Regulatory adenylnucleotide-mediated binding of the PII-like protein SbtB to the cyanobacterial bicarbonate transporter SbtA is controlled by the cellular energy state

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ABSTRACT

Cyanobacteria have evolved one of the most powerful CO₂ concentrating mechanisms (CCM), supporting high photosynthetic rates with limiting inorganic carbon (Ci), which makes their CCM a desirable system for integration into higher plant chloroplasts to enhance photosynthetic yield. The CCM is driven by active Ci uptake, facilitated by bicarbonate transporters and CO₂ pumps, which locally elevates the CO₂ concentration and carboxylation rate of the primary CO₂ fixing enzyme, Rubisco, inside cytoplasmic micro-compartments (carboxysomes). Ci uptake responds allosterically to Ci supply and light, but the molecular signals and regulators of protein function are unknowns. Functional analyses of sodium-dependent bicarbonate transporters classified as SbtA in *E. coli* support the hypothesis that SbtA activity is negatively regulated through association with its cognate PII-like SbtB protein. Here, we demonstrate that the association of SbtA with SbtB from two phylogenetically distant species, *Cyanobium sp.* PCC7001 and *Synechococcus elongatus* PCC7942, depends on the relative amounts of ATP or cAMP compared to ADP or AMP. Higher ATP over ADP or AMP ratios decreased the formation of SbtA:SbtB complexes, consistent with a sensory response to the cellular adenylate energy charge ($AEC = [ATP + 0.5 ADP] / [ATP + ADP + AMP]$) and the different binding affinities of these adenylates to SbtB protein trimers. Based on evidence for adenylate ligand-specific conformation changes for the SbtB protein trimer of *Cyanobium sp.* PCC7001, we propose a role for SbtB as a curfew protein locking SbtA into an inactive state as safe-guard against energetically futile and physiologically disadvantageous activation during prolonged low cellular AEC and photosynthetically unfavourable conditions.

INTRODUCTION

Cyanobacteria have evolved one of the most efficient CO₂ concentrating mechanisms (CCM) known to date, which has been fundamental to their adaptability and successful competition in environments providing limited inorganic carbon (Ci) supply for oxygenic photosynthesis and accounts for cyanobacteria contributing 30-80% of the global photosynthetic productivity in the oceans (Liu et al., 1997; Field et al., 1998). This efficiency has fuelled recent efforts of introducing a synthetic, functional cyanobacterial CCM into chloroplasts of staple crop species, predicted to increase photosynthesis and crop yield significantly (Price et al., 2013; McGrath and Long, 2014; Long et al., 2016; Meyer et al., 2016). The cyanobacterial CCM enables active uptake of two Ci species, CO₂ and bicarbonate (HCO₃⁻), resulting in the accumulation of large cytoplasmic bicarbonate pools (20-40 mM) (Badger and Andrews, 1982; Price and Badger, 1989) that support carbonic anhydrase (CA)-mediated elevation of the CO₂ concentration around the primary CO₂-fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Consequently, Rubisco carboxylation is

enhanced over oxygenation, and energetically costly photorespiration suppressed (for review see (Badger and Price, 1992; Kaplan and Reinhold, 1999; Price et al., 2008; Espie and Kimber, 2011; Price, 2011)). To date, five Ci uptake systems have been verified, varying with respect to energization, substrate affinity, flux rates and induction by low Ci conditions (Price, 2011). Two light-dependent, thylakoid-located CO_2 pumps or energy-coupled CAs, Ndh-1₃ and Ndh-1₄ (Maeda et al., 2002; Ohkawa et al., 2002), convert external and internally recaptured CO_2 to HCO_3^- , energized by NADPH and reduced ferredoxin (Ogawa et al., 1985; Maeda et al., 2002; Price et al., 2008). The three types of plasma membrane-located bicarbonate transporters, the ATP-dependent BCT1 complex (Omata et al., 1999) and the $\text{HCO}_3^-/\text{Na}^+$ symporters BicA (Price et al., 2004) and SbtA (Shibata et al., 2002), transfer bicarbonate directly from the outside into the cytoplasm upon activation by low Ci conditions and light (Sültemeyer et al., 1998; McGinn et al., 2003). Of particular interest for a synthetic CCM is SbtA due to its ability to function in non-photosynthetic *E. coli* (Du et al., 2014). SbtA requires a Na^+ gradient directed from the outside to the inside of the cell for activity (Shibata et al., 2002; Price et al., 2004). Both CO_2 uptake and HCO_3^- transport are activated by light within seconds (5 and 30 s, respectively) (Badger and Andrews, 1982; Price et al., 2011) and gradually inactivated in the dark, suggesting a link to the state of photosynthetic electron transport or a redox signal (Kaplan et al., 1987), but the molecular mechanisms regulating the activity of specific bicarbonate transporters are largely unknown.

An intriguing, highly conserved feature of cyanobacterial genomes is the co-occurrence of the *sbtB* gene, encoding the soluble, PII-like SbtB protein, in the immediate gene neighbourhood of the *sbtA* gene (Rae et al., 2011). Both genes are co-expressed under Ci limitation in *Synechococcus elongatus* PCC7942 and *Synechocystis* sp. PCC 6803 (Woodger et al., 2003; Schwarz et al., 2011), suggesting a functional connection between the SbtA and SbtB proteins. This has been underpinned by constitutive inactivation of SbtA-mediated bicarbonate uptake upon co-expression with the cognate SbtB proteins in *E. coli* and by evidence for specific binding of SbtA (SbtA7942) to SbtB (SbtB7942) from *Synechococcus elongatus* PCC7942 (Du et al., 2014). Similarly, a study by Selim et al. (Selim et al., 2018) indicated SbtA (SbtA6803) and SbtB (SbtB6803) from *Synechocystis* sp. PCC 6803 interacted based on SbtA6803-dependent recruitment of SbtB6803 into membrane fractions under Ci starvation, which was enhanced by addition of ADP or AMP and diminished by cAMP. The authors suggested the cAMP:AMP ratio may indirectly signal the Ci levels in the cell and thus regulate SbtA6803:SbtB6803 complex formation and Ci uptake. However, the HCO_3^- uptake activity of SbtA6803 was not directly measured.

SbtB proteins have been classified as non-canonical PII-like members of the multi-functional PII and PII-like protein superfamily consisting of transcriptional and protein activity regulators,

environmental signal transducers or sensors, which are widely-distributed across bacteria, archaea and plants. Crystal structures revealed PII and PII-like protein are homotrimers with clefts between monomers forming binding sites for small effector molecules. Typically, effectors are bound in a competitive manner and may bind cooperatively. Common ligands for PII proteins are ATP, ADPs, Mg^{2+} and 2-oxoglutarate (2OG) which impact on protein conformation, particularly the T-loop, and interaction with target proteins (Radchenko et al., 2010; Huergo et al., 2013; Zeth et al., 2014; Forchhammer and Lüddecke, 2016). Ligands identified for cyanobacterial PII-like proteins include ADP and bicarbonate for the carboxysome-associated CPII from *Thiomonas intermedia* (Wheatley et al., 2016), and primarily adenyl nucleotides for the SbtB6803 protein (cAMP, AMP, ADP, ATP) (Selim et al., 2018) and SbtB (SbtB7001) protein from *Cyanobium sp.* PCC7001 (cAMP, AMP, ADP, ATP and Ca^{2+} ATP) (Kaczmarek et al., 2019). SbtB6803 and SbtB7001 share the basic architecture, but differ in ligand-coordinating residues, ligand affinities and ligand-induced conformation changes.

The energy status of living cells is reflected in the relative abundance of the adenyl nucleotides ATP, ADP and AMP and referred to as the cellular adenylate energy charge (AEC), described quantitatively as $([ATP]+0.5[ADP])/([ATP]+[ADP]+[AMP])$ (Chapman et al., 1971). The differential binding affinities for ATP, ADP and AMP suggest SbtB may sense the AEC and dynamically interact with SbtA in response to changes in adenylate nucleotide ratios. Here, we investigated (i) the effects of the adenyl nucleotides ATP, ADP, AMP and cAMP on the association of SbtA (SbtA7001) from *Cyanobium sp.* PCC7001 and SbtA7942 with their cognate SbtB proteins *in vitro*, based on the hypothesis that the adenyl nucleotide ligand-induced structural changes observed for SbtB7001 will determine whether SbtA and SbtB form a complex inactivating SbtA, and (ii) *in vivo* effects of SbtB deletions on SbtA activity in response to CO_2 availability and light.

RESULTS

The impact of small effector molecules on the association of either SbtA7001 or SbtA7942 with their cognate SbtB proteins in stable complexes was investigated *in vitro* with membrane-enriched, native protein extracts from the *E. coli* strain DH5 α (*E. coli*) and from the bicarbonate transporter deletion strain ΔCS of *Synechococcus elongatus* PCC7942 (*Synechococcus* ΔCS), both co-expressing SbtA7001 and SbtB7001 or SbtA7942 and SbtB7942 from plasmids. The membrane-integral SbtA proteins and the soluble, cytoplasmic SbtB proteins were both present in the membrane-enriched fraction (Fig. S1A), but, contrary to the study by Selim et al. (Selim et al., 2018), membrane co-localization was not an unequivocal indicator of SbtA:SbtB complex formation as substantial amounts of either SbtB protein accumulated in the membrane fractions in the absence of SbtA protein (Fig. S1B). Specific binding was therefore evaluated by metal immobilized

chromatography (IMAC) purification of SbtA:SbtB complexes with immobilized SbtB as bait and immunological analysis of relative amounts of SbtA and human influenza hemagglutinin (HA) epitope-tagged SbtB proteins with specific antisera. Note that the tagged SbtB7001 and SbtB7942 protein monomers are detected at 13 kD consistent with their calculated molecular weights, whereas the apparent molecular weights of SbtA7001 and SbtA7942 monomers are approximately 11 kD lower than their calculated molecular weights (23 vs 35 kD and 29 vs 40 kD, respectively) as has been quite commonly observed for membrane proteins and was attributed to altered protein-SDS detergent interactions (Rath et al., 2009).

Effects of small charged molecules on SbtA:SbtB complex formation

In the absence of additional effectors, both SbtA-SbtB pairs were dissociated in IMAC binding assays (Fig. 1, S2), which we attributed to the strong dilution effect of extraction and assay buffers on effectors present in intact cells below levels of efficacy. Potential effector molecules reported to interact with PII and PII-like proteins were tested for their ability to facilitate the association of SbtA:SbtB complexes, with particular focus on the adenyl nucleotides ATP, ADP, AMP and cAMP and Ca^{2+} that bind to SbtB7001 with micromolar affinities. Since Ca^{2+} enhanced binding of ATP to SbtB7001 *in vitro* (Kaczmarek et al., 2019), and Ca^{2+} and ATP co-occur *in vivo*, we simulated the basal Ca^{2+} levels in cyanobacterial cells (Leganés et al., 2009) including 200 nM CaCl_2 as a potential cofactor in binding analyses. On their own, Ca^{2+} and Mg^{2+} (another common cofactor for binding of ATP) did not facilitate SbtA7001:SbtB7001 association (Fig. S2). Individual adenyl nucleotides had distinctive effects on the association of both SbtA7001: SbtB7001 and SbtA7942:SbtB7942 protein complexes. SbtA and SbtB remained fully dissociated in the presence of either 2 mM ATP or cAMP, whereas stable SbtA:SbtB complexes formed in the presence of 2 mM ADP or AMP (Fig. 1), showing similar effects on binding interactions for both SbtA-SbtB pairs. Importantly, SbtA-SbtB-effector interactions were qualitatively identical for proteins expressed in *E. coli* and *Synechococcus* ΔCS, ruling out confounding effects by potential species-dependent post-translational modifications of either binding partner, adding validity to integrating findings from heterologous expression systems.

Amongst small molecule effectors tested, those unable to bind to SbtB7001 were equally ineffective in mediating the binding of either SbtA-SbtB pair. Calvin-Benson-Basham (CBB) cycle intermediates indicative of photosynthetic activity, the substrate ribulose-1,5-bisphosphate (RuBP) and product, 3-phosphoglycerate (3PGA) of the Rubisco reaction, as well as the tricarboxylic acid (TCA) cycle mediate 2OG, a master regulatory metabolite coordinating carbon and nitrogen metabolism (Huergo and Dixon, 2015), did not promote SbtA:SbtB complex formation. A synergistic

effect between equimolar 2OG and ATP or ADP, analogous to the prerequisite binding of Mg^{2+} ATP to the *E. coli* PII protein prior to 2OG to become effective, could also be ruled out, as 2OG did not alter the ATP- and ADP-specific effect on the SbtA:SbtB interaction (Fig. 2). HCO_3^- does not bind to the SbtB7001 protein *per se* (Kaczmariski et al., 2019), but considering HCO_3^- is the primary substrate for SbtA, we tested whether bicarbonate levels exerted feedback regulation onto SbtA activity, potentially by varying amounts of inactivate SbtA:SbtB complexes. However, SbtA7001 and SbtB7001 interactions proved to be independent of HCO_3^- across a wide concentration range (0 to 100 mM) and irrespective of combinations with 10 mM Mg^{2+} , nano- to millimolar Ca^{2+} concentrations and/or 2 mM ATP or 2 mM AMP (Fig. S2, S3). Interestingly, 10 mM inorganic phosphate (PO_4^{2-}) generally enhanced the formation of SbtA:SbtB complexes (Fig. S4). It is subject to further investigation though, as to whether physiological concentrations of free phosphates affect the interaction of SbtA7001 and SbtB7001 *in vivo*.

The adenyl nucleotide ratios correlate with the level of SbtA:SbtB association

A link to the adenylate energy charge, suggested by the opposing effects of ATP versus ADP or AMP on SbtA:SbtB complex formation, was explored *in vitro*, simulating different energy charge levels with different molar ratios of ATP:ADP or ATP:AMP within a constant total adenyl nucleotide pool of 2 mM (Fig. 3). Irrespective of protein expression in *E. coli* and *Synechococcus* ΔCS, both SbtA-SbtB pairs showed a decrease in amounts of SbtA bound to SbtB with the increasingly higher proportion of ATP over ADP (Fig. 3A-D) and AMP (Fig. S3E, F). Association of SbtA with SbtB and ATP:ADP and ATP:AMP ratios were non-linearly correlated and well fitted by a sigmoidal logistic model with mean adjusted R^2 values of 0.926 ± 0.023 (Fig. S5). Adenyl nucleotide ratios supporting 50% (AR50), 25% (AR25) or 10% (AR10) of the maximum levels of SbtA bound to SbtB derived from curve fits showed no statistically significant differences between the two SbtA-SbtB pairs and in response to the different adenyl nucleotide ratios tested (Table 1, $P > 0.05$). However, slightly but consistently lower AR values for SbtA74942 indicated lower ATP:ADP and ATP:AMP ratios prevented binding to SbtB than required for the same effect on binding of SbtA7001 and SbtB7001. This may suggest differences in the sensitivity to the intracellular AEC signalled by the molar fraction of ATP in the adenylate pool for the SbtA:SbtB interactions in different cyanobacterial species, but requires more detailed investigation. Similar to ATP, cAMP alone prevented association of both SbtA-SbtB pairs (Fig. 2), and showed increasingly less SbtA was bound to SbtB with higher cAMP:ADP and cAMP:AMP ratios (Fig. 4). Subject to further investigation, the same cAMP levels affected comparatively less SbtA7942 to associate with its cognate SbtB protein than observed for SbtA7001 (Table 1). Taken together, when analysed separately, SbtA:SbtB binding appeared to respond to

both, molar fractions of ATP and cAMP. One caveat in the experimental design was the application of the same concentration ranges of ATP and cAMP. While ATP concentrations up to 2 mM corresponded closely to the intracellular ATP pools in several bacteria (Thauer et al., 1977; Yaginuma et al., 2014), estimates of cAMP levels in cyanobacterial cells have been up to 1000-fold lower than the intracellular ATP levels (Ohmori and Okamoto, 2004), which suggests the molar fraction of ATP rather than cAMP in the adenylate pool predominantly determines the extent of SbtA:SbtB association *in vivo*.

Light-regulated SbtA7942 function *in vivo* is independent of SbtB7942 in *Synechococcus*

Regulation of SbtA activity in *E. coli* was closely linked to the presence of the cognate SbtB, where constitutive bicarbonate uptake by SbtA was inactivated when co-expressed with SbtB (Du et al., 2014). In cyanobacteria, however, SbtA and SbtB proteins are both present under low C_i conditions (Fig. 1) and yet SbtA is active to support photosynthesis in the light, which suggests additional levels of regulation tune SbtA function. Potential effects of SbtB on SbtA activity in cyanobacteria were assessed by comparison of photosynthetic performance between *Synechococcus* ΔCS expressing either SbtA7942 and SbtB7942 or SbtA7942 only. Specific SbtA-dependent photosynthetic oxygen evolution and C_i uptake rates were determined by inhibiting both CO_2 pumps with 500 μM ethoxymylamide (EZ) and performing measurements at pH 8.0 where the prevalent C_i species was HCO_3^- . Direct measurements of radioactively labelled [^{14}C] HCO_3^- uptake by SbtA7942 showed bicarbonate uptake rates were unaffected by the presence of up to 1 mM EZ, ruling out inhibitor-induced side effects (Fig. S5). In constant light, SbtA7942-dependent photosynthetic rates were the same in low C_i -induced cell cultures with (SbtAB) or without (SbtA) the SbtB7942 protein expressed (Fig. 5) and indistinguishable with respect to substrate affinities ($k_{0.5}$ 26 ± 5 and 29 ± 4 μM C_i , respectively) and maximum rates of photosynthetic O_2 evolution (V_{max} 179 ± 33 and 164 ± 19 $\mu mol\ mg\ Chl^{-1}\ h^{-1}$, respectively) (Fig. 5, inset). Cell lines co-expressing SbtA and HAHis-tagged SbtB had a 19% lower, but statistically not significant ($P > 0.05$), mean photosynthetic rate (V_{max} 145 ± 14 $\mu mol\ mg\ Chl^{-1}\ h^{-1}$) and HCO_3^- affinity ($k_{0.5}$ 39 ± 15 $\mu M\ C_i$), potentially due to variations in SbtA protein levels. Similarly, steady state HCO_3^- uptake and photosynthetic O_2 evolution rates of SbtA compared to SbtAB showed little differences in response to a change of light intensities from 1400 to 200 to 1400 $\mu mol\ photons\ m^{-2}\ s^{-1}$ in the presence of saturating 250 and 500 μM external C_i (Fig. S7), supporting the notion that SbtB is not involved in modulation of SbtA7942 activity in response to light-dependent changes in photosynthesis that alter Rubisco activity and C_i demand. Since many photosynthetic processes are almost instantaneously inactivated in the dark, bicarbonate uptake by SbtA7942 in light to dark transition and the potential regulatory role of SbtB were assessed by direct

uptake measurements of [^{14}C]HCO $_3^-$. Fully active SbtA7942 was inactivated 15 s after transition from light into darkness, and the initial, fast dark inactivation dynamics of SbtA7942 were unchanged irrespective of the presence of the SbtB7942 protein (Fig. 6). However, this does not rule out a likely role for SbtB in longer dark periods.

Reduced growth of the SbtB6803 knockout mutant in *Synechocystis* sp. PCC6803 had been observed under low Ci condition and in low light previously (Selim et al., 2018). Hence, *Synechococcus* ΔCS expressing SbtA7942 and tagged or untagged SbtB7942 or SbtA7942 only were for 10. In contrast to *Synechocystis*, we observed *Synechococcus* ΔCS cells expressing only SbtA7942 grew to similar or slightly higher densities compared to cells expressing SbtA7942 and tagged or untagged SbtB7942 on solid media at pH 8 in air under a 12/12 h low light/dark regime (Fig. S8). This suggests SbtB7942 is not essential for autotrophic growth of *Synechococcus* ΔCS and, consistent with the photosynthetic performance (Fig. 5), is not required for optimal growth under these conditions.

DISCUSSION

Regulation of cyanobacterial bicarbonate transporters has been an unresolved question for quite some time with patchy evidence for diverse mechanisms and co-factors that may be involved. Here, we elucidated the potentially regulatory protein-protein interaction between two SbtA bicarbonate transporters and their cognate PII-like SbtB proteins depend on the relative concentrations of the adenyl nucleotides ATP, ADP, AMP and cAMP. The SbtA-SbtB pairs originated from the transitional α -cyanobacterium *Cyanobium* sp. PCC7001 and the β -cyanobacterium *Synechococcus elongatus* PCC7942 which are phylogenetically relatively distant species (Sánchez-Baracaldo et al., 2019) and share limited overall sequence homology at the amino acid level, with 60% identity between the two SbtA (Fig. S9, S10) and 58% identity between the two SbtB (Fig. 7C, S11) proteins, yet the interaction profiles for both SbtA-SbtB pairs were convincingly similar to conclude both protein pairs share the same overall regulatory mechanisms.

Is ATP or cAMP a primary effector?

In vivo, the intracellular adenyl nucleotide pools invariably contain ATP, ADP, AMP and cAMP, albeit relative concentrations may vary rapidly dependent on metabolic activity in response to environmental and developmental factors (De la Fuente et al., 2014). In cyanobacteria, cAMP is predominantly a secondary messenger as part of different signalling events, whereas the relative abundances of ATP, ADP and AMP pool defines the energy status of a cell. Notwithstanding, both processes could translate Ci availability and Ci demand to SbtB-mediated regulation of SbtA. In addition, relative abundance of cAMP, ATP and AMP in the adenylate pool are interdependently

influenced by the activities of adenylate cyclase and phosphodiesterases which catalyze cAMP synthesis from ATP and hydrolytic degradation to AMP, respectively. Depending on the species, adenylate cyclase activity is stimulated by N and P deficiency, osmotic stress, light quality and in some cases also bicarbonate or CO₂ (Cann, 2004; Hammer et al., 2006; Xu and Su, 2009; Agostoni and Montgomery, 2014), potentially linking cAMP and Ci sensing.

Evidence from *Synechocystis* sp. PCC6803 suggests the cAMP:AMP ratios determined formation of SbtA:SbtB complexes and SbtA activity. The SbtB6803 protein from *Synechocystis* binds cAMP with higher affinity than other adenylates, and was proposed to be a novel cAMP receptor sensing the Ci status and mediating acclimation of the CCM to high Ci levels (Selim et al., 2018). High Ci acclimated cells were characterized by elevated [cAMP], a high cAMP:AMP ratio and downregulated Ci uptake. Under these conditions, SbtB6803 was dissociated from SbtA6803 which argued SbtA in the unbound state was inactive. Conversely, bicarbonate uptake is upregulated in low Ci acclimated cells, which coincided with a low cAMP:AMP ratio and high [AMP] favouring co-localization of SbtB with SbtA in the membrane (Selim et al., 2018), and therefore suggested SbtA in the bound state was active. These findings are difficult to reconcile with the simple interaction model based on SbtB-mediated SbtA regulation in *E. coli*, where SbtA-dependent bicarbonate uptake measurement consistently showed that SbtA alone was clearly active and co-expression of SbtA and SbtB inactivated bicarbonate uptake, including the SbtA6803-SbtB6803, SbtA7001-SbtB7001 and SbtA7942-SbtB7942 pairs (Du et al., 2014). The binding analyses performed here (Fig. 3) support the view that low [ATP] and low AEC dominate association of SbtA7001-SbtB7001 and SbtA7942-SbtB7942 pairs. We need to consider that the experimentally applied cAMP, but not ATP, ADP or AMP concentrations, were almost certainly orders of magnitude higher than *in vivo* levels (Ohmori and Okamoto, 2004). Therefore, it seems unlikely the intracellular [cAMP] required for effective competition with [ATP], and thus affecting SbtA:SbtB binding, would occur under physiological conditions. Species-related differences in cAMP-mediated regulation may provide a partial explanation for differences between studies, such as the loss of cAMP receptor proteins (CRPs) from the *Synechococcus elongatus* PCC7942 genome, amongst other *Prochlorococcus* and *Synechococcus* species (Xu and Su, 2009). CRPs are transcriptional activators or repressors upon binding of cAMP, involved in the regulation of multiple target genes. Possibly, roles of cAMP in post-translational regulation, such as the interaction of SbtA7942 and SbtB7942, may also be affected. In addition, the 6-12 times higher binding affinity of ATP over cAMP, ADP or AMP to SbtB7001 (Kaczmarek et al., 2019) as opposed to highest affinity for cAMP by SbtB6803 (Selim et al., 2018) suggests the interaction of SbtB7001 and SbtA7001 may be more sensitive to the AEC.

The SbtA and SbtB proteins interact dynamically in response to the adenylate energy charge

Universally, ATP, ADP and AMP form the core of the energy system of living cells. The majority of biochemically available energy is linearly correlated with the molar fraction of ATP and $\frac{1}{2}$ molar fraction of ADP of the total adenylate pool, but was experimentally simplified by testing effects of ATP:ADP and ATP:AMP ratio effects separately. Adenylate pools measured from a diverse range of tissues, organisms and environmental conditions suggest active growth requires an energy charge between 0.8 and 0.9, while a drop of the energy charge below 0.5 is unsustainable (Chapman et al., 1971), which sets the expected response margins expected for AEC-regulated processes. The AEC is often sensed by regulatory proteins through competitive binding of ATP or ADP causing opposite directed responses (Atkinson and Walton, 1967). In fact, PII proteins have been shown to sense the energy status of the cell and regulate target enzyme activity by undergoing specific conformational changes in response to binding ATP or ADP which altered the interaction between the PII protein and its target and modulated the activity of the target protein (Radchenko et al., 2013; Zeth et al., 2014; Selim et al., 2019). This principle applied to binding of the PII-like SbtB protein and SbtA from both cyanobacterial species investigated. The simulated AEC supporting more than 90% unbound (and active) SbtA was 0.74 to 0.91 (Table 1), and corresponds well with the measured ATP/(ATP+ADP) ratios of 0.8 in the light at pH 8 reported for a thermophile *Synechococcus* strain (Kallas and Castenholz, 1982), 0.8 to 0.9 for *Synechococcus elongatus* (Rust et al., 2011) and 0.8 for *Anacystis nidulans* (recalculated from (Bornefeld and Simonis, 1974)), which suggests physiologically active cyanobacterial cells maintain an almost fully active pool of SbtA protein dissociated from SbtB. This was corroborated by the *in vivo* activity of SbtA7942 in the *Synechococcus* Δ CS strains expressing SbtA or both SbtA and SbtB, supporting the same photosynthetic and bicarbonate uptake rates (Fig. 5, 6, S7) and growth at low Ci (Fig. S8). In comparison, co-expression of SbtB and SbtA fully inhibited SbtA function in actively growing *E. coli*, where all carbon sources had been removed prior to measuring bicarbonate uptake. This inhibition had been attributed to the formation of SbtA:SbtB complexes (Du et al., 2014). If SbtA:SbtB association was AEC-dependent, experimental conditions must have caused a sufficiently lower AEC than 0.8, reported for *E. coli* in exponential growth phase (Chapman et al., 1971). One possibility is that the high expression levels of a foreign membrane and soluble protein depleted internal energy and N resources, and further removal of exogenous C sources led to stressed cells operating at a substantially lower energy charge. Therefore, future analyses should explore whether artificially raised intracellular [ATP] and AEC levels can dissociate SbtA:SbtB complexes and restore SbtA activity in *E. coli*.

Adenylnucleotide-dependent conformation of SbtB for regulatory interaction with SbtA

In PII proteins the surface-exposed, flexible T-loop has been identified as the central region that mediates the regulatory interaction of the PII protein with its target protein(s). The T-loop undergoes ligand-induced conformation changes upon binding small effector molecules in a co-operative or competitive manner which determines whether PII binds to its target. For example, binding of ATP to the PII protein from *Synechococcus elongatus* PCC7942 induced T-loop conformation changes facilitated association of N-acetyl-glutamate kinase (NAGK) and arginine synthesis (Zeth et al., 2014; Selim et al., 2019), and the ADP-stabilized T-loop of the PII protein GlnK from *E. coli* enable formation of an inactivating complex with the ammonium transporter AMTB (Radchenko et al., 2010). There seems to be a direct correlation, analogous to PII proteins, between changes to the flexible T-loop of SbtB7001 trimers ligands occupying the intermolecular nucleotide binding clefts (Kaczmariski et al., 2019) and the opposing effects of ATP and ADP or AMP on SbtA:SbtB binding and implicit SbtA regulation, which is summarized in Fig. 8. The probability for each adenylate ligand to occupy a nucleotide binding site depends on the binding affinity and the local concentration of the ligand. At high ATP:ADP or AMP ratios, when Ca^{2+}ATP occupies the nucleotide binding sites of the SbtA trimers, the T-loops are stabilized through interactions of specific residues with the γ -phosphate of ATP, in a position close to the core ('closed' conformation) that limits further access to the site ((Kaczmariski et al., 2019), Fig. 7A). It appears that the Ca^{2+}ATP -induced stable T-loop conformation prevents the SbtB7001 trimers from binding to SbtA, promoting full SbtA activity. In contrast, at low ATP:ADP or AMP ratios, occupation of the binding site by ADP or AMP leaves the T-loops in their inherent, disordered ('open') conformation ((Kaczmariski et al., 2019), Fig. 7A), and SbtB is able to form a stable, inactivating complex with SbtA. The interaction between SbtB7942 and SbtA7942 is likely to be modulated by a similar adenylnucleotide-induced T-loop conformation change, inferred from highly homologous modelled structures of the two SbtB proteins, their overall amino acid sequence identity of 57.7% and almost complete conservation of the T-loop and ATP-binding residues (Fig. 7B, C). However, until the nature of the molecular interactions between SbtB and SbtA are resolved, relationships between structure and function of SbtB, particularly the T-loop, remain speculative.

Implications for regulation of SbtA activity in cyanobacteria

For cyanobacteria, it is a selective advantage to maximize photosynthetic CO_2 fixation and carbon gain for growth in the light. In the dark, photosynthetic CO_2 fixation ceases making Ci acquisition redundant and energetically futile. Additionally, continued accumulation of large amounts of bicarbonate may disrupt intracellular pH and ion homeostasis, particularly if $[\text{Na}^+]$ were

to build up by $\text{HCO}_3^-/\text{Na}^+$ symport due to SbtA activity. Therefore, the co-ordinated regulation of Ci acquisition and photosynthetic activity may be imperative for survival and success of cyanobacteria. A hypothetical scenario for the regulation of bicarbonate uptake by SbtA in cyanobacteria is depicted in Fig. 8. SbtA and SbtB are both presented as trimers, which is supported by evidence from crystal structures, size exclusion chromatography and homology to PII proteins for SbtB (Selim et al., 2018; Kaczmarek et al., 2019), and is highly likely for SbtA (Du et al., 2014) but needs further verification. We postulate two types of regulatory mechanisms for SbtA-mediated Ci uptake may have evolved that differ in time scales, fast inactivation-activation within seconds to match rapid changes in photosynthetic CO_2 -fixation and longer-term inactivation as safe-guard against activation under photosynthetically/metabolically unfavourable conditions which involves SbtB. In the light, under low Ci conditions, bicarbonate uptake by SbtA may be limited by light intensity and substrate availability, but SbtA functions independent of the SbtB protein (Fig. 5, S7). The AEC of the cell under otherwise favourable environmental conditions would remain steady near 0.8 to 0.9 (Rust et al., 2011), preventing SbtB from binding to SbtA. Upon onset of darkness, SbtA-mediated bicarbonate transport is inactivated within seconds (Fig. 6). At this time scale involvement of SbtB is unrealistic. Apart from evidence for SbtA inactivation in SbtB-less *Synechococcus*, inactivation is likely to occur more quickly than the exchange of ATP for ADP or AMP from the SbtB trimers and subsequent binding to SbtA even though the AEC may change rapidly. As shown for *Anacystis nidulans*, a dark-induced drop in AEC can occur within seconds, reducing the ATP/(ATP+ADP) ratio from 0.85 to 0.4 and gradually stabilizing at about 0.6 after 30 minutes in the dark (calculated from (Bornefeld and Simonis, 1974)). A similar immediate decline in ATP levels was observed in *Synechococcus* after a light-to-dark shift, but ATP levels were restored to 85% of those in the light within 10 minutes (Takano et al., 2015). In contrast, a one-hour lag in the dark was observed in *Synechococcus elongatus* (Rust et al., 2011) before the ATP/(ATP+ADP) ratio decreased from 0.85 to 0.5. As such, the nature of the fast signals and signal transduction pathways that trigger SbtA inactivation upon the light-to-dark transition and, in reverse, its activation after dark-to-light remain virtually a black box of unknowns (Fig. 8) with ample possibilities for speculation.

Light-dark transitions generate rapidly reversible changes in cellular redox status driven by rapid photochemistry and physical properties of photosynthetic electron transport, known to regulate CBB cycle enzyme activity via Chloroplast protein (CP)12 protein and ferredoxin-thioredoxin system redox signalling cascades (Tamoi et al., 2005). It is intriguing to consider similar redox regulation for SbtA. Although SbtA lacks highly conserved Cys residues that could form sulfhydryl bonds with canonical CP12 or thioredoxins, a CP12 variant without sulfhydryl bonds identified in *Synechococcus elongatus* PCC7942 may be a light and Ci responsive regulator. Gene expression of

the CP12 variant changed from high to low Ci conditions and with light intensity (Stanley et al., 2013), and a deletion mutant exerted reduced growth under light-dark cycles (Tamoi et al., 2005). Alternatively, potential redox-signalling may involve Ca^{2+} . Light-dark transitions produced specific, short-lived 30 s $[\text{Ca}^{2+}]$ spikes in the cyanobacterium *Anabaena sp.* PCC7120, which were suggested to arise from influx of Ca^{2+} through plasma membrane located Ca^{2+} channels triggered by a redox change within the photosynthetic electron transport chain (Torrecilla et al., 2004). Similarly, a dark-induced change in membrane potential could have triggered opening of Ca^{2+} channels as observed in phototaxis (Murvanidze and Glagolev, 1982) or the release of Ca^{2+} from the many Ca^{2+} binding proteins present in cyanobacteria (Domínguez et al., 2015). A change in protein phosphorylation is an efficient option for rapid regulatory post-translation modifications, illustrated by phospho-relays able to transduce a stimulus into the motor response within 200 ms in regulation of chemotactic motility in *E. coli* (Parkinson, 2003). The two major phospho-relay signal transduction systems are two-component signalling systems and eukaryotic-type Ser/Thr and Tyr kinases and phosphatases. Studies of cyanobacterial phospho-proteomes suggest extensive regulatory networks exist involving phosphorylation cascades and relays for many biological processes, some of which combine two-component signalling and Ser/Thr/Tyr phosphorylation (Dworkin, 2015). The extent of SbtA phospho-regulation by light and Ci availability is unknown, but several highly conserved, cytoplasmic exposed Ser, Thr, Tyr and Arg residues (Fig. S9) could be targets of redox and/or Ca^{2+} signalling-coupled kinase-phosphatase systems, involving (i) light-dark/redox-responsive Ser, Thr or Tyr phosphorylation/de-phosphorylation, (ii) Arg-phosphorylation by a redox/ Ca^{2+} sensory His kinase analogous to two-component signalling, and (iii) a combination of phospho-transfer from a sensory His kinase to a Ser/Thr or Tyr kinase/phosphatase acting on SbtA.

The relatively longer time constants for binding of SbtB to SbtA, exceeding initial SbtA inactivation, and the AEC-dependence involved in SbtB-mediated SbtA regulation, clearly favour a role for SbtB as “curfew” protein in longer-term inactivation of SbtA. Depending on the analysis, the cellular adenylate energy charge dropped to about 0.6 within minutes to an hour in the dark. Based on the *in vitro* binding analyses (this work), this AEC is sufficiently low to replace ATP with ADP and AMP in the nucleotide binding sites in SbtB trimers and to sequester substantial amounts of SbtA into stable inactive complexes. The stoichiometry of SbtA and SbtB trimers within complexes remains to be elucidated. The association with SbtB shields SbtA from fast re-activation and would overwrite any environmental and intrinsic cues that may otherwise induce futile activation/inactivation cycles and activation of SbtA under conditions causing pro-longed inhibition of photosynthetic CO_2 assimilation such as darkness and a low AEC at night. In addition, there may be conditions requiring stable inactivation of SbtA in the light during prolonged nutrient or

environmental stress energy if the AEC falls significantly below 0.8 and resources are invested in protective processes instead of photosynthesis and Ci accumulation. After transition from dark to light the AEC rises very rapidly and bicarbonate uptake is activated after about 30 s (Badger and Andrews, 1982). This suggests the majority of the SbtA:SbtB complexes must dissociate within this time frame for SbtA to be active. It is conceivable that the 6-12 times higher binding affinity of ATP to SbtB7001 and greater ease of access to the nucleotide binding site with ADP or AMP bound facilitate a more rapid replacement of the low affinity ligands ADP and AMP by ATP than in reverse.

Conclusions

The interactions of the SbtB7001 and SbtB7942 proteins with their corresponding SbtA bicarbonate transporters are the first examples among PII-like proteins that provide evidence for SbtB as a sensor and regulatory protein in response to the adenylate energy charge of the cell, similar to canonical PII proteins. The two SbtA-SbtB pairs used here appear to be subject to the same adenylnucleotide regulation despite their origin from two distinct genera, *Cyanobium* and *Synechococcus*. It will be of interest to assess SbtA-SbtB interactions across a wide range of species to evaluate to which extent AEC-mediated regulation has been adopted across cyanobacterial species. The light-dark regulation of SbtA *in vivo* raises many questions. The light signalling pathway(s) and the molecular mechanism(s), independent of SbtB, need to be addressed, perhaps with redox- and post-translational regulation of SbtA as a starting point for further investigations. Likewise, the basis of energy charge-sensing of SbtB and its interaction with SbtA is clear, though the precise details are far from being fully understood. The elucidation of molecular and biochemical mechanisms underlying the interaction between SbtA and SbtB, causality of AEC and SbtA:SbtB complex formation *in vivo* and differential effects of environmental conditions are challenges for the future. However, the link between SbtB as a sensor of the cellular energy status and response regulator of SbtA activity is an important insight which aids informed strategies for expression of the SbtA-SbtB systems from *Synechococcus* and *Cyanobium* in higher plant chloroplasts.

MATERIALS AND METHODS

Detailed information on protocols and materials are provided as Supplementary Information.

E. coli and cyanobacterial cell lines

Nucleotide and amino acid sequences are referred to by gene ID and Locus_tags from IMG (Integrated Microbial Genome database; <https://img.jgi.doe.gov>). The HCO₃⁻ uptake deficient cell line *Synechococcus* ΔCS was derived from wild-type *Synechococcus elongatus* PCC794 by eliminating

expression of the bicarbonate transporters BCT1 and SbtA. The *cmpA* (IMG ID: 637799921, Synpcc7942_1488) and *cmpB* (IMG ID: 637799922, Synpcc7942_1489) genes were inactivated with a kanamycin resistance (*kan^R*) marker (Omata et al., 1999), and both the *sbtA* (IMG ID: 637799907, Synpcc7942_1475) and *sbtB* (IMG ID: 637799908, Synpcc7942_1476) genes were replaced with a chloramphenicol resistance (*cm^R*) gene (Dzelzkalns et al., 1984) (Table 1). For protein expression, *sbtA* and *sbtB* genes from *Synechococcus elongatus* PCC7942 and *Cyanobium sp.* PCC7001 (IMG ID: 647590134, CPCC7001_1784 and IMG ID: 647590133, CPCC7001_1671, respectively) were synthesized (GenScript, New Jersey, USA) and inserted into plasmids pSE2-1 and pSE4-1 carrying a spectinomycin resistance (*sp^R*) marker (Price et al., 2004; Du et al., 2014). Gene expression is controlled by under the isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible *lacZ* promoter in pSE2-1 and native *sbtA* and *sbtB* promoters in pSE4-1. The SbtB proteins were C-terminally tagged with a HAHis epitope for immunodetection and affinity purification. For a full list of cell lines and expression vectors refer to Table S2.

Growth of *E. coli* and cyanobacteria

For maintenance, mutant selection and protein expression, *E. coli* DH5 α derived cell lines were grown in Lysogeny broth (LB; sp100 μ g ml⁻¹) as described (Du et al., 2014). Expression of SbtA and SbtB proteins was induced for 3 h with 1 mM IPTG in *E. coli*. *Synechococcus* Δ CS cell lines were grown in modified BG-11 medium buffered with 20 mM HEPES-KOH pH 8.0 (Maeda et al., 2002; Woodger et al., 2003) in high Ci (2-4% (v/v) CO₂ enriched air) or low Ci (ambient air, 400 ppm CO₂) conditions under constant light (70 μ mol m⁻² s⁻¹). Antibiotics were supplied as appropriate (kan 100 μ g ml⁻¹, cm 8 μ g ml⁻¹, sp 100 μ g ml⁻¹). Protein expression of SbtA and SbtB was induced by exposing high Ci-grown for 4 h to low Ci. For growth assays, a dilution series of low Ci-induced cultures were spotted onto agar plates and grown in air under 12 h light (50 μ mol m⁻² s⁻¹)/12 h dark cycles.

Extraction of native proteins

The isolation of membrane-enriched fractions of native proteins from *E. coli* and cyanobacteria was adapted from (Du et al., 2014). Membrane proteins were resuspended in IMAC HEPES binding buffer (50 mM HEPES, 100 mM NaCl, 25 M imidazole, pH 8.0) supplemented with 1% (w/v) *n*-Dodecyl β -D-maltoside (DDM) for further analysis.

Immobilized metal affinity chromatography (IMAC) protein binding assays and quantification of SbtA:SbtB binding

The association of SbtA and HAHis-tagged SbtB proteins was analysed in IMAC binding assays adapted from (Du et al., 2014). Native protein extracts were incubated in IMAC HEPES binding buffer supplemented with 200 nM CaCl_2 and a range of potential effector molecules, and Ni-charged Profinity™ IMAC resin (Biorad, USA) for 1 h at 23 °C to allow HAHis-tagged SbtB to bind to the resin and SbtA to associate with SbtB. Details on effectors and adenylnucleotide ratios are provided in the figures and captions. IMAC purified SbtA:SbtB complexes were subjected to immunoblot analysis as described in (Du et al., 2014). SbtA protein was quantified by densitometry of Western blot images (ImageLab software (BioRad, USA) and correlated with the adenylnucleotide ratio (AR) in the binding reaction. The AR corresponding to 50% (AR_{50}), 25% (AR_{25}) and 10% (AR_{10}) of SbtA associated with SbtA:SbtB complexes were estimated from sigmoidal logistic curve fits (OriginPro v2020, OriginLab Corp., USA).

Photosynthetic analysis by membrane inlet mass spectrometry (MIMS) measurements

Net photosynthetic O_2 evolution and Ci uptake rates were calculated from changes in the concentrations of dissolved CO_2 and O_2 in low Ci induced cyanobacterial cell suspensions measured in a closed cuvette attached to a mass spectrometer as described previously (Badger and Andrews, 1982; Badger et al., 1985, Price, 2004 #32). CO_2 uptake was inhibited with 500 μM EZ and HCO_3^- -dependent photosynthetic O_2 evolution rates determined after incremental additions of bicarbonate with cell densities of 4 $\mu\text{g ml}^{-1}$ chlorophyll *a* (Chl *a*). Chl *a* content was determined spectrophotometrically in 90% (v/v) methanol extracts (Porra et al., 1989). Maximum velocity of O_2 evolution (V_{max}) and affinity for HCO_3^- ($k_{0.5}$) were derived from Michaelis-Menten kinetics.

Ci uptake measurement by silicon oil centrifugation-filtration

Light- and dark stimulated uptake of active species of $[^{14}\text{C}]\text{HCO}_3^-$ in cyanobacterial cell cultures was measured by using the silicon oil centrifugation-filtration method as described previously (Kaplan et al., 1980; Price and Badger, 1989). Low Ci -induced cell cultures at cell densities equal to 4 $\mu\text{g ml}^{-1}$ Chl *a* were treated with 500 μM EZ, and $[^{14}\text{C}]\text{HCO}_3^-$ uptake determined for a 30 s-interval at various time points after a light to dark shift. HCO_3^- uptake rates were calculated based on the specific activity of the $[^{14}\text{C}]\text{HCO}_3^-$ substrate.

Bioinformatics

Homologous SbtA and SbtB proteins were identified in BLASTP (Altschul et al., 1990) searches and multiple pairwise amino acid sequence alignments generated in Geneious Prime 2020.2.4 (<https://www.geneious.com>) with MUSCLE software (Edgar, 2004). Protein identifiers are

the gene IDs and Locus_tags from IMG (further detail in Tables S3 and S4). The domain homology model of the SbtB7942 monomer was generated in PSIPRED workbench (<http://bioinf.cs.ucl.ac.uk/psipred/>) and the fully automated homology modelling workflow Domserf 2.1 (Buchan and Jones, 2019). Template models were identified from PSI BLAST searches against the PDB and CATH databases and parsed into domains for optimal matching against the query sequence. Final models were constructed by MODELLER software based on a subset of best domain matches to SbtB7942. The overall folds of SbtB7942 and SbtB7001 chain A with ADP (PDB ID: 6MMC) or Ca²⁺ and ATP ligands (PDB ID: 6MM2) proteins were compared by superimposition, using Chimera v1.14 (<http://www.cgl.ucsf.edu/chimera>) and MatchMaker (Meng et al., 2006).

Statistics

Statistically significant differences between data sets were determined by performing a one-way analysis of variance (ANOVA) and post-hoc Tukey's Honest Significant Difference (HSD) test (Tukey, 1949), using a type I error level $\alpha=0.05$ as threshold ($P < 0.05$).

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Fig. 1. Effects of adenyl nucleotides on the association of SbtA and cognate SbtB proteins. The SbtA7001-SbtB7001 and SbtA7942-SbtB7942 pairs were expressed in *E. coli* (A, C) and *Synechococcus* ΔCS (*; B, D). During *in vitro* IMAC binding assays with 2 mM ATP, ADP, AMP, or cAMP, the HAHis-tagged SbtB proteins were immobilized for interaction with SbtA proteins and effectors. The SbtB protein and corresponding amounts of SbtA retained in SbtA:SbtB complexes were detected immunologically. The composite Western blot image is representative; n=3-8 per adenyl nucleotide treatment.

Fig. 2. The association of SbtA and cognate SbtB proteins is not linked to key metabolites of the CBB cycle, RUBP and 3PGA, and the TCA cycle, 2OG. The SbtA₇₀₀₁-SbtB₇₀₀₁ (A) and SbtA₇₉₄₂-SbtB₇₉₄₂ (B) pairs were expressed in *E. coli*. *In vitro* IMAC binding assays were performed with 2 mM RUBP, 3PGA or 2OG, and 2 mM 2OG and 2 mM ADP, or ATP combined (2OG ADP and 2OG ATP, respectively). The HAHis-tagged SbtB proteins and the SbtA proteins retained in SbtA:SbtB complexes were detected immunologically. Representative Western blot images; n=2.

Fig. 3. Effects of ATP:ADP and ATP:AMP ratios on the association of SbtA and SbtB proteins. The SbtA₇₀₀₁-SbtB₇₀₀₁ (A, C, E) and SbtA₇₉₄₂-SbtB₇₉₄₂ (B, D, F) pairs were expressed in *E. coli* (A, C, E, F) and in *Synechococcus* Δ CS (*) (B, D). Variation in energy charge was simulated in *in vitro* IMAC binding assays by different molar ratios of ATP:ADP (A-D) and ATP:AMP (E, F), with the total adenylnucleotide concentration at 2 mM. The HAHis-tagged SbtB proteins and the SbtA proteins retained in SbtA:SbtB complexes were detected immunologically. The 17 kD-protein above SbtB₇₉₄₂ is a fragment specific to SbtA. Representative Western blot images; n=3-4 per adenylnucleotide treatment.

Fig. 4. Effects of the cAMP:ADP and cAMP:AMP ratios on the association of SbtA and SbtB proteins. The SbtA7001-SbtB7001 (**A, C**) and SbtA7942-SbtB7942 (**B, D**) pairs were expressed in *E. coli*. *In vitro* IMAC binding assays were performed with a range of cAMP:ADP (**A, B**) and cAMP:AMP (**C, D**) ratios, with a total adenyl nucleotide concentration of 2 mM. The HAHis-tagged SbtB proteins and the SbtA proteins retained in SbtA:SbtB complexes were detected immunologically. Representative Western blot images; n=3 per adenyl nucleotide treatment.

Fig. 5. Net photosynthetic O₂ evolution rates of *Synechococcus* ΔCS supported by SbtA-dependent bicarbonate uptake in the presence and absence of SbtB. The SbtA7942 protein was expressed without SbtB7942 (SbtA) or with untagged (SbtAB) or HAHis-tagged SbtB7942 (SbtABH) from plasmids in low Ci induced *Synechococcus* ΔCS . Steady state photosynthetic O₂ evolution rates (V_{O_2}) were determined over a range of external Ci concentrations, while CO₂ uptake was inhibited by EZ, with MIMS. Maximum O₂ evolution rates (V_{max} , $\mu\text{mol mg Chl } a^{-1} \text{ h}^{-1}$) and the Ci concentrations required for half-maximum photosynthetic rates ($k_{0.5}$, μM) were derived from Michaelis-Menten kinetics (inset). Statistically significant differences ($P < 0.05$) are indicated by different letters. Values are means $\pm$ SE; n=11 (ΔCS), n=9 (SbtA), n=7 (SbtAB), n=3 (SbtABH).

Fig. 6. Dark-inactivation of SbtA-mediated bicarbonate uptake in *Synechococcus* ΔCS in the presence and absence of SbtB. Bicarbonate transport activity of SbtA7942 was estimated from the accumulation of ¹⁴C in *Synechococcus* ΔCS cells after silicon oil centrifugation-filtration. Gross HCO₃⁻ uptake rates normalized to Chl *a* content are shown at various time points following a shift from light into the dark for SbtA7942 only (SbtA) and SbtA7942 co-expressed with SbtB7942 (SbtAB). Residual HCO₃⁻ uptake rates for ΔCS represent passive, intracellular ¹⁴C accumulation and extracellular ¹⁴C trapped in the water shell adhering to each cell. Values are means ± SE; n=3-5.

Fig 7. Structural comparison of the overall fold of SbtB7942 and SbtB7001 protein models.

(A) Superimposition of the three-dimensional models derived from crystal structures of SbtB7001 with Ca^{2+} and ATP ligands (SbtB7001-CaATP; PDB ID 6MM2) and SbtB7001 binding ADP (SbtB7001-ADP; PDB ID 6MMC). The grey circles indicate the location of the nucleotide binding cleft. Ligand-induced structural differences affect primarily the T-loop which assumed a closed conformation in SbtB7001-CaATP and an open, disordered state in SbtB7001-ADP. (B) Superimposition the PSIPRED generated domain homology model of the apo-SbtB7942 monomer on the SbtB7001-ADP structure. The core structures of both SbtB proteins are highly similar, independent of ligands bound to SbtB7001. The configuration of the T-loop of SbtB7942 (grey coil) was modelled without ligand present. Therefore the open state was assumed. (C) Pairwise amino acid sequence alignment of SbtB7001 and SbtB7942 (57.7 % identity). Identical residues are highlighted in colour. The T-, B- and C-loop residues are framed orange, black and blue, respectively. Black arrows indicate critical residues for binding ATP phosphate groups (Kaczmarzski et al., 2019).

Fig. 8. Proposed regulation of SbtA bicarbonate transport activity by environmental stimuli and the adenylate energy charge (AEC). Expression of SbtA and SbtB in *Synechococcus elongatus* is induced by low Ci availability in the light. Plasmamembrane (pm)-integral SbtA is actively transporting bicarbonate from the external environment (ext) into the cytoplasm (cyto) independent of SbtB, but depends on Na⁺-symport and requires a cell-inward-directed Na⁺ gradient. Functional SbtA is likely a homotrimer (SbtA³) that transports HCO₃⁻ and Na⁺ at a 1:1 stoichiometry through each monomer. Light-stimulated photosynthetic electron transport sustains high levels of intracellular ATP over ADP and AMP (high AEC), which facilitates binding of Ca²⁺ATP to SbtB trimers (SbtB³), stabilization of the SbtB T-loops, and SbtB remains dissociated from SbtA. However, causality between T-loop conformation and SbtA:SbtB interaction has not been proven yet. Upon transition from light to dark, a change from high to low AEC is likely to take minutes, yet SbtA-mediated bicarbonate uptake is inactive within 15 s. Potential, fast signals for SbtA dark-inactivation (and light-activation) remain speculative and may include changes in membrane potential, redox status, and/or post-translational modifications of SbtA and other regulatory proteins specific to photosynthetic cyanobacteria. In prolonged darkness, cells operate at a lower AEC. The relatively higher amounts of ADP and AMP replace ATP as ligands in SbtB trimers, which destabilizes the T-loops, and stable SbtA:SbtB complexes of unknown stoichiometry are formed. Thus, SbtB may have a role as curfew protein, locking SbtA into its inactive state and preventing energetically wasteful and ineffective bicarbonate accumulation under photosynthetically unfavorable conditions.

Table 1. Correlations between association of SbtA and SbtB proteins and adenylnucleotide ratios.

Adenylnucleotides	SbtA	AR ₅₀ ^a	AR ₂₅ ^a	AR ₁₀ ^a
ATP:ADP	SbtA7001	0.55 ± 0.10	0.72 ± 0.06	0.86 ± 0.03
	SbtA7942	0.41 ± 0.14	0.57 ± 0.11	0.74 ± 0.08
ATP:AMP	SbtA7001	0.77 ± 0.03	0.85 ± 0.03	0.91 ± 0.04
	SbtA7942	0.53 ± 0.11	0.69 ± 0.09	0.85 ± 0.07
cAMP:ADP	SbtA7001	0.63 ± 0.11	0.81 ± 0.07	0.91 ± 0.04
	SbtA7942	0.62 ± 0.06	0.73 ± 0.05	0.83 ± 0.04
cAMP:AMP	SbtA7001	0.85 ± 0.08	0.91 ± 0.05	0.95 ± 0.03
	SbtA7942	0.58 ± 0.01	0.71 ± 0.01	0.83 ± 0.01

^a Adenylnucleotide ratios corresponding to 50% (AR₅₀), 25% (AR₂₅) and 10% (AR₁₀) of SbtA bound to SbtB were estimated from sigmoidal logistic curve fits (Fig. S5). AR values (± SE) are averages of 3 to 4 independent experiments. AR values were statistically not significantly different at $P \leq 0.05$ between SbtA-SbtB pairs and for different adenylnucleotide combinations tested (AR₅₀ $P = 0.232$, AR₂₅ $P = 0.211$, AR₁₀ $P = 0.275$).