_i amounts in lakes, in agreement with its low-affinity, high-flux characteristics [41]. Picoplanktonic cyanobacteria living in the open oceans are characterized by large genome reductions. Consistently, the genomes of *Prochlorococcus* spp. and marine *Synechococcus* spp. have lost several C_i uptake systems [42]. The molecular basis of C_i acquisition in these ecological important cyanobacteria, which are believed to perform approximately 20% of annual CO_2 fixation, is not well understood and needs further investigations. Bioinformatic analysis showed that their genomes harbor many yet uncharacterized proteins belonging to protein families of characterized C_i uptake systems. Hence, these proteins represent excellent candidates for the further study on new C_i uptake systems among cyanobacteria [39].

1.2.2 Carboxysome and RubisCO

The final C_i fixation by RubisCO is performed in the bacterial micro-compartments called carboxysomes (Figure 1.1). Carboxysomes are polyhedral bodies, resembling phage capsids, which became visible in all cyanobacterial cells via transmission

electron microscopy. Their specific function in CO_2 fixation was demonstrated in attempts to localize RubisCO and the site of $^{14}CO_2$-incorporation in cyanobacterial cells, both are confined to the carboxysome structures. Despite a similar function, two phylogenetically distinct carboxysomes and RubisCO types are present among cyanobacteria, distinguishing the so-called *β*-cyanobacteria and the so-called *α*-cyanobacteria [43, 44]. *β*-cyanobacteria, to which the majority of the cyanobacterial phylum belongs, harbor RubisCO proteins of the subclass 1B and *β*-carboxysomes (Ccm-type). In the model cyanobacterium *S. elongatus*, most carboxysomal proteins are encoded in one large *ccm* operon that forms a superlocus also comprising operons encoding RubisCO and some C_i uptake systems [45]. The *α*-cyanobacteria, which form a phylogenetic separate clade of picoplanktonic cyanobacteria dominated by oceanic *Prochlorococcus* spp. and *Synechococcus* spp. [46], contain RubisCO type 1A and *α*-carboxysomes (Cso-type), which were most probably acquired via horizontal gene transfer from proteobacteria [47]. Because most cyanobacteria, including all well-investigated model strains, belong to the class of *β*-cyanobacteria, we will here shortly describe only this type.

The structure and genesis of *β*-carboxysomes were intensively characterized over the past 20 years. These carboxysomes assemble from the inside out [48]. First, hexadecameric RubisCO molecules and CA assemble together with CcmM to a procarboxysome, which is subsequently surrounded by the shell proteins. In the case of *S. elongatus* and *Synechocystis*, a β-type CA named as CcaA has been identified, which is responsible for the rapid conversion of bicarbonate into CO_2 inside the carboxysomes [49, 50]. Structural analyses revealed that CcaA forms well-packed trimer-of-dimers organization. Moreover, it binds with high affinity to CcmM [51]. However, the CA-type inside carboxysomes is variable among cyanobacteria [47, 52]. CcaA homologs are only found in a rather low number of cyanobacterial genomes. This implied the question, which CA is replacing the CcaA in other cyanobacterial strains. In the filamentous strain *Nostoc* (*Anabaena*) sp. PCC 7120, one isoform of CcmM has been shown to exhibit CA activity [53]. This CA-like domain is well conserved in cyanobacterial CcmM proteins, in addition to RbcS-like domains. The structure showed that this CcmM domain resembles γ-type CAs. It became obvious that CcmM-mediated CA activity seems to be more widespread among cyanobacteria than CcaA. In addition to its CA function, the CcmM protein plays a major role as a scaffold protein for the semicrystalline order of RubisCOs inside carboxysomes. Recently, the structural basis for CcmM and RubisCO interaction has been resolved, which showed unexpectedly that the RbcS-like domain of CcmM did not directly interact with RbcS in RubisCO, but CcmM rather binds to a region between RbcL dimers [54]. The RubisCO-CcmM structures are then anchored by CcmN to the inner carboxysomal shell [45].

The polyhedral protein shell of *β*-carboxysomes is mainly composed by CcmK proteins that form hexameric structures with a central pore, which is believed to facilitate the flux of bicarbonate and RuBP toward the carboxysome lumen and of 3PGA out of it [55]. CcmKs are the main constituents of the shell. In *Synechocystis*, four different CcmK proteins are present, which can be expressed to different extents under fluctuating C_i levels. The variable composition of carboxysome shell

by different CcmKs is anticipated to impact the function of the carboxysome because the CcmK homologs mostly differ in sequences around the pore-forming part, which influence their size and charge and possibly provide a range of selectivity toward RuBP and 3PGA as well as possibly bicarbonate, CO_2 and O_2 [45]. However, this assumption needs experimental support, which is hampered because of the difficulty to isolate intact, functional carboxysomes from cyanobacteria. Another hexameric shell protein is CcmP, which is found in lower abundances compared to CcmKs. The CcmP protein can form stacked dimers with a central chamber and gated pores, which are believed to allow a controlled permeation of larger molecules such as RuBP and 3PGA across the carboxysomal shell [45, 56]. Finally, the pentameric shell protein CcmL forms the vertices in the icosahedral carboxysomal shell [57].

As mentioned above, mutation of genes for structural carboxysomal proteins or for CA always resulted in the HCR phenotype, which shows that the compartmentalization of RubisCO and CA inside the carboxysome is an essential part of the cyanobacterial CCM (e.g. [58]). The *Synechocystis* Δ*ccmM* mutant accumulated high amounts of photorespiratory intermediates because RubisCO is exposed to much lower CO_2 and higher O_2 partial pressure than in intact carboxysomes [59].

1.3 Regulation of C_i Assimilation

Environmental fluctuations in the available C_i must be measured by cyanobacteria in order to coordinate the CCM activity and the downstream utilization of fixed carbon by the primary carbon metabolism. This regulation is achieved at different levels, including transcriptional but also posttranscriptional control. Using model cyanobacteria, the activity of the CCM and the carbon metabolism has been compared mainly under two conditions. Usually, cells grown under CO_2-supplemented conditions (1–5% CO_2, defined as high carbon, HC) have been challenged by shifts to ambient air, LC (0.04% CO_2) conditions. This shift scenario has been selected because many mutants with defined mutations in CCM components are characterized by the HCR phenotype and can be only cultivated and characterized at HC conditions. However, the HC to LC shift protocol usually needs to include a medium change, i.e. HC-grown cells are harvested by centrifugation or filtration to remove the C_i-enriched medium at time point zero before cells are then suspended in C_i-poor fresh medium. This treatment disturbs the steady-state light conditions and might be stressful for the cells, i.e. it is probably more physiological to shift from LC into HC conditions, which simply involves a change in the composition of the gas stream through the cultures.

1.3.1 Regulation of the CCM

The activity of the CCM is clearly higher in LC- than in HC-grown cyanobacterial cells, which is directly seen in the enhanced photosynthetic C_i affinity of LC cells [34]. This change is mainly regulated at the transcriptional level, influencing the differential expression of C_i uptake systems, while RubisCO and carboxysome

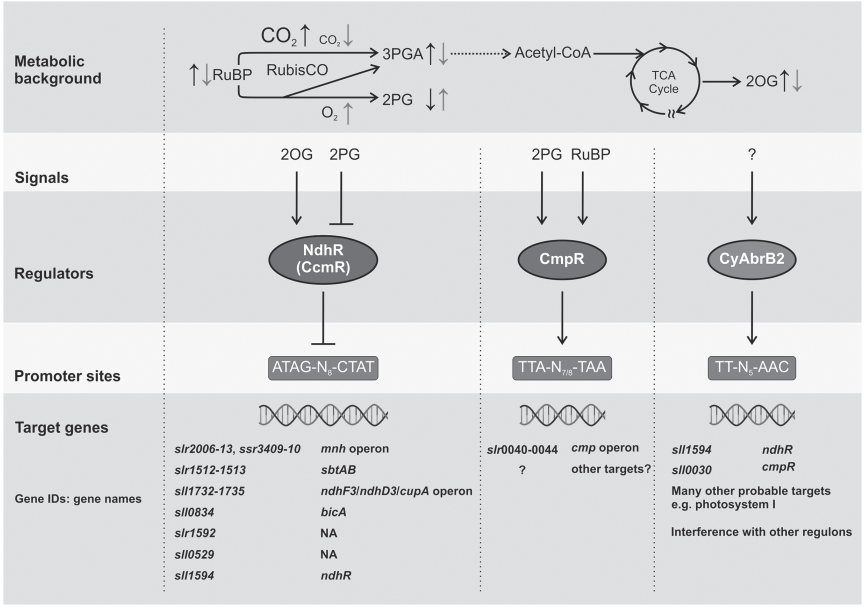

Figure 1.2 Regulation of carbon-concentrating mechanism (CCM) on the transcriptional level via the repressor protein NdhR (CcmR) and the activator proteins CmpR or CyAbrB2 in *Synechocystis* sp. PCC 6803. Metabolites such as 2-phosphoglycolate (2PG), 2-oxoglutarate (2OG), and ribulose 1,5-bisphosphate (RuBP) serve as corepressors or inducers by changing the binding affinity of the transcription factors NdhR and CmpR for their corresponding binding motifs present in the promoter regions of different target genes. These metabolite changes are related to high CO_2 (black arrows) or low CO_2 (relatively high O_2, grey arrows) conditions and regulate the expression of different sets of CCM genes according to the available C_i. The signals involved in the activation of CyAbrB2 are not known. Further details can be found in Section 1.3.1.

protein expression is less variable. Three transcription factors, NdhR, CmpR, and cyAbrB2, have been identified (see Figure 1.2), involved in the transcriptional CCM regulation to different extents [15].

The LysR-type transcription factor NdhR (sometimes named as CcmR) is clearly the most important regulator because it targets the largest C_i-regulon in *Synechocystis* and probably many more cyanobacteria [60–63]. NdhR homologs are encoded in virtually all cyanobacterial genomes. The genes for the high-affinity Ndh1-3 CO_2 uptake system (*sll1732-1735*) are among the targets, which led to its naming. The *sbtAB* (*slr1512-1513*) and *mnh* (*slr2006-2013*, *ssr3409-3410*) operons, *bicA* (*sll0834*), and the genes *sll0529* and *slr1592* for two hypothetical proteins are also part of the regulon. Probably, *ndhR* (*sll1594*) itself also belongs to its regulon because two NdhR-binding motifs have been identified in front of the *ndhR* promoter sequence [60]. NdhR acts as a repressor, which is active under HC conditions and becomes released from the promoters under LC, allowing RNA-polymerase binding and strong expression (Figure 1.2). NdhR binds specifically to the target DNA sequence ATAG-N_8-CTAT, which is present near the core promoter of the genes in the C_i-regulated NdhR regulon [60].

The DNA-binding activity of LysR-type transcription factors is often regulated by low-molecular mass effector molecules. In the case of NdhR, it was first verified that binding of 2-oxoglutarate (2OG) increases the promoter affinity of NdhR. Hence, 2OG acts as a corepressor and signals the HC situation to NdhR because 2OG is accumulated in cells under excess carbon as well as nitrogen limitation [64]. Initially, binding of $NADP^+$ was reported for NdhR, which was thought to signal the redox state [65]. However, this binding was not verified when the structure of the *Synechocystis* NdhR was solved in its apo- and holo-forms with different potential effector molecules [66]. This study revealed the structural basis of how 2OG-binding as a HC signal improves the DNA-binding of NdhR and verified 2OG as a classical corepressor. Interestingly, the structural study identified 2PG as a new specific binding partner of NdhR. 2PG is the product of RubisCO's oxygenation reaction and accumulates under LC conditions; hence, 2PG signals C_i limitation to the NdhR system. The 2PG-binding deactivates NdhR by changing its structure in such a way that promoter binding is not possible; hence, the LC signal 2PG acts as an "inducer" of the NdhR regulon. Together, the corepressor 2OG and the inducer 2PG perfectly allow fine-tuning of the NdhR activity by sensing the cellular carbon, as well as nitrogen status [67].

The transcription factor CmpR has been identified as the activator of the BCT1 transporter [17]. In *Synechocystis*, it is encoded just upstream (*cmpR* gene *sll0030*) of the *cmp* operon (*slr0040-0044*) harboring the structural proteins of BCT1. Like NdhR, CmpR belongs to the LysR group of transcription factors. Its promoter binding is activated by the effector molecules 2PG and RuBP, signaling a LC situation to CmpR [68, 69]. 2PG accumulates under LC because of the relative increase of the RubisCO oxygenase activity, whereas RuBP accumulation results from the overall lower CBBC activity. The activated CmpR protein binds to the sequence TTA-$N_{7/8}$-TAA upstream of the *cmp*-operon promoter, which promotes RNA-polymerase binding and activates *cmp* expression under LC. Whether or not CmpR has additional target genes is not known yet. However, there are some hints that it may also participate in the regulation of some genes from the NdhR and especially CyAbrB2 regulons [70].

The third regulator, at least partly involved in the LC induction of the CCM, is cyAbrB2 [70, 71]. It belongs to a widespread AbrB class of bacterial transcription factors that are involved in many different regulatory networks. *Synechocystis* encodes for at least two AbrB-like proteins. The cyAbrB1 encoded by *sll0359* has been found to be essential for cell viability, which made functional analyses difficult. However, cyAbrB2 encoded by *sll0822* can be mutated, which promoted its functional assignment. Mutation of *cyabrB2* in *Synechocystis* revealed a decreased expression of many genes encoding CCM components after HC to LC shifts. Interestingly, the LC-induced expression stimulation of *ndhR* was completely abolished, which might indicate that cyAbrB2 specifically regulates this repressor. This finding also implies that possibly many C_i-related expression changes in the Δ*cyabrB2* mutant are rather indirect, i.e. they possibly result from the transcriptional changes of *ndhR* (and likely *cmpR*) [70]. However, cyAbrB2 has also direct targets because many genes affected in the C_i-regulated transcription in the Δ*cyabrB2* mutant

do not belong to the NdhR and CmpR regulons. For example, genes encoding photosystem 1 subunits are no longer LC regulated in the Δ*cyabrB2* mutant, which led to the conclusion that this transcription factor integrates C_i and light signals in *Synechocystis* [70] and, furthermore, is also sensing the nitrogen status [72].

Interestingly, the CCM can also be regulated by the available light intensity and possibly even light quality on transcriptional level [73]. Mutation of the light-sensing protein RcaE had strong impact on the CCM in *Fremyella diplosiphon*. A Δ*rcaE* mutant exhibits altered carboxysome size and number, *ccm* gene expression, and carboxysome protein accumulation relative to the wild type. This RcaE-based sensing mechanism obviously coordinates light signals with C_i assimilation in this cyanobacterium. Furthermore, CCM-related genes were also found in the RpaB regulon, which mainly regulates light- and redox-controlled stoichiometry of the photosystems in *Synechocystis* [74].

1.3.2 Further Regulation of Carbon Assimilation

Investigations of *Synechocystis* mutants defective in different regulatory proteins such as *ndhR*, *cyabrB2*, and *glnB* (P_{II}) regarding LC acclimation revealed that a number of LC-induced genes encoding proteins not directly involved in the CCM remained regulated in all of these mutants like in the wild type [60, 70, 75]. In addition, none of these mutants showed significantly reduced growth at LC conditions. All these results point to the existence of further regulatory mechanisms involved in the acclimation toward LC conditions.

The P_{II}-paralog SbtB could represent a promising candidate. In *Synechocystis*, SbtB (*slr1513*) is encoded downstream of the SbtA bicarbonate transporter, in the same LC-induced operon, and is currently annotated as a hypothetical protein in CyanoBase. The importance of SbtB became obvious when *sbtA* was expressed in *Escherichia coli*. SbtA-based bicarbonate transport activity could be detected in *E. coli*; however, it was highly downregulated when *sbtA* and *sbtB* were co-expressed. The authors concluded that SbtB may be an inhibitor of the transporter SbtA [76]. Interestingly, the SbtB protein shows weak sequence similarities to P_{II} proteins, which are well characterized in their role of sensory circuits, particularly important for the nitrogen acclimation of different bacteria [77]. It has been shown that the P_{II} paralog GlnK regulates the activity of the ammonia transporter AmtB in *Bacillus subtilis* by direct protein–protein interaction [78]. The three-dimensional structure of SbtB was indeed very similar to the "classical" P_{II} protein GlnB of cyanobacteria [79]. Moreover, the specific binding of adenyl-nucleotides by SbtB was shown, whereby cAMP was bound with highest affinity. This finding was exciting because cAMP has been proposed as potential signal involved in C_i acclimation because the activity of soluble adenylate cyclase, the enzyme producing cAMP, is specifically regulated by bicarbonate in different organisms including cyanobacteria [80]. Furthermore, the mutant Δ*sbtB* showed distinct differences in growth and photosynthetic C_i affinity when cultivated at HC or LC conditions [79]. Collectively, these features qualify the widespread P_{II} paralog SbtB as a new sensor protein somehow involved in the acclimation of cyanobacteria toward

different C_i conditions, thereby implicating cAMP as another cellular C_i sensory molecule.

1.3.3 Metabolic Changes and Regulation During C_i Acclimation

In addition to changes at the transcriptional level, *Synechocystis* cells shifted from HC to LC conditions show a defined metabolic response. These cells are characterized by a transient accumulation of photorespiratory intermediates, 2PG and glycine, whereas glycogen and many intermediates of the carbohydrate metabolism strongly declined, as well as most amino acids with the exception of several branched chain amino acids [81, 82]. This metabolic signature points to a changed flux pattern when cells are grown in air [83]. At excess C_i, i.e. under HC conditions, organic carbon fixed via CBBC is predominantly exported into the carbohydrate metabolism and stored as glycogen. Furthermore, it supports high growth rate and an efficient nitrogen assimilation. When shifted to LC conditions, the lower activity of CBBC due to decreased RubisCO carboxylation, which is obvious from the accumulation photorespiratory intermediates, is transiently compensated by the breakdown of glycogen. In the long-term LC acclimation, carbon from CBBC is mostly exported via glycolysis into the tricarboxylic cycle to maintain a reduced level of nitrogen assimilation and growth. Remarkably, the metabolic changes in LC-shifted cyanobacteria resemble metabolic changes in *Arabidopsis thaliana* leaves after shifts from low to high photorespiratory conditions [82].

These changes in the carbon flux of LC-shifted *Synechocystis* cells seem to be mostly regulated at the biochemical level because no significant transcriptional changes in genes encoding enzymes of the primary carbon metabolism have been detected during LC shifts [60]. Similarly, proteomics showed that proteins belonging to the C_i fixation and metabolism section did not significantly change at varying degrees of CO_2 limitation, while this group of proteins slightly responded to different light intensities [84]. Moreover, this metabolic response is largely conserved in *Synechocystis* mutants defective in the regulatory proteins *ndhR*, *cyabrB2*, and *glnB* (P_{II}) [60, 70, 75]. The biochemical regulation may involve different specificities of isoenzymes, which are found at branching points in the cyanobacterial primary metabolism, such as glyceraldehyde 3-phosphate dehydrogenases or phosphoglycerate mutases [85–87]. Furthermore, many enzymes involved in the primary carbon metabolism have been identified as phosphoproteins [88, 89]; hence, phosphorylation of key enzymes might also contribute to the changed carbon flux in LC-shifted cells. However, it should be noted that the regulation of primary carbon metabolism might be different among cyanobacteria. In contrast to *Synechocystis*, clear C_i-related transcriptional changes of several genes encoding enzymes involved in primary metabolism were found in *S. elongatus* [90]. This cyanobacterium has a smaller genome compared to *Synechocystis*, consistently the number of isoenzymes is smaller in *S. elongatus* than in *Synechocystis*, which might partially explain the use of different strategies for the regulation of primary carbon metabolism among these two cyanobacteria [91].

1.3.4 Redox Regulation of C_i Assimilation

Cyanobacteria as plants have to switch between autotrophic carbon assimilation during the day and heterotrophic sugar catabolism during the night, when stored glycogen or starch reserves are used. In contrast to plant cells, the enzymes for carbon anabolic and catabolic routes are all situated in the cyanobacterial cytoplasm. Hence, their activity needs to be carefully separated to avoid futile cycles. Although transcriptional regulation is slow and occurs during long-time acclimation, post-translational protein modifications mediate fast response to environmental changes. Light and C_i availability are tightly coupled as the generation of reductants occurs mainly by photosynthesis and its consumption in CBBC. Thus, a rapid sensing of the redox status of the cell is of central importance to regulate the balance between photosynthesis and carbon allocation [92].

It has been shown decades ago that glucose utilization after glycogen breakdown occurs mainly via the oxidative pentose phosphate pathway [93], which overlaps to a large extent with the reductive pentose-phosphate cycle, i.e. the regenerative phase of CBBC. Four enzymes of the plant CBBC are known to be post-translationally activated by light through thioredoxin (TRX)-dependent reduction of disulfide bonds – namely, GAPDH, PRK, FBPase, and SBPase. This TRX-dependent regulation was exclusively found in plants and is absent in cyanobacteria and eukaryotic algae [94–97], suggesting that the light-dependent regulation mediated by TRX has been progressively introduced during evolution. Indeed, the regulatory active cysteines in plant enzymes are located in N- or C-terminal extensions (e.g. GAPDH) or inserted in the sequence (e.g. FBPase) [98–100]. However, extensive redox proteome studies with cyanobacteria such as *Synechococcus* sp. PCC 7002 revealed over 350 putative redox-regulated proteins in response to high light or C_i depletion [92]. Among them, proteins of the carbon metabolism (e.g. PGM) were activated by ferredoxin-TRX reductase/TRX reduction systems under light conditions and CCM proteins (e.g. CcmM) are also targeted by TRX [101, 102]. In contrast to plants, the cyanobacterial TRX-targeting process is focused on light-dependent activation of CCM and glycogen synthesis, whereas in plants, the CBBC enzymes became prime targets. Furthermore, the complete pathway of glycogen synthesis belongs to the Trx-targets in *Synechocystis* and not only the rate-limiting enzyme, ADP-glucose pyrophosphorylase, as seems to be the case in plastids [103].

Additional proteins involved in the regulation of CBBC enzymes are also activated by TRX in a light-dependent manner. In *S. elongatus* as in plants, it has been shown that the small protein CP12 binds to the $NADP^+$-dependent GAPDH and subsequently forms a ternary complex with PRK ($NADP^+$-GAPDH/CP12/PRK). In this conformation, both enzyme activities are strongly inhibited. Complex dissociation and rapid recovery of enzyme activity can be induced by reduced TRX, 1,3-bisphosphoglycerate (substrate of GAPDH), $NADPH_2$, or ATP, all products of the light-activated photosynthesis in plants [104–106]. However, in cyanobacteria, it has been shown that the GAPDH/CP12/PRK complex formation is rather influenced by the NAD(H)/NADP(H) ratio than TRX during day–night cycles [106]. CP12 from *S. elongatus* lacks two cysteine residues that have been shown to be

involved in complex formation and TRX-dependent reduction in plants. Thus, the complex formation in cyanobacteria is due to electrostatic interactions between CP12 and $NADP^+$-GAPDH and PRK during dark. This interaction is thought to be disrupted by the negatively charged phosphate groups of $NADPH_2$ [105], which is roughly threefold higher than NADH in light-exposed cyanobacterial cells [106].

1.4 Engineering the Cyanobacterial CCM

During the past years, cyanobacteria attained increasing attention as the so-called green cell factories for the biotechnological production of fuels or feedstocks [107]. The possible application stimulated efforts to analyze whether C_i assimilation can limit cyanobacterial growth or productivity. Therefore, strains were generated overexpressing RubisCO, CBBC enzymes, and C_i uptake systems. For example, overexpression of the BicA bicarbonate transporter in *Synechocystis* resulted in almost doubled growth and biomass production under LC conditions, which point at C_i transport limitation at ambient air despite induced CCM. However, the *bicA* overexpressing strains suffered at HC conditions because the surplus-fixed carbon changed the carbon allocation toward extracellular polysaccharides [108]. The enhanced expression of endogenous sedoheptulose 1,7-bisphosphate/fructose 1,6-bisphosphate phosphatase in *Synechococcus* sp. PCC 7002 improved growth up to 50% because of reprogramming of carbon metabolism, while the elevated levels of RubisCO did not show a growth promoting effect [109]. However, overexpression of RubisCO as well as different CBBC enzymes improved the productivity of an ethanol-producing *Synechocystis* strain, approximately 50% more ethanol was obtained [110]. These examples show that there is space for growth and productivity enhancement because of the manipulation of the CCM, CBBC, and associated processes in cyanobacterial biotechnology, but the effects may differ between the used chassis strains and product.

In contrast to cyanobacteria, most land plants including crops using the C3-type of photosynthesis do not exhibit a CCM, which results in loss of photosynthetically fixed CO_2 because of the occurrence of photorespiration. Under ambient conditions, roughly 25–40% of the RubisCO reactions lead to RuBP oxygenation in plants. In contrast, only 1% of RubisCO reactions are estimated to be oxygenation in *Synechocystis* [111]. This discrepancy initiated attempts to express the efficient cyanobacterial CCM in crop plants, with the hypothesis that increased C_i assimilation would suppress photorespiration [112, 113]. The first successful steps have been reported, for example, on the expression of cyanobacterial bicarbonate transporters in the chloroplast envelope [114, 115] as well as in the establishing of carboxysome-like structures inside tobacco chloroplasts [116, 117]. These results show impressively that it is possible to express cyanobacterial CCM components in plants and, furthermore, that carboxysome-like structures can easily be formed in the chloroplast with a minimal set of structural proteins. However, so far, none of these recombinant plants showed improved C_i assimilation, and it remains to be seen if this approach will improve the yield of crop plants.

1.5 Photorespiration

Photorespiration is the process of light-dependent O_2 consumption coupled to the release of CO_2, which is closely related to CBBC [118]. It is initiated by the oxygenation of RuBP via RubisCO as aforementioned, producing 2PG that, at least in plant chloroplasts, inhibits the activity of CBBC enzymes triosephosphate isomerase [6, 119], phosphofructokinase [120], and sedoheptulose 1,7-bisphosphate phosphatase [7]. Hence, the biochemical photorespiratory cycle (C2 oxidative photosynthetic carbon cycle) metabolizes the toxic 2PG into useful intermediates and replenishes the metabolites of CBBC (Figure 1.3). The canonical photorespiratory pathway, which was discovered and investigated in plants [121], employs eight enzymes in its core cycle – namely, 2PG-phosphatase (PGLP), glycolate oxidase (GOX), glutamate:glyoxylate aminotransferase (GGT), glycine decarboxylase (GDC), serine hydroxymethyltransferase (SHMT), serine/alanine:glyoxylate aminotransferase (SGT), hydroxypyruvate reductase (HPR), and finally glycerate 3-kinase (GLYK) that consumes one ATP per cycle [122]. These enzymes convert two molecules of 2PG into one molecule of the CBBC intermediate 3PGA (Figure 1.3, black lines). Hence, 75% of the organic carbon from 2PG is salvaged into 3PGA, and 25% of the carbon is lost as CO_2. Furthermore, the GDC reaction releases NH_3, which needs to be refixed by N-assimilatory reactions that consume energy and reducing power. Because of these losses, photorespiration is often seen as a "wasteful" process [123]. However, it is clearly an essential salvage process allowing operation of oxygenic photosynthesis with CBBC in the O_2-rich Earth atmosphere. This essential function of photorespiration has been demonstrated in studies with mutants affected in core photorespiratory steps. Like the aforementioned CCM mutants, photorespiratory mutants are always characterized by the HCR phenotype, i.e. cyanobacterial [124], plant [125, 126], and algal [127] mutants blocked in the glycolate oxidation reaction could not grow at ambient air but are rescued under HC conditions.

1.5.1 Cyanobacterial Photorespiration

Initially, it was assumed that cyanobacteria do not depend on photorespiration because the CCM activity was believed to completely eliminate RubisCO's oxygenase activity. In fact, the cyanobacterial CCM is very efficient. It reduces the flux into the photorespiratory metabolism to approximately 1% under ambient conditions [83, 111] compared to 25–40% measured in plants. The remaining RubisCO oxygenase activity indicates that the carboxysomal shell does not completely prevent O_2 diffusion. Moreover, during carboxysome assembly, RubisCO is not always caged and may be partly active. *In silico* analysis of cyanobacterial genomes implied the frequent occurrence of homologs to enzymes catalyzing the plant-like C2 cycle (Figure 1.3, black line). In most cyanobacteria, glycolate is converted to glyoxylate by glycolate dehydrogenase(s) (GDH), which in contrast to GOX in plants is not using oxygen [122].

In addition to the canonical photorespiratory cycle, these analyses gave hints for the existence of two other routes to detoxify 2PG in cyanobacteria and

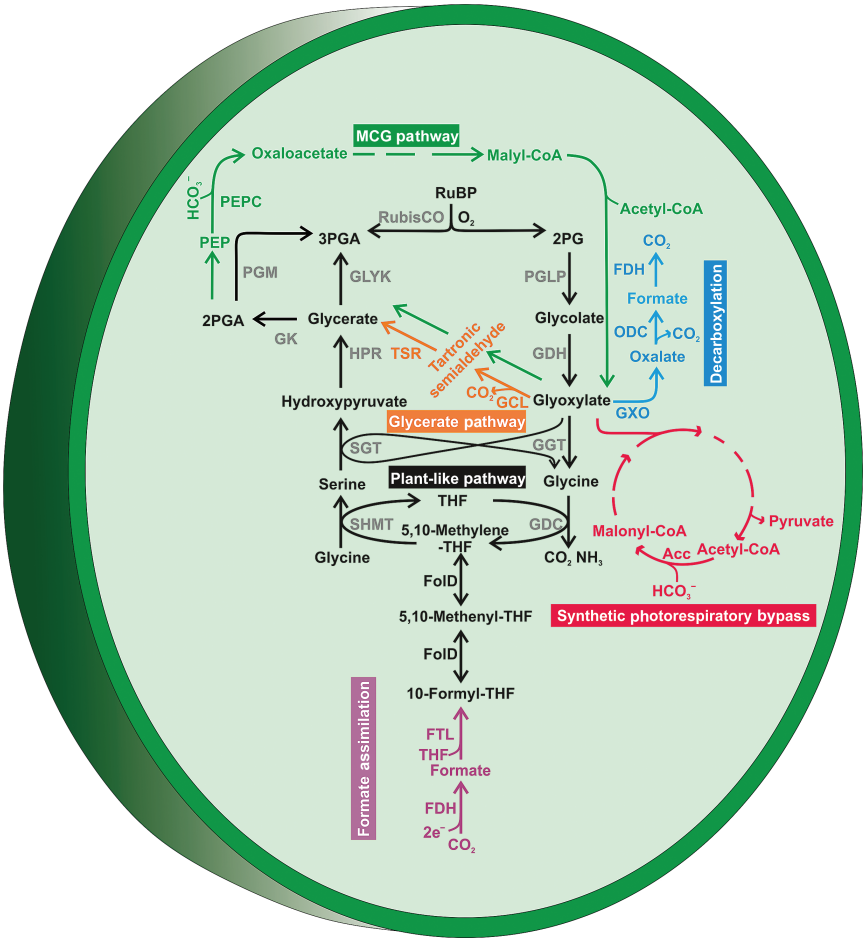

Figure 1.3 Cyanobacterial photorespiratory metabolism and synthetic bypasses. The black line represents the plant-like C2 cycle, the orange line represents the bacterial-like glycerate pathway, the blue line shows a complete decarboxylation route, the purple line represents the formate assimilation pathway, the red line shows the synthetic photorespiratory bypass, and the green line shows the MCG pathway. RuBP: ribulose 1,5-bisphosphate; 2PG: 2-phosphoglycolate; 3PGA: 3-phosphoglycerate; THF: tetrahydrofolate; RubisCO: ribulose 1,5-bisphosphate carboxylase/oxygenase; PGLP: 2-phosphoglycolate phosphatase; GDH: glyoxylate dehydrogenase; GGT: glutamate:glyoxylate aminotransferase; GDC: glycine decarboxylase complex; SHMT: serine hydroxymethyltransferase; SGT: serine:glyoxylate aminotransferase; HPR: hydroxypyruvate reductase; GLYK: glycerate 3-kinase; GK: glycerate 2-kinase; PGM: phosphoglycerate mutase; GCL: glyoxylate carboligase; TSR: tartronic semialdehyde reductase; GXO: glyoxylate oxidase; ODC: oxalate decarboxylase; FDH: formate dehydrogenase; FTL: 10-formyl-THF ligase; FolD: 5,10-methylene-THF dehydrogenase/cyclohydrolase; ACC: acetyl-CoA carboxylase; PEPC: phosphoenolpyruvate carboxylase.

particularly *Synechocystis*. A second possible pathway, the bacterial glycerate pathway (Figure 1.2, orange line), can metabolize glyoxylate via tartronic semialdehyde into glycerate [122, 128]. Investigations of *Synechocystis* mutants also revealed a third potential route, the decarboxylation branch (Figure 1.2, blue line). Here, glyoxylate is stepwise completely decarboxylated via oxalate and formate to CO_2 [124]. The functionality and interaction of these three routes were revealed using a genetic approach with *Synechocystis* mutants. None of the single or double mutants, where only one or two of the three routes was affected, showed the HCR phenotype, while mutants with defects in all three routes or mutants with completely abolished glycolate oxidation (just upstream of the branching into the three routes) showed the characteristic HCR phenotype [124]. However, it should be noted that for many enzymes proposed to be involved in the glycerate pathway and particularly the decarboxylation branch, the biochemical verification is still missing.

Diversity of photorespiratory pathways has been reported in different cyanobacterial species. For example, most cyanobacterial genomes do not possess the genes for the decarboxylation branch. Moreover, the majority of cyanobacteria uses plant-type GLYK to produce 3PGA from glycerate. However, *Synechocystis* and *S. elongatus* do not harbor *glyk* genes and use the 2PGA-forming glycerate 2-kinase (GK) in combination with phosphoglycerate mutase (PGM) to convert glycerate into 3PGA [86, 129].

1.5.2 Attempts to Engineer Photorespiration

Because of the high photorespiratory losses in C3 plants, diverse attempts were undertaken to diminish photorespiration. The initial strategies to directly disrupt this process have not improved photosynthetic efficiency in plants because of the accumulation of toxic intermediates (e.g. 2PG and glyoxylate) and diminished regeneration of the carbon acceptor molecule RuBP [130]. More promising approaches to engineer photorespiratory bypasses were proposed later. Most of them aim to avoid or decrease the release of ammonia diminishing its costly re-assimilation or shift the CO_2-releasing step from plant mitochondria to chloroplast elevating the CO_2 concentration at the site of RubisCO [131–133]. More radical redesigns have been reported recently because of the development of synthetic biology, which can be tested in the cyanobacterial model before engineering the complex plant system. By freely combining naturally evolved enzymes from various organisms with newly evolved enzyme activities, novel photorespiration bypasses are drafted, which not only decrease the energy loss but could also turn photorespiration into a carbon-positive process.

For example, an oxygen-insensitive, carbon-fixing synthetic photorespiratory pathway (Figure 1.3, red cycle) based on the 3-hydroxypropionate (3-HP) cycle was designed and introduced into *S. elongates*. The 3-HP cycle is used by anoxygenic phototrophs for C_i assimilation [134]. This synthetic pathway could function as both a photorespiratory bypass and an additional carbon fixation pathway [135]. This pathway starts with the photorespiratory intermediate glyoxylate to produce pyruvate, which is then coupled with the additional fixation of bicarbonate by

acetyl-CoA carboxylase (ACC). Although no obvious physiological benefit was observed with this synthetic metabolic cycle in the *S. elongatus* wild type, the authors pointed out that improvement may be achieved with an increased activity of ACC because the endogenous abundance of this enzyme might be insufficient to support the synthetic bypass [135]. Probably, expression of this or related pathways in a CCM-mutant background with enhanced photorespiratory flux would be more beneficial for the cyanobacterial cell. Recently, the synthetic malyl-CoA-glycerate (MCG) cyclic pathway (Figure 1.2, green line) was proposed, which can not only metabolize glyoxylate to acetyl-CoA but also provides an additional route for CO_2 fixation by phosphoenolpyruvate carboxylase (PEPC) [136]. Implementation of the complete MCG pathway increased the acetyl-CoA pool, C_i assimilation, and cell growth of *S. elongatus* under ambient air when fed with 50 mM sodium bicarbonate [136]. PEPC is one of the most favorable carboxylating enzymes with a very high affinity for bicarbonate [137]; hence, its function might also reduce the dependence on RubisCO and CBBC.

Another approach is based on the implementation of formate assimilation (FA) as an additional C_i assimilation step (Figure 1.2, purple line). CO_2 can be reduced by formate dehydrogenase (FDH) to the simplest organic molecule formate, which is then introduced into the natural C1 metabolism by ligation to tetrahydrofolate (THF) to produce 10-formyl-THF, that can be further metabolized to 5,10-methylene-THF used for serine biosynthesis via SHMT [138]. Compared with the most natural C_i-fixing pathways, the FA-reducing CO_2 pathway shows advantages in terms of energy efficiency and feasibility [139]. This route relies on an efficient FDH capable of reducing CO_2 to formate. As the first attempt, a light-driven formate production system based on engineered FDH was generated, which allowed the production of formate in the micro-oxic environment of *Nostoc (Anabaena)* sp. PCC 7120 heterocysts [140]. In a separate study, the expression of the downstream enzymes for FA, including FTL and FolD, enabled *E. coli* to synthesize proteinogenic serine from external formate [141]. These studies provide a platform for the future introduction of FA utilizing pathways into photosynthetic organisms to improve carbon assimilation efficiency. The implementation of these synthetic routes might pose a challenge because of their interference with the endogenous metabolisms, which is a noteworthy point for researcher's consideration. Nevertheless, these novel pathways could be easily tested in the cyanobacterial background before their introduction into plants to improve crop yield and feed growing population sustainably in the future.

1.6 Concluding Remarks

As reviewed here, cyanobacteria evolved many measures to successfully acclimate to fluctuating C_i conditions. Especially, the efficient CCM make them largely independent of the external C_i, permitting growth even at low CO_2 partial pressures. Our understanding of the CCM, its function, and regulation has now reached a system level. However, most of this knowledge was attained with a few unicellular

model cyanobacteria. Variations from this picture might exist in the cyanobacterial phylum, which is already depicted from the analyses of the currently existing more than 300 available genome sequences. It is likely that the study of cyanobacterial strains from ecological niches very poor in C_i will provide new insights into carbon assimilation. Environmental concerns about the use of fossil fuels and increasing CO_2 in the Earth atmosphere brought cyanobacteria into the focus of green biotechnology. Many attempts to produce different biofuels and feedstocks have been published, but generally, the obtained product values are rather low. This situation will be certainly changed if the limited understanding of regulatory processes involved in carbon partitioning improves. Here, post-translational mechanisms are probably of higher importance than transcriptional regulation, so a revitalization of physiological and biochemical studies on key enzymes involved in carbon assimilation is required. With the advent of synthetic biology, new methods and concepts are available for general redesign of metabolic routes. Cyanobacteria can here serve as ideal chassis to test such concepts because of their relatively easy genetic manipulation. The close evolutionary relation of cyanobacterial and plant photosynthetic metabolism will then likely permit transfer of successful concepts from the cyanobacterial system to crop plants. Finally, cyanobacterial genetic resources, for example, CCM modules, offer great opportunities to improve carbon assimilation in the future.

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