On the role and regulation of glycogen catabolic isoenzymes in *Synechocystis* sp. PCC6803

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Abstract

Glycogen is the main carbon storage polymer in many organisms and widespread throughout all domains of life. In cyanobacteria glycogen degradation plays an essential role when metabolism changes to a heterotrophic mode during dark phases or the resuscitation from nitrogen starvation. Like many other cyanobacteria the genome of *Synechocystis* sp. PCC 6803 encodes for several homologues of glycogen catabolic enzymes. The role and regulation of this isoenzymes has so far been only partly understood. Here we show via biochemical analysis that the glycogen phosphorylase GlgP1 is regulated by a C-terminal redox switch unique to certain cyanobacteria. This is the first time a redox regulation of a glycogen degrading enzyme has been shown in prokaryotes. Remarkably, GlgP1 is inactivated in its reduced state and gets activated via oxidation by ROS. We conclude that GlgP1 is especially important for rapid glycogen degradation in certain stress conditions during photoautotrophic growth. We suggest that thereby carbon is channeled through the OPP pathway, to stabilize photosynthesis during situations of high ribulose-1,5-BP demands. Furthermore, we suggest that the concomitant formation of NADPH within this pathway is essential to regenerate ROS scavenging compounds like GSH to reduce potential oxidative damage and to improve survivability under prolonged phases of oxidative stress. In addition, we show for the first time the role of the two glycogen debranching isoenzymes where GlgX1 turned out to be the essential glycogen debranching enzyme during resuscitation from chlorosis, while the role of GlgX2 remains elusive.

Introduction

Glycogen is the major carbon storage compound in animals, fungi and most bacteria. It's a glucose polymer linked at position α-1,4 with branches at position α-1,6. In bacteria, particularly in free living strains, glycogen is usually built up under conditions of carbon surplus and degraded when energy or carbon supplies are limited [1-3]. In cyanobacteria glycogen plays a key role in carbon metabolism and is tightly coupled to a photoautotrophic lifestyle where excess fixed CO_2 is stored as glycogen during the day which thereon gets catabolized during the night when metabolism switches to a heterotrophic mode. This essential role is further underlined by the fact that glycogen metabolic enzymes are conserved in all known cyanobacterial strains [4]. Next to providing energy during dark phases, glycogen degradation is also known to play a crucial role during acclimation to environmental stress conditions, such as the lack of available combined nitrogen sources, frequently occurring in marine and terrestrial ecosystems [5]. Adaptation to nitrogen starvation in non-diazotrophic cyanobacteria is characterized by the initiation of a genetically determined program, called chlorosis, where cells enter a state of dormancy, enabling them to outlive long periods of nitrogen deprivation. From this state the cells can resuscitate within 48 hours once combined nitrogen sources are available again [6]. This resuscitation process is supported by a rapid degradation of the previously accumulated high amounts of glycogen, which provides the building blocks for re-establishing the metabolic processes [7, 8]. Glycogen breakdown requires the coordinated action of glycogen phosphorylase (GlgP) and glycogen debranching enzyme (GlgX): The GlgP reaction releases Glc-1P by transferring orthophosphate to the non-reducing end of glucose residues which is considered the rate limiting step in glycogen catabolism [9]. This processive reaction stops once GlgP reaches the last four glucose residues left on a branch. In prokaryotes those remaining glucose units are hydrolyzed by "direct" debranching enzymes, releasing maltotetraose which can be further metabolized by maltodextrin phosphorylase (MalP) and by a-1,4-glucanotransferase (MalQ) [10]. The released Glc-1P is then turned to Glc-6P by phosphoglucomutase (PGM) and can fuel different metabolic pathways including the Embden–Meyerhof–Parnas (EMP), the Entner-Doudoroff (ED) and the oxidative-pentose-phosphate (OPP) pathway.

Like many other cyanobacteria, the genome of the unicellular model organism *Synechocystis* sp. PCC6803 encodes for several isoenzymes involved in glycogen catabolism, including the glycogen phosphorylases *sll1356* (*glgP1*) and *slr1367* (*glgp2*) and the glycogen debranching enzymes *slr0237* (*glgX1*) and *slr1857* (*glgX2*). Of the two phosphorylases it has been shown that only GlgP2 is responsible for glycogen degradation during the dark and during resuscitation from chlorosis. GlgP1 on the other hand does not contribute to glycogen degradation under those conditions but has been shown to be relevant for growth at high temperatures [8, 11]. The roles of GlgX1 and GlgX2 have so far not been investigated at all and it is unclear if these enzymes also diverge in their activity and role in *Synechocystis*. The need to switch between autotrophic and heterotrophic modes in cyanobacteria implies that glycogen catabolism must undergo a strict regulation to prevent a premature degradation of

glycogen. While our previous work showed that PGM plays an essential role in the regulation of glycogen metabolism during chlorosis and resuscitation, the diverging roles of GlgP1 and GlgP2 also imply a direct regulation of these enzymes [12].

In this study we show that the activity of GlgP1 but not GlgP2 is strongly regulated by a C-terminal isoenzyme specific redox switch, unique in certain cyanobacterial strains. We assume that GlgP1 activity is closely linked to stress conditions during photosynthetic growth. Thereby, carbon flow through the OPP can provide a way for fast ribulose-1,5-BP regeneration and the formation of reduction equivalents, which are highly demanded during certain stress conditions. Furthermore, we show that GlgX1 is the major glycogen debranching enzyme essential for resuscitation from chlorosis and significantly enhances GlgP activity. This enhancement is particularly evident with glycogen originating from *Synechocystis*.

Results

GlgP1 activity is inhibited under reducing conditions

Synechocystis expresses two isoforms of glycogen phosphorylases, the product of sll1356 (*glgP1*) and slr1367 (*glgP2*). Previous studies showed that GlgP2 is essential for glycogen degradation during the night and resuscitation from chlorosis while GlgP1 does not contribute to glycogen degradation under those conditions. However, the enzymatic activity and regulation of GlgP1 and GlgP2 has so far not been investigated.

To determine the enzymatic activity of GlgP1 and GlgP2 we purified the respective recombinant proteins overexpressed in *Escherichia coli.* Since GlgP requires pyridoxal phosphate (PLP) as an essential co-factor, we measured UV/Vis absorbance to ensure that both enzymes show equal loading with PLP **(Figure S1)**. We assessed the GlgP enzymatic activity by measuring the release of Glc-1P in an assay that couples Glc-1P interconversion to Glc-6P by phosphoglucomutase (PGM) followed by oxidation using glucose-6-phosphate-dehydrogenase (G6PDH) and $NADP^+$. As substrates we used a fixed concentration of 10 mM KH_2PO_4 (saturating conditions, data not shown) and glycogen derived from oyster at final concentrations between 0.1 and 1 % to start the reaction. We could show that both purified GlgPs were active and were able to degrade glycogen *in vitro*. Interestingly, GlgP1 and GlgP2 have similar K_M values for glycogen, but GlgP1 shows a higher overall catalytic efficiency (**Figure 1**, **Table 1**).

Inspired by the findings of Florencio, Pérez-Pérez [13] who identified GlgP2 as a possible target of TrxA, we tested if performing the GlgP assay under reducing conditions modulates the activity of GlgP1 and GlgP2. Therefore, we incubated the GlgP1 and GlgP2 master mix with 5 mM of the reducing agent dithiothreitol (DTT) for 30 minutes before starting the enzymatic assay by addition of glycogen. Strikingly, we observed that under reducing conditions GlgP1 did not follow standard Michaelis-Menten kinetics anymore but showed an allosteric/sigmoidal kinetic curve. Catalytic efficiency of GlgP1 dropped to 7.5 % of the original activity as a result of a strongly increased $K_{0.5}$ and a reduced K_{cat}. GlgP2 activity was not markedly changed under reducing conditions (**Figure 1**, **Table 1**).

Next, to find out which concentrations of DTT are needed for GlgP1 to show maximum inhibition we performed a redox titration where we treated GlgP1 with different concentrations of DTT, ranging from 1 to 10 mM for 30 min. As shown in **Figure 1C**, treatment with 5 mM DTT for 30 min is sufficient for maximum inhibition of GlgP1 and higher DTT concentrations do not further reduce enzymatic activity. GlgP1 treated with 5 mM DTT was therefore used as a reference for a fully reduced enzyme in all upcoming experiments. Treatment of the purified GlgP1 with H_2O_2 on the other hand did not increase enzymatic activity, suggesting that our purified, untreated GlgP1 is already fully oxidized.

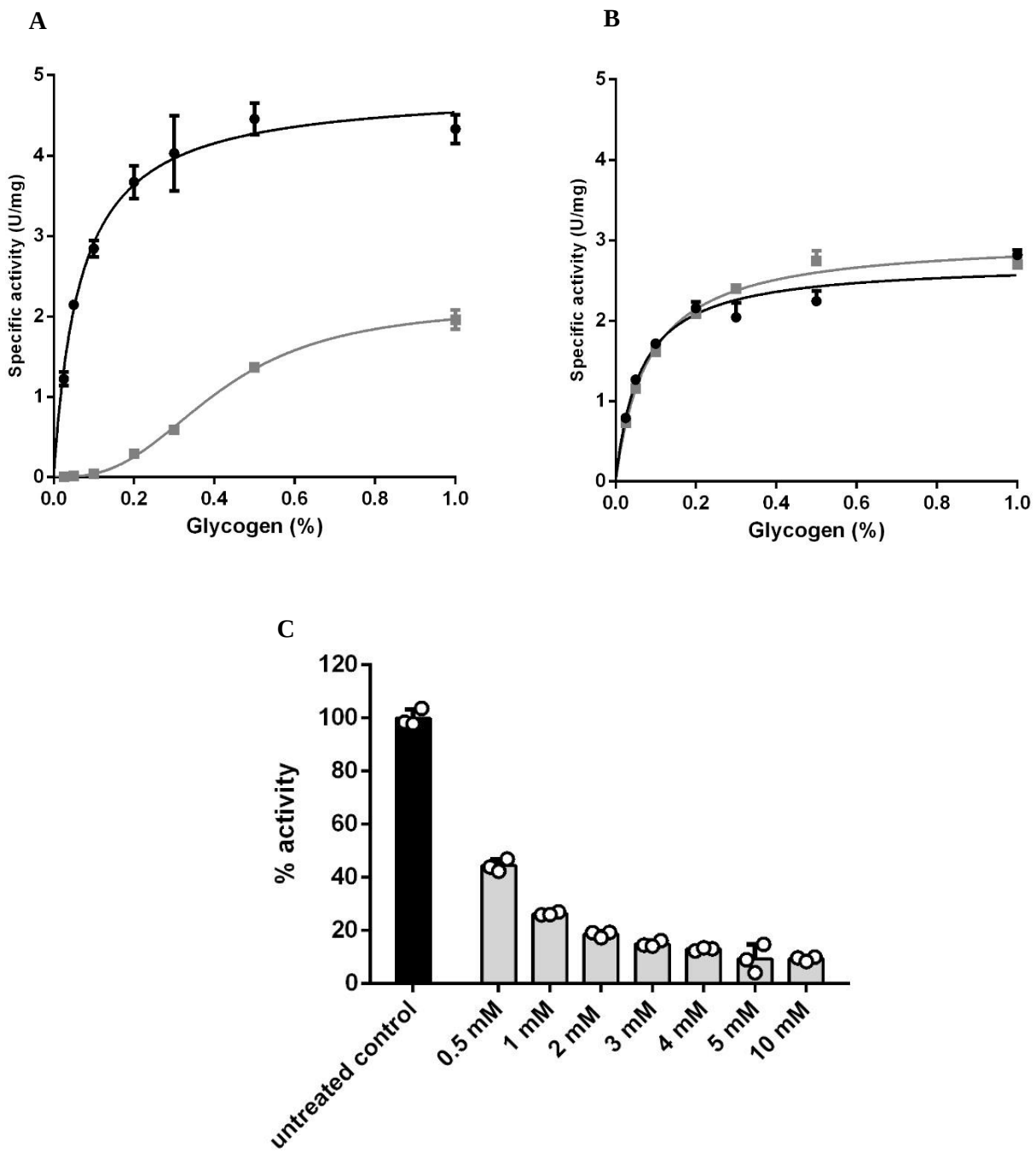

Figure 1: GlgP1 is strongly inhibited in presence of DTT

Shown is the Michaelis-Menten kinetics of GlgP1 (A) and GlgP2 (B): In black, without addition of effectors; in light grey, after 30 min incubation with 5 mM DTT. At least three replicates were measured for each data-point. Error bars represent the SD. (C): Shown is the activity of GlgP1 at 0.1% glycogen: In black, an untreated control which was considered 100%; in light grey bars, activity at different concentrations of DTT as indicated on the x-axis.

	GlgP1			GlgP2		
Treatment	**Kcat**	**K_M or $K_{0.5}$**	**Catalytic efficiency Kcat/K_m or $K_{0.5}$**	**Kcat**	**KM**	**Catalytic efficiency Kcat/Km**
Untreated	6.56 ± 0.16	0.07 ± 0.01	**93.71**	4.44 ± 0.14	0.06 ± 0.01	**74.00**
5 mM DTT	2.90 ± 0.08	0.41 ± 0.09	**7.07**	4.94 ± 0.09	0.08 ± 0.01	**61.75**

Table 1: Kinetic parameters of GlgP1 and GlgP2 with and without 5 mM DTT

K_M/$K_{0.5}$ and Kcat values are means of triplicates with ± SD.

GlgP1 activity is modulated by TrXA and gets activated by oxidation

To gain deeper insights in the apparent redox regulation of GlgP1, we tested the enzymatic activity of GlgP1 in comparison to GlgP2 under the influence of different reducing agents to see if we observe a similar effect as with DTT before. Therefore, we compared the treatment of GlgP1 and GlgP2 (25 nM each) with 5 mM DTT with the treatment of the thiol independent reducing agent TECEP. Furthermore, we tested the intracellular present redox active molecules glutathione (GSH) and cysteine (all in concentrations of 5 mM). In addition, we also purified the product of *slr0623* encoding thioredoxin A (TrxA) and tested treatment of GlgP1 and GlgP2 with previously reduced TrxA (TrxA_red) and oxidized TrxA (TrxA_ox) at concentrations of 2.5 µM respectively. As substrates we used 10 mM KH_2PO_4 and 0.1% of oyster glycogen. The activity of untreated GlgP1 and GlgP2 served as a reference. The results showed that in addition to 5 mM DTT, TrxA_red showed the strongest effect on GlgP1 by decreasing its activity to about 20% of the original activity. With about 40 % remaining activity, treatment with TECEP showed a weaker inhibition than DTT and TrxA even though TECEP and DTT have comparable reduction potentials (-0.29 V vs. -0,33 V). GSH, cysteine and TrxA_ox showed no marked effect on GlgP1 (**Figure 2A**). Furthermore, GlgP2 activity was not markedly affected by any of these treatments (**Figure 2B**).

These results clearly demonstrate inhibition of GlgP1 activity by the presence of certain reducing agents. The fact that TrXA_red is highly efficient in reducing GlgP1 at low concentrations, whereas other intracellular reducing compounds like GSH and cysteine show no effect even at high concentrations, indicates a specific modulation of GlgP1 activity by TrXA.

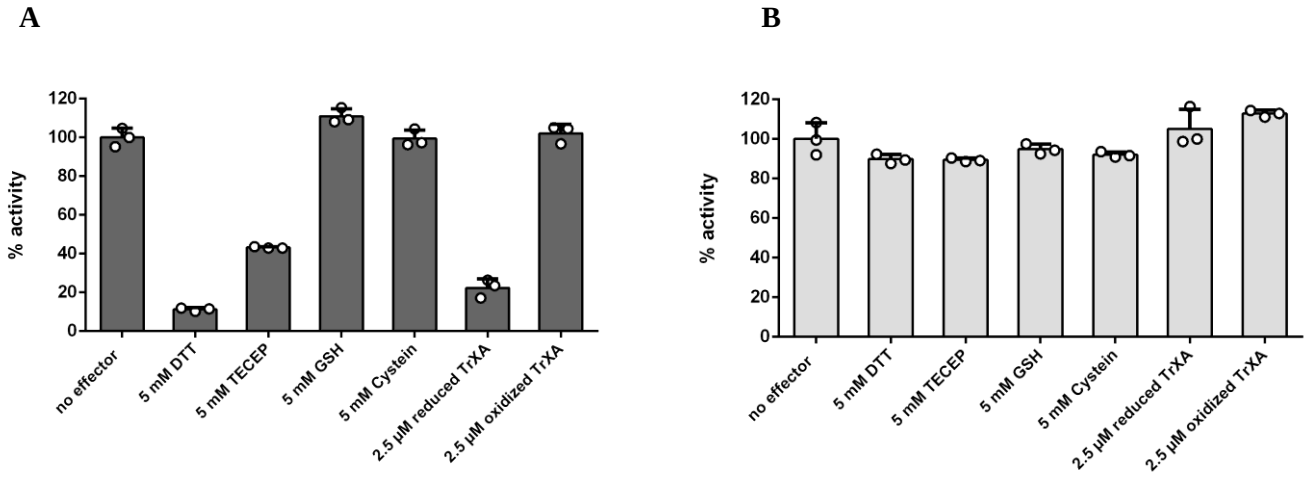

Figure 2: GlgP1 activity is specifically modulated by TrXA:

(A) shows the activity of GlgP1 after 30 min incubation with different reducing agents. Values represent relative activity and are compared to the reaction with no effectors which was considered 100%. (B) shows the same experiment as a for GlgP2. At least three replicates were measured for each datapoint. Error bars represent the SD.

Activity of reduced GlgP1 can be restored by H_2O_2 and glutathione disulfide (GSSG)

Do determine if reduced GlgP1 can be re-activated by oxidation, we incubated the previously DTT-reduced GlgP1 with the following oxidizing agents for 30 minutes: oxidized DTT (DTTox) glutathione disulfide (GSSG) (5 mM each) and TrXA_ox (10 µM). For the stronger and potentially protein damaging oxidizing agents $CuCl_2$ and H_2O_2 we determined the ideal concentration in a previous titration experiment (data not shown). This turned out to be 5 µM of $CuCl_2$ and 0.08% of H_2O_2. We considered the activity of untreated GlgP1 as 100% while the reduced GlgP1 showed about 10% remaining activity. The strongest re-activation of GlgP1 could be achieved by treatment with H_2O_2 and GSSG leading to an increase in activity to 65% and 50% of the original activity, respectively. Treatment with $CuCl_2$ lead to an increase in activity to about 30% of original activity. Treatment with DTT_ox only led to an increase to about 20 % of original activity while the treatment with TrX_ox showed no marked increase in activity at all (**Figure 3**). Altogether, activity of reduced GlgP1 could be restored by certain oxidizing conditions, indicating the presence of a specific redox switch that is lacking in GlgP2.

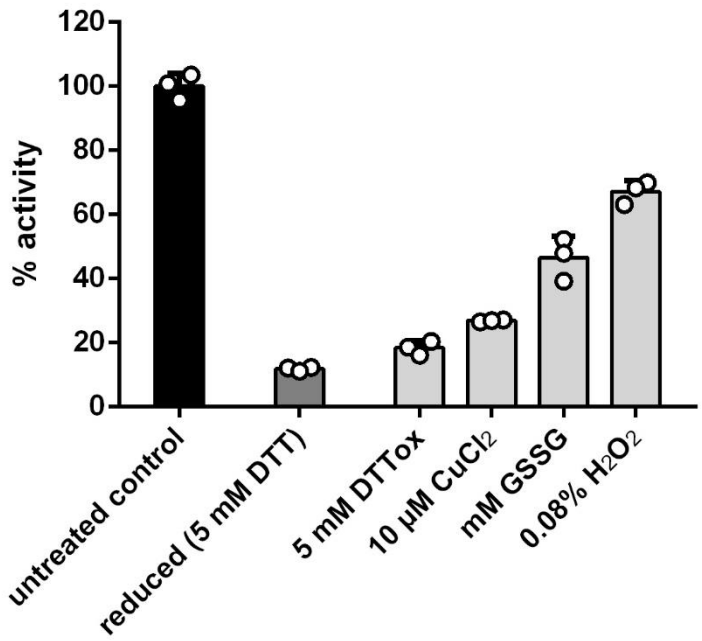

Figure 3: Activity of reduced GlgP1 can be restored by oxidation.

Shown is the activity of GlgP1: in black the untreated control which was considered 100%; in dark grey, fully reduced with 5 mM DTT; in light grey fully reduced with subsequent treatment with different oxidizing agents. At least three replicates were measured. Error bars represent the SD.

GlgP1 activity is regulated by a C-terminal redox switch.

To determine the regulatory site involved in redox regulation of GlgP1 we aligned the aa sequence of GlgP1 and GlgP2, revealing a high overall sequence identity of 62 %. By searching for prominent cysteine residues which might form disulfide bonds, we identified a characteristic motif at the C-terminal domain of GlgP1 containing two cysteine residues at position 837 and 842. By searching for this domain in other organisms we also identified that this domain is unique to certain strains of cyanobacteria. (**Figure 4A**). Prediction of the respective protein structure by AlphaFold suggests a surface exposed hairpin structure with a potential disulfide formation between the cysteines C837 and C842 (**Figure 4B**). To analyze whether this domain is in fact responsible for redox regulation, we created a point mutation by exchanging the cysteine at position 837 with a serine residue to mimic a permanently reduced state of the enzyme. The activity of the GlgP1 C837S variant corresponded precisely to the activity of the fully reduced GlgP1 WT, but in contrast to the WT enzyme this variant did not respond to treatment with oxidizing agents **(Figure 4C, Figure S1)**. This result clearly demonstrates that the C-terminal segment of GlgP1 acts as a redox sensitive element to tune the activity of this enzyme.

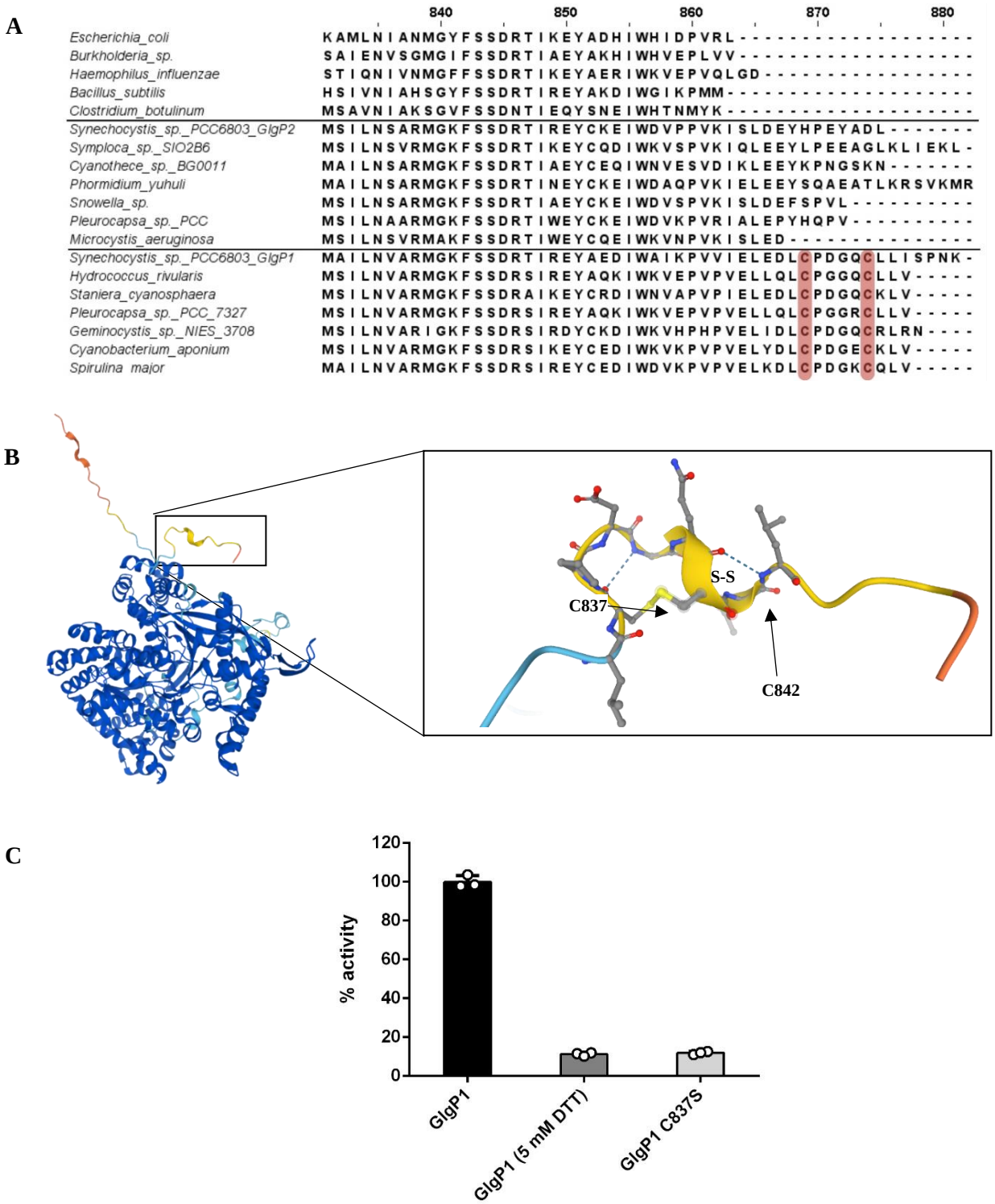

Figure 4: GlgP1 is regulated by a C-terminal redox switch exclusive to cyanobacteria.

(A): Alignment of the C-terminal domain of different bacterial GlgP homologues. Marked in red is the characteristic cysteine motif in GlgP1 homologues.
(B): Structure of *Synechocystis* GlgP1 predicted by AlphaFold. Shown in detail is the unique C-terminal domain with a predicted disulfide bond formation of Cys837 and Cys842.
(C): Shown is the GlgP1 activity: in black, without effector which was considered 100%; in dark grey, in the fully reduced state after treatment with 5 mM DTT; in light grey, activity of the GlgP1 C837S variant without any effectors. At least three replicates were measured. Error bars represent the SD.

A *glgp1* knockout mutant shows growth inhibition when light intensity is increased

Regulation of Glgp1 by TrXA and ROS implies a connection to photosynthetic activity. To determine if a *glgp1* deficient mutant shows growth impairment when light intensity is increased, we compared growth of a *glgp1* and a *glgp2* deficient mutant to the wildtype at elevated light intensities. For this experiment the cultures were first grown at 50 µE for 24 hours followed by an increase in light intensity to 120 µE. Strikingly, we observed an immediate growth impairment for *glgp1* which resulted in a fully restricted growth after 72h while WT and the *glgp2* mutant were unaffected (**Figure 5**). Since ROS formation should be increased after changing light intensity to 120 µE this finding supports our hypothesis in *glgp1* being important under situations of increased oxidative stress.

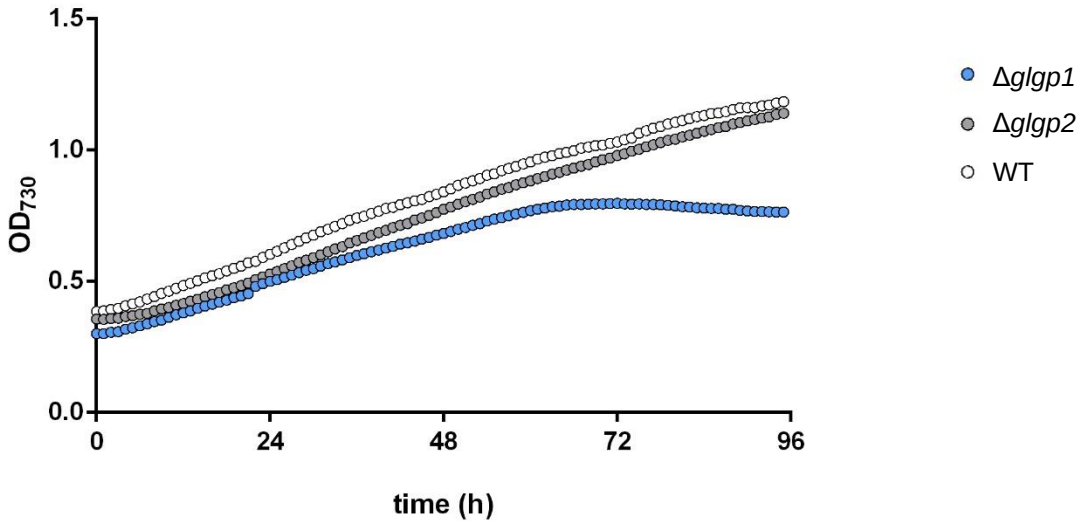

Figure 5: GlgP1 plays a role in growth at moderate light intensities

Shown are the growth curves of Δ*glgp1* (blue) and Δ*glgp2* (grey) deficient mutants compared to the WT (white). Strains grown with ambient air at 28°C with constant illumination at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 24 hours, before elevation to 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

A *glgx1* knockout mutant is impaired in recovery from chlorosis

For a complete breakdown of glycogen, the activity of the glycogen phosphorylase GlgP needs to be accompanied by a glycogen debranching enzyme. Like with GlgP1 and GlgP2, *Synechocystis* possesses two annotated isoenzymes of glycogen debranching enzymes, Slr0237 (GlgX1) and Slr1857 (GlgX2), which have so far not been investigated regarding their role and activity. To gain deeper insights into the physiological role of GlgX1 and GlgX2 we created knockout mutants of *glgx1* and *glgx2* and investigated their viability during diurnal growth and during resuscitation from chlorosis by estimating viable cells counts using the drop-plate assay. When cells were grown at diurnal rhythm, none of the mutants showed growth impairment compared to the wildtype. However, during resuscitation from nitrogen chlorosis, a strong growth impairment of the *glgx1* knockout mutant became apparent while the *glgx2* knockout was unaffected. This phenotype got even more pronounced when resuscitation was done in day night rhythm or after a prolonged period of chlorosis (**Figure 6**). This experiment clearly shows that GlgX1 appears to be the crucial glycogen debranching enzyme in resuscitating chlorotic cells, while GlgX2 does not seem to be important under this condition.

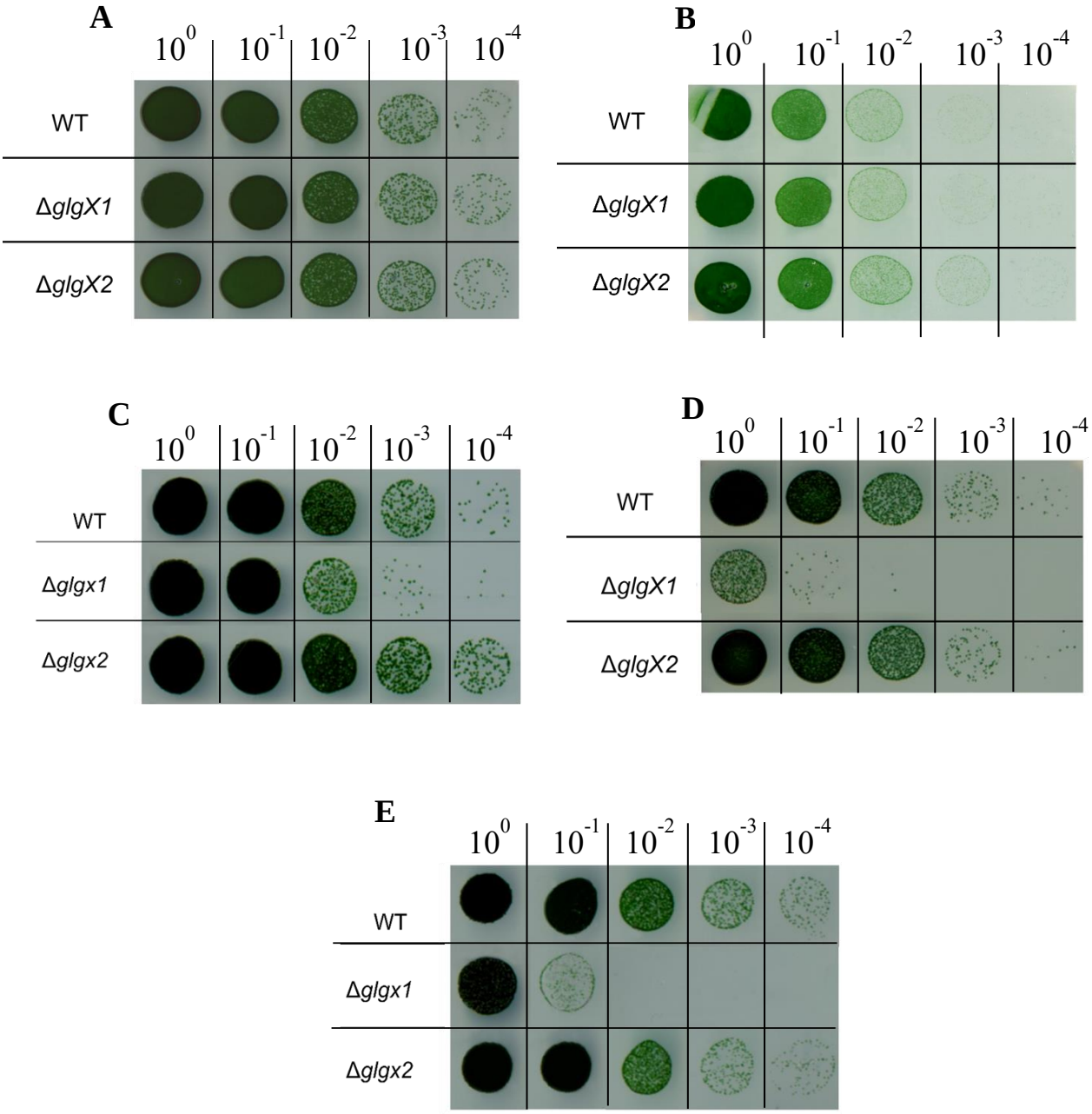

Figure 6: GlgX1 is essential for recovery from long term chlorosis.

Shown are spot viability assays on solid BG11 agar of *glgx1* and *glgx2* deficient mutants compared against the wildtype (WT). Dilutions are indicated in the top row. The following conditions have been tested:

(A) Growth under constant light; (B) growth at light-dark cycle (12h – 12h); (C) Resuscitation after 14 days of chlorosis under constant light. (D) Resuscitation after 14 days of chlorosis at light-dark cycle (12h-12h); (E) Resuscitation after 21 days of chlorosis under constant light.

GlgX1 enhances GlgP1 and GlgP2 activity in degrading glycogen originating from *Synechocystis*

To further investigate GlgX1 and GlgX2 *in vitro*, we overexpressed strep-tagged versions of the enzymes in *E. coli* and assessed the activity of the purified enzymes by adding them to the GlgP assay. Next to glycogen from oyster we also included glycogen purified from nitrogen starved *Synechocystis* cultures (both at a final concentration of 0.1%) to see how the properties of the glycogen itself influences enzymatic activity. When oyster glycogen was used as a substrate, GlgX1 enhanced GlgP1 and GlgP2 activity both by around 25% whereas GlgX2 had no effect on GlgP activity. Repeating the experiment with glycogen purified from nitrogen starved *Synechcoystis* cells yielded two major findings:

First, compared to oyster glycogen, *Synechocystis* glycogen as substrate gives rise to generally lower GlgP activities, in particular 60% for GlgP1 and 40% for GlgP2 (without GlgX addition). Second, addition of GlgX1 enhanced the overall degradation of *Synechocystis* glycogen much stronger than degradation of oyster glycogen. In combination with GlgP1, GlgX1 increased the overall activity by around 65% while with GlgP2, the activity increased by 105%. However, since GlgP2 is less efficient with *Synechocystis* glycogen, the total activity of GlgP2 with GlgX1 was still lower than for oyster glycogen (**Figure 7**).

This result agrees well with the observed phenotype of the corresponding mutants in resuscitation from chlorosis, where GlgX1, similar to GlgP2, appeared to be essential, while GlgX2 and GlgP1 were redundant.

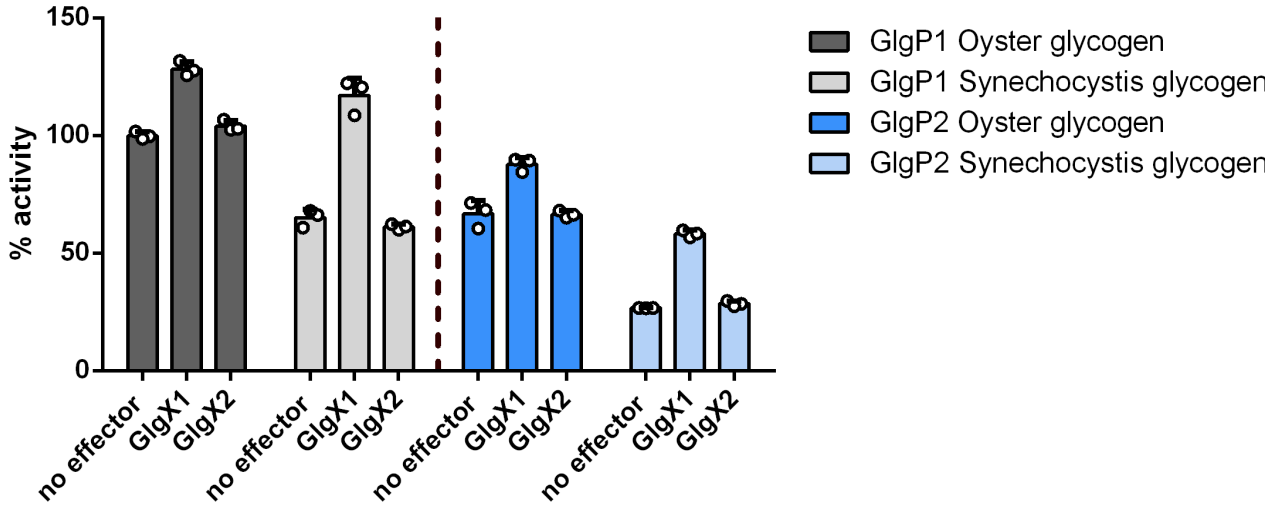

Figure 7: GlgX1 enhances GlgP1 and GlgP2 activity.

Shown is the GlgP1 and GlgP2 activity in presence of GlgX1 or GlgX2 or without any effector: In dark grey GlgP1 activity with oyster glycogen; in light grey GlgP1 activity with *Synechocystis* glycogen; in dark blue, GlgP2 activity with oyster glycogen; in light blue GlgP2 activity with *Synechocystis* glycogen. Values represent relative activity compared to the no effector oyster glycogen measurement which was considered 100%. At least three replicates were measured for each data point. Error bars represent the SD.

Discussion

Regulation of glycogen metabolism is essential for cyanobacteria to enable survivability in an ever-changing environment. Therefore, investigating the role and regulation of glycogen associated enzymes is essential to gain a deeper understanding of cyanobacteria in general. In this study we investigated the role and regulation of the glycogen catabolic enzymes in *Synechocystis*. We could show that GlgP1 is strongly regulated via a C-terminal redox switch, which is the first time this mode of regulation for glycogen phosphorylases was shown in bacteria. Activation of GlgP1 by oxidation connects glycogen degradation by GlgP1 to the formation of ROS from which we conclude that GlgP1 plays an essential role in adaptation to certain stress conditions. Furthermore, we show that GlgX1 is essential during recovery from chlorosis and enhances glycogen degradation by GlgP activity in dependence of the used glycogen source, while the role of GlgX2 remains elusive.

GlgP1 shows a novel mode of redox regulation for bacterial glycogen phosphorylases

It has been shown before that GlgP2 is the essential glycogen phosphorylase for glycogen degradation during the night and resuscitation from chlorosis, while GlgP1 was not contributing to glycogen degradation under those conditions [8]. This suggests a different mode of regulation for the two isoenzymes. We could show that both purified GlgP isoenzymes are active *in vitro* and follow standard Michaelis-Menten kinetics, with GlgP1 showing a higher overall catalytic efficiency compared to GlgP2. In the presence of reducing agents, GlgP1 activity strongly decreases and kinetics show an sigmoidal kinetic profile. Compared to GlgPs from other bacteria, cyanobacterial GlgPs have a C-terminal extension of up to 15 amino acids. We discovered that the redox regulation of GlgP1 was located at this C-terminal extension which carries two characteristic cysteine residues at position 837 and 842, not present in GlgP2, that are predicted to form a disulfide bond. Formation of a C837S variant of GlgP1 showed a strongly diminished activity on the level of a fully reduced GlgP1. From these results we conclude that the disruption of this disulfide bond leads to a conformational change of the C-terminal extension, inactivating the enzyme. The change of the kinetic profile in the reduced state implies a strongly reduced affinity for glycogen from which we conclude that the altered conformation of the C-terminal extension impairs binding of glycogen to the active site. Another explanation could be a hindered oligomerization since glycogen phosphorylases are known to be active as homodimers. While the C-terminal extension seems to be a general feature in cyanobacterial GlgPs, the double cysteine motif in this domain is only found certain strains including non-diazotrophic and diazotrophic strains alike. This raises the question on how this mechanism has developed over the course of evolution. To our knowledge the only indication of a redox dependent regulation of an enzyme involved in glycogen degradation in bacteria has been observed for a specific amylase of the cyanobacterium *Nostoc* sp. PCC7119 [14]. In humans an isoenzyme-specific redox regulation of the brain glycogen phosphorylase

was shown, affecting binding of its activator AMP, which is not known to be an activator for bacterial GlgPs [15]. However, conversely to this known redox regulations GlgP1 gets activated by oxidation making this a unique mode of GlgP regulation.

Activation of GlgP1 by ROS is connected to photosynthesis and high light stress

Being active only in the oxidized state, can explain why GlgP1 is inactive during resuscitation from chlorosis or during the night but also raises the question on the potential role of GlgP1 and under which situations it gets activated. By testing different intracellularly present reducing agents we could show that reduction and inactivation of GlgP1 was especially effective with TrXA, an m-type thioredoxin which has been shown to be essential in *Synechocystis* [16]. Thioredoxin has been assumed before to play a role in regulation of glycogen metabolism: A proteomic approach by Pérez-Pérez et al. revealed that TrxA potentially interacts with several glycogen associated enzymes in *Synechocystis*. However, GlgP1 was not identified in this study Florencio, Pérez-Pérez [13]. Inactivation of GlgP1 was reversible by oxidation which was most effective with H_2O_2 and GSSG. While H_2O_2 is a common ROS species, GSSG is formed as a product of ROS scavenging by GSH and therefore closely connected to ROS formation. Thereby, a low GSH/GSSG ratio is an indicator for high oxidative stress. Being exclusive to cyanobacteria and the fact that regulation was especially effective with TrXA and H_2O_2/GSSG strongly indicates a connection of GlgP1 activity to photosynthetic activity: TrxA as a part of the ferredoxin/thioredoxin system is known to provide the biochemical link between light reactions and the regulation of carbon metabolism in photoautotrophic organisms [17]. ROS on the other hand are a byproduct of the photosynthetic electron transport chain, whose formation increases with light intensity and are encountered by all photosynthetically active organisms.

Linking GlgP1 to photosynthetic activity is consistent with data on its expression levels, where it was shown that *glgp1* expression is upregulated during the day and downregulated during the night which is the reverse for *glgp2* [18]. The only study that directly addressed the potential role of GlgP1 by Fu and Xu discovered that GlgP1 is important for survivability at high temperatures but also that GlgP1 is only active during illumination. High temperature has been shown to cause strong ROS formation in bacteria and in nature high temperature and high light are often associated with one another [19]. We could show that independently from temperature an increase in light intensity also leads to growth impairment of a *glgp1* deficient mutant **(Figure 5)**.

While the formation of ROS is unfavorable due to oxidative damage it is also known that they contribute to signaling during several stress conditions [20-22]. This suggests that glycogen degradation by GlgP1 signaled by ROS could be a measure to counter different environmental stress condition, including heat stress and higher light intensities.

It has been shown before that high temperatures have negative influence on photosynthetic rate by making RuBisCo more likely to incorporate O_2 instead of CO_2 and due to the fact that CO_2 shows a worse water solubility at higher temperatures then O_2 [23]. This increases the demand of ribulose-1,5-BP due to a higher rate of photorespiration. Several studies have shown that glycogen degradation can contribute to replenish the ribulose-1,5-BP pool by shuffling carbon through the OPP pathway to stabilize photosynthesis [24]. This mechanism appears to be especially important during the "restart" of photosynthesis after the night phase where it has been shown that glycogen degradation at the end of the dark phase can fill up the depleted ribulose-1,5-BP pools, leading to a faster start of photosynthesis while glycogen free mutants show a strong delay [25]. We assume that the same pathway contributes to ribulose-1,5-BP formation during heat stress. Furthermore, it was shown that the survivability under higher temperature and high ROS formation is dependent on the availability of GSH [26, 27]. Reduction of GSSG by glutathione reductase is dependent on NADPH which is mainly formed at the end of photosynthetic linear electron transport, but also in the OPP. The oxygen evolving complex of PSII has also been shown to be prone to heat damage which would inhibiting the NADPH formation by linear electron transport, making the OPP the only source of NADPH.

GlgX1 is the essential glycogen debranching enzyme during resuscitation from chlorosis

On average, one third of the glycogen granule is presumed to be accessible for GlgP without the need to cleave alpha-1,6 branches. [28]. Therefore, GlgX activity is especially important for a full and efficient breakdown of the glycogen granule. Data on the contribution of debranching enzymes to cyanobacterial glycogen degradation are scarce and the role of the two isoenzymes in *Synechocystis* has so far not been investigated. With our findings we show that GlgX1 is the main glycogen debranching enzyme. A knockout mutant of *glgx1* shows strong impairment in resuscitation from chlorosis while a *glgx2* knockout mutant appears to be unaffected. The phenotype of a *glgx1* deficient mutant gets more pronounced during prolonged periods of chlorosis and also when recovery is done in day/night rhythm. We assume that under those conditions the cells have to rely longer on respiration and therefore glycogen must get degraded more thoroughly. During day/night neither *glgx1* nor *glgx2* KO mutants showed a visible phenotype. From this we conclude that glycogen breakdown by GlgP alone is sufficient under these conditions or the two debranching enzymes can replace each other under those conditons. The time period of a heterotrophic type of metabolism is longer during resuscitation from chlorosis than during the night (24h vs 12h). Additionally, it can be considered that during resuscitation from chlorosis the rate of glycogen degradation must be higher compared to dark phases due to a much higher demand of carbon building blocks. Nevertheless, day/night experiments with longer dark phases might also reveal a phenotype for *glgx1* or *glgx2*. In our experiments we couldn't detect any phenotype for a *glgx2* deficient mutant under the tested conditions and in our *in vitro* enzymatic assays GlgX2 didn't show any enzymatic activity. Compared to glycogen phosphorylases debranching enzymes are difficult to classify

and many enzymes possess a similar hydrolase domain. Therefore, GlgX2 could in fact have a different role than annotated.

Enhancement of GlgP activity by GlgX strongly depends on the type of glycogen used

We could clearly show that GlgX1 increases GlgP activity. Thereby, the enhancement of GlgP by GlgX1 appears to be strongly dependent on the used glycogen source and is markedly higher for glycogen originating from *Synechocystis* than for the commercially available oyster glycogen. Interestingly, glycogen degradation by GlgP2 shows a stronger dependance on GlgX1 than degradation by GlgP1 but only for glycogen from *Synechocystis*. The reason for this difference could be due to a difference in branching pattern but also other forms of glycogen modification might play a role as it was shown for eukaryotic glycogen [29]. In case of *Synechocystis* two glycogen synthases equally contribute to glycogen synthesis [30]. A study showed that the branching pattern slightly differs for glycogen from single glycogen synthase knockout mutants, while the WT appears to be a mixture of the two [31]. Future experiments have to elucidate if degradation by GlgP differs between the glycogen produced by the two synthases. Unlike for GlgP1 we could not detect any regulatory effect of glycogen debranching enzymes by treatment with reducing and oxidizing agents, indicating that the main regulation of degradation is achieved via the GlgP enzymes **(Figure S3)**.

The fact that GlgX1 particularly enhances GlgP2 activity and that a knockout of each enzyme shows a similar phenotype in resuscitation from chlorosis indicates that GlgX1 and GlgP2 form a functional pair.

Methods

Isothermal, Single-Reaction DNA Assembly (Gibson Cloning)

Cultivation of *Escherichia coli*

If not otherwise stated *E. coli* was grown in Luria-Bertani medium at 37°C. For growth on plates, 1.5% (w/v) agar-agar was added. For cells containing plasmids, the appropriate concentration of antibiotics was used. All *E. coli* strains used in this study are listed in **Table S5**.

Cultivation of *Synechocystis*

All *Synechocystis* sp. PCC 6803 strains used in this study were grown in BG_{11} supplemented with 5 mm $NaHCO_3$, as described previously [32]. A list of the strains used is provided in **Table 2**. Two kinds of wild-type strains, Glc sensitive and Glc tolerant, were used; both strains responded equally during nitrogen starvation and resuscitation. Cultivation was performed with continuous illumination (40–50 µmol photons m^{-2} s^{-1}) and shaking (130–140 rpm) at 27°C if not stated otherwise. Induction of nitrogen starvation and resuscitation was induced as described previously [33, 34]. If mutants or strains containing antibiotic markers were used, the precultures were propagated with the appropriate concentration of antibiotics. Biological replicates were inoculated with the same precultures but propagated, nitrogen starved, and resuscitated independently in different flasks under identical conditions.

Cultivation of *Synechocystis* under moderate light

Growth curves of WT and *glgP1*- and *glgP2* deficient mutants were aquired via liquid cultures in a Multi-Cultivator MC 1000-OD (Photon Systems Instruments, Czech Republic). All *Synechocystis* sp. PCC 6803 strains were grown as described above, before being transferred to fresh standard BG11 with respective antibiotics in Multi-Cultivator flasks for growth measurements, where the starting optical density of all cultures was adjusted to OD750 ~0.3. Cultivation was carried out with constant aeration using ambient air 28°C under constant illumination for 24 hours at 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ before being raised to 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Optical density readings were taken every hour for 96 hours using the instruments' inbuilt function.

Spot viability assay

Serial dilutions of chlorotic cultures were prepared (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}), starting with an OD_{750} of 1.5 µL of these dilutions, dropped on solid BG_{11} agar plates, and cultivated at 50 µmol photons m^{-2} s^{-1} and 27°C for 5 to 7 d.

Protein purification

The plasmids used for protein overexpression are shown in **Table 4**. *Escherichia coli* Rosetta-gami (DE3) (details **Table 2**) was used for the overexpression of all proteins. For this, cells were cultivated in 2xYT (3.5% tryptone, 2% yeast extract, 0.5% NaCl; 1L of culture in 5L flasks) at 37 °C until reaching exponential growth (OD_{600} 0.6-0.8). Protein overexpression was induced by adding 75 µg/L anhydrotetracycline, followed by incubation at 20°C for 16 h. Cells were harvested by centrifugation at 4000 g for 10 min at 4 °C. Cell disruption was performed by sonication in 40 mL of lysis buffer (100 mM Tris-HCl pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 20 mM Imidazole for His-tagged proteins and 100 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM $MgCl_2$ for Strep-tagged proteins) Lysis buffers were supplemented with DNAse I, and cOmplete™ protease inhibitor cocktail (Roche, Basel)). The cell lysate was centrifuged at 40,000 g for 45 min at 4°C and the supernatant was filtered with a 0.22 µM filter.

For the purification of His-tagged proteins, 5 mL Ni-NTA HisTrap columns (GE Healthcare, Illinois, USA) were used. The cell extracts were loaded onto the columns, washed with wash buffer (50 mM Tris-HCl pH 7.4, 500 mM NaCl, and 50 mM Imidazole) and eluted with elution buffer (50 mM Tris-HCl pH 7.4, 500 mm NaCl, and 500 mM Imidazole).

GlgP and GlgX enzymatic assays

Buffer for enzymatic reactions was composed of 50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 1 mM $NADP^+$, 5mM KH_2PO_4, 1U/µl phosphoglucomutase from rabbit muscle, 10 µM Glucose-1,6-bisphosphate and 1 U/mL G6PDH from *Saccharomyces cerevisiae* (G6378, Sigma Aldrich). For GlgP1/2 activity, 2,5 µg of Strep-tagged purified protein was added to each reaction. Reaction was started by the addition of glycogen. Reactions were carried out in a total of 300 µl in a 96-well microplate. Absorption change at 340 nm was continuously measured for 15 min at 30 °C in a TECAN Spark® Multiplate reader (Tecan Group AG, Männedorf, Switzerland). At least three replicates were measured. For enzymatic assays with reducing or oxidizing agents, GlgP1 and GlgP2 were incubated with the given amount of effector for 30 minutes at RT.

For determining GlgX activity, 15 µg of GlgX1 and GlgX2 Strep-tagged purified protein were added to the GlgP assay mastermix respectively.

Table2: List of strains used in this study

E. coli NEB10β	Δ(ara-leu) 7697 araD139 fhuAΔlacX74 galK16 galE15 e14-Φ80dlacZΔM15 recA1 relA1 endA1 nupGrpsL(StrR) rphspoT1 Δ(mrr-hsdRMS-mcrBC)
E. coli Rosetta gami (DE3)	Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F'[lac^+ $lacI^q$ pro] gor522::Tn10 trxB pLysSRARE (Cam^R, Str^R, Tet^R)
Synechocystis sp. PCC 6803	Wildtype
Δ*glgp1*	*Sll1356::Kan*^{R}
Δ*glgp2*	*Slr1367::Spec*^{R}
Δ*glgx1*	*Slr0237::Spec*^{R}
Δ*glgx2*	*Slr1857::Kan*^{R}

Table3: List if used primers

Primer	**Sequence (5'- 3')**
pASKC_glgp1_fw	CTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGGAGCACCTTCCCATGGC
pASKC_glgp1_rev	GGTCTTATTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTTTATTAGGAGAAATTAACAAAC
pASKC_glgp2_short_fw	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGATTGATCAATCTACCCTAAAC
pASKC_glgp2_rev	GTCTTATTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTAAATCGGCGTATTCGGGATGG
pASKC_glgx1_fw	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAGTGCCACAGTTGATATCTGTTC
pASKC_glgx1_rev	GGTCTTATTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTTTTGCCATTAACACCACAG
pASKC_glgx2_fw	TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACAAATGGAACGCATAGATATTCATC
pASKC_glgx2_rev	GGTCTTATTTTTCGAACTGCGGGTGGCTCCAGCTAGCCATTTTAGCCAGTAAAATAACAAC
pET15b_trxA_fw	CAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCTCGAGATGAGTGCTACCCCTCAAGTTTC
pET15b_trxA_rev	CCCTCAAGACCCGTTTAGAGGCCCCAAGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAAC
Glgp1_C837S_fw	AGAAGACCTCtctCCCGATGGTC
Glgp1_C837S_rev	AATTCAATTACCACGGGTTTG
GlgX1_DS_fw	CAAGGTAGTCGGCAAATAATCTAATTTACTGAGTTTTTGCCC
GlgX1_DS_rev	CGGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGCAATGTTAGATAAACTACATTG
GlgX1_Spec_fw	GAATCTGAACAATTTTTGGAATTGCCTGAGCTCTTGACCGAACGCAGCGGTGGTAAC
GlgX1_Spec_rev	GTTACCACCGCTGCGTTCGGTCAAGAGCTCAGGCAATTCCAAAAATTGTTCAGATTC
GlgX1_US_fw	GACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTTGGGGCAGGCAAGGCCATTGAC
Glgx1_US_rev	CCACTGCGCCGTTACCACCGCTGCGTTCGGTCAAGAGCTCAGGCAATTCCAAAAATTGTTC

GlgX2_DS_fw	CATTTGATGCTCGATGAGTTTTTCTAAATTGTCTTAAATTTCCCTTTCCCTTC
GlgX2_DS_fw_redesigned	GTTATCTTGGCAAATCATCTATTGTCTTAAATTTCCCTTTC
GlgX2_DS_rev	ATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCATTCGCCATTCAGGCTGC
GlgX2_Kan_fw	GAGGCATTTTAGCAGGACACCCGAACCGAACAGGCTTATGTC
GlgX2_Kan_rev	CATTACTTTCCAAGTTGTGCCTCAGAGTTTGTAGAAACGCAAAAAG
GlgX2_US_fw	GACCATGATTACGCCAAGCTTGCATGCCTGCAGGTCGACTATCCCCGGATATTCTTCCCC
GlgX2_US_rev	GACATAAGCCTGTTCGGTTCGGGTGTCCTGCTAAAATGCCTC
GlgX2_prom_fw	TGCGTTTCTACAAACTCTGAGGCACAACTTGGAAAGTAATG
GlgX2_prom_rev	GAAAGGGAAATTTAAGACAATAGATGATTTGCCAAGATAAC

Table4: List of used plasmids

Plasmid	**Purpose**
pASKC_glgp1	Expression of C-terminal Strep-tagged *Synechocystis* GlgP1 in *E.coli*
pASKC_glgp2	Expression of C-terminal Strep-tagged *Synechocystis* GlgP2 in *E.coli*
pASKC_glgp1_C837S	Expression of C-terminal Strep-tagged *Synechocystis* GlgP1 C837S in *E.coli*
pASKC_glgx1	Expression of C-terminal Strep-tagged *Synechocystis* GlgX1 in *E.coli*
pASKC_glgx2	Expression of C-terminal Strep-tagged *Synechocystis* GlgX2 in *E.coli*
pASKC_trXA	Expression of C-terminal Strep-tagged *Synechocystis* TrXA in *E.coli*
pUC19_glgx1_KO	Replacement of *slr0237* for $Spec^R$ in *Synechocystis* sp. PCC 6803
pUC19_glgx2_KO	Replacement of *slr1857* for Kan^R in *Synechocystis* sp. PCC 6803

Statistical analysis

Statistical details for each experiment can be found in the figure legends. GraphPad PRISM was used to perform one-sided ANOVA to determine the statistical significance. Asterisks (*) in the figures symbolize the p-value: One asterisk represents $p \leq 0.05$, two asterisks $p \leq 0.01$, three asterisks $p \leq 0.001$, and four asterisks $p \leq 0.0001$

Author contributions

NN performed experiments and wrote manuscript

KL performed growth curves measurements.

KF conceived study and edited manuscript

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