Redox regulation of glycogen biosynthesis in the cyanobacterium *Synechocystis* sp. PCC 6803. Analysis of the AGP and glycogen synthases.

Runing title: Cyanobacterial glycogen synthesis regulation

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Abstract

Glycogen constitutes the major carbon storage source in cyanobacteria, as starch in algae and higher plants. Glycogen and starch synthesis is linked to active photosynthesis and both of them are degraded to glucose in the dark to maintain cell metabolism. Control of glycogen biosynthesis in cyanobacteria could be mediated by the regulation of the enzymes involved in this process, ADP-glucose pyrophosphorylase (AGP) and glycogen synthase, which were identified as putative thioredoxin targets. We have analyzed whether both enzymes were subjected to redox modification using purified recombinant enzymes or cell extracts in the model cyanobacterium *Synechocystis* sp. PCC 6803. Our results indicate that both AGP and glycogen synthases are sensitive to copper oxidation. However, only AGP exhibits a decrease in its enzymatic activity, which is recovered after reduction by DTT or reduced thioredoxin (TrxA), suggesting a redox control of AGP. In order to elucidate the role in redox control of the cysteine residues present on the AGP sequence (C45, C185, C320 and C337), they were replaced with serine. All AGP mutant proteins remained active when expressed in *Synechocystis*, although showed different electrophoretic mobility profiles after copper oxidation, reflecting a complex pattern of cysteines interaction.

Introduction

Cyanobacteria constitute a large group of oxygenic photosynthetic prokaryotes able to fix atmospheric CO_2 utilizing the ATP and the reducing electron power generated by the photosynthetic process. Cyanobacteria are widespread on different environments including freshwater, oceans and terrestrial habitats. They are responsible for a significant part of the global biomass and the oxygen in the atmosphere (Blankenship and Hartman, 1998; García-Pichel, 2008). Furthermore, they are also evolutionary precursors of chloroplast by an endosymbiotic process (Criscuolo and Gribaldo, 2011; Price et al., 2012). Carbon assimilation in cyanobacteria is a typical photosynthetic process and most of the carbon fixed is usually combined with nitrogen, via the glutamine synthetase-glutamate synthase pathway, to produce amino acids and subsequently proteins (Muro-Pastor et al., 2005). In addition to proteins, carbon is also used to synthetize other compounds including sugars, fatty acids, nucleotides and polysaccharides (Machado and Atsumi, 2012). Polysaccharides represent an interesting way to storage carbon for its use under low-energy conditions, as occurs in darkness (Steuer et al., 2012; Suzuki et al., 2010; Wyman and Thom, 2012; Xu et al., 2013). In cyanobacteria, glycogen is the main polysaccharide accumulated inside the cell under certain metabolic situations, such as nitrogen deprivation (Ball and Morell, 2003; Grundel et al., 2012; Suzuki et al., 2010). Recently, the synthesis of a polysaccharide much more similar to starch than to glycogen have been reported in some cyanobacterial strains (Suzuki et al., 2013). This fact supports the view of a cyanobacterial origin of plastid starch in algae and plants (Ball et al., 2011; Ball and Morell, 2003). In addition, many cyanobacteria are able to produce exopolysaccharides (EPS) that are exported outside the cell in response to environmental stresses or unbalanced carbon/nitrogen status (Pereira et al., 2009). Other soluble sugars, such as trehalose, sucrose or glucosylglycerol are also produced as osmoprotectants by cyanobacteria in response to osmotic stress adaptation (Klahn and Hagemann, 2011).

The biosynthesis of glycogen in cyanobacteria requires the participation of different enzymes. The first step is the conversion of glucose-6P into glucose-1P by phosphoglucomutase, followed by the action of ADP-glucose pyrophosphorylase (AGP) that synthesizes ADP-glucose, using glucose-1P and ATP. This reaction generates PPi, which is converted into phosphate by a soluble pyrophosphatase. The glucose moiety of the ADP-glucose is then transferred to the non-reducing end of a linear α-1,4 glucan by glycogen synthase. Finally, glycogen branches are introduced by the branching enzyme, which adds α-1,6 glycosidic bonds (Ball and Morell, 2003; Miao et al., 2003; Suzuki et al., 2010; Xu et al., 2013; Yoo et al., 2002).

In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) glycogen biosynthesis requires the participation of phosphoglucomutase (encoded by *sll0726*), AGP (*glgC: slr1176* gene), two different glycogen synthases (encoded by *glgA1*: *sll0945* and *glgA2: sll1393*), and the branching enzyme (*glgB: sll1058*). Mutant strains lacking AGP protein or both glycogen synthases are unable to produce detectable glycogen (Carrieri et al., 2012; Grundel et al., 2012; Miao et al., 2003). In addition, the AGP mutant presents a pleitropic phenotype that includes, among others, impaired cell-viability under different stress conditions and the lack of bleaching, a process that allows the cells to degrade phycocyanin after being transferred to a nitrogen-free medium (Carrieri et al., 2012; Grundel et al., 2012; Miao et al., 2003). A similar phenotype has also been described for a double mutant lacking both glycogen synthases suggesting that glycogen is essential under particular environmental conditions (Grundel et al., 2012).

The key control step in glycogen/starch synthesis in photosynthetic organisms seems to be the regulation of AGP enzyme. In this regard, it is well known that AGP is finely regulated by the allosteric effect of 3-phosphoglycerate in higher plants, algae and cyanobacteria (Ballicora et al., 2004; Crevillen et al., 2003; Iglesias et al., 1991; Ventriglia et al., 2007). In addition to allosteric regulation, redox regulation has been also documented in the case of higher plants, showing a stimulation of AGP activity by thioredoxins, preferentially thioredoxin f (Buchanan

and Balmer, 2005; Hendriks et al., 2003; Sanz-Barrio et al., 2013; Thormahlen et al., 2013). Such redox regulation of AGP was related to the formation of a disulfide bridge between the two small subunits of AGP, APS1, with the reduced form being the active one. In this regard, replacement of Cys81 with Ser impaired the formation of the disulfide between the two APS1 subunits in *Arabidopsis* AGP (Hadrich et al., 2012). Transgenic plants containing the APS1$_{C81S}$ accumulated more starch during the day than plants expressing the WT version, which correlated with the absence of the dimeric form (Hadrich et al., 2012). However, other reports indicated that there were no differences in the amount of starch stored by these transgenic plants compared to that containing the wild-type APS1 version (Li et al., 2012; Thormahlen et al., 2013). By contrast, data concerning the redox regulation of cyanobacterial AGP is scarce and indicated a lack of this type of regulation (Fu et al., 1998; Iglesias et al., 2006). Up to date no data is available concerning a possible regulation of the glycogen synthase activity in cyanobacteria. Glycogen synthesis is active in the light, especially in cells subjected to nitrogen starvation. However, only glycogen content was determined but not the glycogen synthase activity (Ball and Morell, 2003; Grundel et al., 2012; Xu et al., 2013).

In this study we analyzed whether glycogen synthesis is redox modulated in the cyanobacterium *Synechocystis*. Our results indicate that none of the two glycogen synthases present in *Synechocystis* undergo redox regulation. In contrast, AGP activity is regulated by oxidoreduction of its cysteines both *in vitro* and in cell extracts. Furthermore, AGP is able to form mixed disulfides with TrxAC35S (a mutant version of the most abundant thioredoxin in *Synechocystis*) and TrxA is also able to reduce the AGP both *in vitro* and in cell extracts. Mutations of the four cysteines present in AGP revealed a complex pattern of oxidation that probably involved all of them. All of these AGP mutant proteins, with the exception of AGP$_{C45S}$, presented activities comparable to the wild type protein. However, when these different versions were reintroduced in the mutant background the resultant strains accumulated less glycogen than the WT strain. This suggests a de-regulation of the whole pathway in the ΔAGP mutant, which could be due to an

unknown secondary mutation. Finally, we analyzed whether thioredoxin mutant strains have a defect in glycogen synthesis regulation but none of the tested strains presented any significant differences in glycogen accumulation, after nitrogen deprivation, when compared to the WT.

Results

Glycogen synthases are not able to interact with TrxA.

Most of the glycogen metabolism enzymes in *Synechocystis* have been identified as glutaredoxin or thioredoxin targets (Li et al., 2007; Lindahl and Florencio, 2003). These included phosphoglucomutase, AGP, glycogen synthases, glycogen branching enzyme and glycogen phosphorylase. Among these enzymes, only phosphoglucomutase has been shown to be redox regulated (Lindahl and Florencio, 2003). In the current study we have tested whether glycogen synthases and AGP were also redox regulated. *Synechocystis* genome contains two genes coding for putative glycogen synthases: *sll0945,* which encodes GlgA1, and *sll1393,* which encodes GlgA2. Of the two glycogen synthases, only GlgA2 was identified as TrxA target (Lindahl and Florencio, 2003), although both of them were identified as GrxA targets (Li et al., 2007). This suggests that these two proteins could be redox regulated. In order to elucidate if both proteins are able to interact with TrxA, TrxAC35S was used. This recombinant version of TrxA lacks the resolving cysteine in the active site, allowing the formation of stable mixed disulfides with its target proteins (Lindahl and Florencio, 2004; Mata-Cabana et al., 2007). Soluble cell extracts were incubated with 50 µM TrxAC35S and analyzed by western blot using non-reducing gels and anti-GlgA1 or GlgA2 specific antibodies (Figures S1 and S2). None of the enzymes were able to form mixed disulfides with TrxAC35S, either in non-treated or oxidized cells extracts (Figure S2). Moreover, both purified recombinant proteins from *E. coli* (Figure S1) were incubated with a 4-fold excess of TrxAC35S for 1h and aliquots were analyzed by western blot. None of the glycogen synthases formed any detectable complexes with TrxAC35S (Figure S2) using anti-TrxA antibodies. These strongly suggest that none of the

glycogen synthases are thioredoxin targets or, at least, not in these recombinant glycogen synthase preparations.

Furthermore, to confirm that glycogen synthases were not redox regulated we have assayed their activities in oxidized and reduced conditions. Purified proteins were assayed as isolated, oxidized (after incubation with 10 μM Cu^{2+}) or after reduction with 5 mM DTT. These conditions were chosen because oxidized and reduced forms of both GlgA1 and GlgA2 could be detected (Figure 1B and C). These two forms were inter-convertible, i.e. copper oxidized proteins were fully reduced by the addition of DTT. Most GlgA1 was oxidized when purified from *E. coli* and its oxidation was only slightly increased by the copper treatment. In contrast, GlgA2 was mostly reduced when purified from *E. coli* and was fully oxidized by copper (Figure 1B and C). Under these conditions, GlgA1 and GlgA2 had specific activities (measured as ADP formation) of 3.33±0.71 and 3.30±0.89 U/mg protein, respectively. Those activities values were not significantly reduced or enhanced by oxidation or reduction (Figure 1A). Taken together these results suggest that none of the glycogen synthases are redox regulated in *Synechocystis*.

AGP is a thioredoxin target.

AGP is another enzyme involved in glycogen synthesis that was identified as putative TrxA target (Lindahl and Florencio, 2003). In order to confirm this interaction, formation of mixed disulfides between AGP and TrxAC35S was analyzed in *Synechocystis* extracts. Soluble cell extracts were pre-oxidized with 25 μM $CuCl_2$ and incubated with 50 μM TrxAC35S. The formation of stable disulfides with AGP was analyzed by western blot using anti-AGP antibodies. Since our antibodies detect two unspecific bands, a cell extract from a *Synechocystis* mutant strain lacking the AGP gene (ΔAGP) was included as control (Figure 2A and Figure S1). Additional protein bands were detected in the lane corresponding to the WT extracts incubated with TrxAC35S that were absent in ΔAGP mutant extracts

(Figure 2A). Thus, this result indicates that AGP is able to form a mixed disulfide with TrxAC35S in cell extracts.

In order to confirm this interaction, purified recombinant AGP was incubated with a four-fold excess of TrxAC35S to obtain stable mixed disulfides. Complex formation was detected by western blot analysis using anti-TrxA antibodies. Two control lanes containing only TrxAC35S or AGP were also included, as these two proteins formed disulfides with themselves (Figure 2B). A protein band with an electrophoretic mobility of ~65 kDa was detected in the non-reducing gel in the lane in which both AGP and TrxAC35S were present but it was absent in all others. This result shows that TrxAC35S can also form a mixed disulfide with AGP *in vitro*.

Plant and bacterial AGP enzymes have been shown to be tetramers (Ballicora et al., 2003; Ballicora et al., 2004) and we have investigated the quaternary structure of *Synechocystis* AGP protein using gel filtration. Extracts from WT cells were size-fractionated on a Superdex 200 column and the eluted fractions were tested for the presence of AGP by western blot. AGP fractionated as 200 kDa molecular mass complexes (Figure 2C). This confirms that native AGP protein forms a tetramer, as previously described for purified AGP from *Synechocystis* (Iglesias et al., 1991). Since glycogen accumulation is dependent on the presence of light, changes in the quaternary structure of AGP were compared between light and dark conditions. For that, the oligomeric state of AGP was analyzed by gel filtration in extracts prepared from cells transferred to the dark for 8 h. In this experiment, AGP eluted as a tetramer in cells extract prepared from light grown cells or dark treated cells (Figure 2C). Moreover, the tetrameric structure of AGP was retained even when cell extracts were reduced with DTT prior to gel filtration (data not shown). This indicates that the tetramer does not require any intermolecular disulfide bridge between subunits for its stabilization.

AGP activity is redox modulated.

Once the interaction between AGP and TrxA was confirmed, we investigated whether this interaction played a role in the modulation of the AGP activity. Analysis of the enzyme activity was carried out with the recombinant purified protein treated in the same conditions as described for glycogen synthases. In the case of AGP, the purified recombinant protein was obtained partially oxidized from *E. coli*. Full enzyme oxidation was achieved by copper treatment (10 µM), which led to a 40% reduction of AGP activity (Figure 3A). In contrast, addition of DTT or reduced TrxA stimulated the activity 3 and 5 times, respectively, compared to the oxidized enzyme (Figure 3A). The treatment conditions used for the enzymatic determination were selected because they were able to produce changes in the redox state of the protein (Figure 3B). The completely oxidized protein forms oligomers and intramolecular disulfides, which were visualized, under non-reducing conditions, as slower and faster migrating bands, respectively (Figure 3B). All of these oxidized forms were removed by DTT or reduced TrxA treatments (Figure 3B). The specific activity of the recombinant protein (10 mU/mg prot) was very low compared to previously described activity for native purified protein from *Synechocystis* (Iglesias et al., 1991). This could be due to the tag used for protein purification in *E. coli*, as it occurs for *Solanum* AGP (Hwang et al., 2004), and/or because the protein would require a post-translational modification that occurs in *Synechocystis* but not in *E. coli*. For this reason, redox regulation of AGP activity was also assayed in *Synechocystis* extracts. Consistently with the *in vitro* data, AGP activity was inactivated by copper treatment and activated by of DTT or reduced TrxA additions (Figure 3C). The specific activity in *Synechocystis* extracts (104 mU/mg protein) was about two times higher than the previously published values (Iglesias et al., 1991), although in this report, enzyme activity was assayed in the opposite direction. Analysis of the AGP oxidation state by western blot showed the formation of oligomers and intramolecular disulfide bonds in the full-oxidized extracts. In contrast, the protein was reduced in non-treated extracts or after DTT or TrxA additions (Figure 3D). Therefore, AGP activity could be correlated with its redox state, being less active in its oxidized form and fully active in the reduced form both *in vitro* and in cell extracts. These results support the view

that AGP activity could be redox regulated in *Synechocystis* and that TrxA could be its reductant *in vivo*.

AGP oxidation involves different cysteine residues.

The amino acid sequence of the *Synechocystis* AGP contains four cysteines: C45, C315, C320 and C337. C45 is conserved in all cyanobacteria, plants and *E. coli* while C315 and C320 are conserved in most cyanobacteria and plants. C337 is only conserved in some cyanobacteria but all sequences contain cysteines in this region of the protein, as stated in Figure S3. In order to elucidate which of the cysteines were involved in the redox regulation, each of them were changed to Ser. The corresponding mutated *glgC* genes, as well as the wild-type, were re-introduced in the genome of the ΔAGP mutant strain, which lacks the complete *glgC* gene, under the control of its own promoter in its endogenous locus. The five different strains obtained after transformation and antibiotic selection expressed the different AGP versions to the same levels of the WT protein as determined by western blot analysis (Figure 5A). AGP activity was measured in cell extracts from these strains and all strains exhibited similar activities to the WT strain (Figure 4A), except the C45S strain which presented half of the activity. The production of glycogen by the five different strains was also determined in cultures grown in light–dark regime (16L/8D). Surprisingly, all complemented strains, including the strain carrying the WT version, synthesized similar amount of glycogen in the light that was degraded in the dark, but the total amount of glycogen produced was half of the WT strain. As expected, the ΔAGP strain lacked glycogen (Figure 4B and C). In order to analyze if each Cys replaced with Ser had an impact in the oxidation state of the protein, cell-extracts from WT and from the mutant AGP strains were oxidized by copper. These extracts were separated in non-reducing SDS-PAGE and AGP was detected by western blot. Oxidized WT protein appeared as a monomer with an intramolecular disulfide (a faster migrating band) on the bottom of the gel and as higher molecular mass complexes on the upper part of the gel. The latter ones could correspond to the dimer and higher order oligomers (Figures

5A), although we cannot rule out that disulfides with other proteins can also be formed in these extracts. Surprisingly, all cysteine substitutions had an effect on AGP oxidation. In particular, C45S mutation impaired the ability to form the dimer, although AGP$_{C45S}$ mutant protein was still susceptible to form oligomers, and an intramolecular disulfide with a different mobility to the one formed in the WT protein (Figure 5A). Both AGP$_{C315S}$ and AGP$_{C320S}$ mutant proteins behaved similarly, being able to form dimer and higher order oligomers almost identical to the WT, although both of them formed an intramolecular disulfide, which was different to the one formed by the WT (Figure 5A). Finally, AGP$_{C337S}$ protein formed a dimer (which had a different mobility to the WT protein) and higher order oligomers as well as an intramolecular disulfide with an altered mobility (Figure 5A). These data suggest that AGP presents a complex pattern of disulfide bridges formation and the only obvious conclusion is that C45 is required for the dimer formation.

When the redox state of AGP was analyzed in cell extracts from light grown *Synechocystis* cultures, most of the AGP appeared in the reduced form (Figure 3D). To further investigate whether the redox state of AGP could be regulated in response to light/dark regimes, cell cultures were grown in the light and transferred to the dark for 24 or 48 h. Cells extracts were resolved in 7% non reducing SDS-PAGE gels and the AGP redox state was analyzed by western blot. Even in long-term dark treated cultures, AGP was present predominantly in the reduced form. However, long film exposures allowed us to detect the dimer and oxidized forms of AGP after extended periods of dark, as shown in Figure 5B.

Thioredoxin mutants are not affected on carbohydrate accumulation after nitrogen starvation.

Glycogen accumulation in cyanobacteria has been described to be maximal when the C/N balance is altered by removal of the nitrogen source from the culture medium (Ball and Morell, 2003; Grundel et al., 2012). In order to test the effect on glycogen accumulation, total carbohydrate content was measured in different

Synechocystis mutant strains in thioredoxin genes. Since total glycogen and total carbohydrate content are highly correlated (Figure S4), glycogen was not measured directly but as total carbohydrates, which facilitated the parallel measurements of all cultures. Furthermore, the ΔAGP strain did not accumulate carbohydrates during nitrogen deprivation, which reinforces that the total carbohydrates content reflects mainly the glycogen content (Figure 6A and S4). We have analyzed single mutants in *trxB* (STXB; *slr1139*), *trxQ* (STXQ; *slr0233*), *trxC* (STXC; *sll1057*), a double mutant *trxBtrxQ* (STXBQ) and a mutant with reduced TrxA levels (STXAc; Figure 6C) together with WT and ΔAGP strains during a nitrogen deprivation period. Nitrogen source (in our case nitrate) was removed by washing the cell cultures twice with a nitrogen-free media and then incubating them in the same nitrogen-free media for 24 h. Afterwards, 10 mM of ammonium chloride (a reduced nitrogen source) was added to the cultures and growth was monitored for another 24 h. After nitrogen deprivation, WT, but not ΔAGP mutant, accumulated carbohydrates, which were rapidly mobilized in WT cells when ammonium was added back to the media. 6h after ammonium addition almost half of the total carbohydrates were already mobilized (Figure 6A). The analysis of the carbohydrates content in thioredoxin mutants indicated that none of them were affected on carbohydrates accumulation and mobilization under these conditions. These data suggest that none of the mutants analyzed are affected in AGP regulation. Furthermore, all strains, except the ΔAGP strain, responded to nitrogen deprivation by activating the bleaching process, which involves the loss of phycocyanin (Figure 6B). This is in agreement with the lack of bleaching phenotype previously reported for other ΔAGP mutant strains (Carrieri et al., 2012; Grundel et al., 2012; Miao et al., 2003).

Discussion

Glycogen and starch constitute the main carbon polymers in which energy is stored to be used when the supply of energy is reduced in a process that is conserved in all living organisms from bacteria to humans. Glycogen synthase activity in yeast

and mammals has been described to be subjected to regulation by phosphorylation as well as by allosteric ligand binding (Roach et al., 2012). In contrast, bacterial glycogen and plant starch synthases appears to be regulated only at the transcriptional level (Ball and Morell, 2003; Tenorio et al., 2003; Wyman and Thom, 2012). In *Synechocystis* the two genes encoding glycogen synthases did not show any substantial transcriptional regulation, even under conditions that induced glycogen accumulation (Osanai et al., 2006; Osanai et al., 2005; Singh et al., 2010). Furthermore, our results show that the activities of both *Synechocystis* glycogen synthases are not redox regulated, even though *Synechocystis* GlgA1 and GlgA2 present 6 and 11 cysteine residues, respectively, and were able to form intra- and intermolecular disulfides (Figure 1B and C). Moreover, none of the glycogen synthases were able to form mixed disulfides with TrxAC35S (neither *in vitro* nor in cell extracts), suggesting that they are not able to interact with TrxA. The interactions detected between these proteins and TrxA or GrxA in previous studies (Li et al., 2007; Lindahl and Florencio, 2003) could be due to an indirect interaction of glycogen synthases with another protein, which is a real TrxA/GrxA target. In this regard, multiple interactions between enzymes involved in starch biosynthesis have been described in plants (Hennen-Bierwagen et al., 2009; Hennen-Bierwagen et al., 2008), including starch synthases, starch branching enzyme or the AGP large and small subunits. Another possibility is that these proteins were regulated by the glutaredoxin/GSH system although this is less likely since oxidation of them did not have any significant effect on their activities (Figure 1A).

In contrast, AGP activity was redox modulated *in vitro* as well as in cell extracts (Figure 3 A and C). In both cases AGP activity was inhibited by oxidation and stimulated by DTT or reduced TrxA additions. This data correlates with the redox state of the protein: it formed intra- and intermolecular disulfides when oxidized, which were lost upon DTT or reduced TrxA treatments both *in vitro* and in cell extracts (Figure 3 B and D). Previous data suggested that *Anabaena* sp. PCC 7120 AGP was not redox regulated since the recombinant purified protein was not activated after DTT reduction. However, its oxidation state prior to this treatment

was not stated (Iglesias et al., 2006). Furthermore, AGP formed mixed disulfides with TrxAC35S both in *Synechocystis* extracts (Figure 2A) and *in vitro* using purified proteins (Figure 2B). Taken together these data reinforce the idea that AGP activity could be modulated *in vivo* by reduction mediated by TrxA. This redox regulation was only analyzed in the presence of 3-phosphoglycerate, which has been shown to be a powerful allosteric activator of AGP activity (Iglesias et al., 1991). It is possible that the redox regulation we have observed has stronger effects at lower concentrations of 3-phosphoglycerate, a condition that would happen in the dark, when CO_2 fixation ceases. In this respect, the *Solanum tuberosum* AGP is more sensitive to the allosteric modulation by 3-phosphoglycerate under non-reducing conditions than under reducing conditions (Iglesias et al., 2006). In addition, when the redox state of AGP was analyzed in dark treated cells, a fraction of the protein was in the oxidized state in the form of dimers and higher order oligomers (Figure 5). All these data suggest that these two levels of regulation could reinforce each other to inhibit glycogen synthesis in darkness. The redox regulation of glycogen accumulation will be further enhanced by the regulation of phosphoglucomutase, which is also inhibited by oxidation (Lindahl and Florencio, 2003). Therefore, under oxidizing conditions less substrate will be available for AGP and it will be less active, decreasing the metabolic flux towards glycogen accumulation. In agreement with the above data, the redox regulation of phosphoglycerate kinase (PGK), another TrxA target in *Synechocystis*, has recently been described (Tsukamoto et al., 2013). Despite there was a clear effect of TrxA on PKG activity and its oligomerization state, this enzyme could not be detected in the oxidized state *in vivo* (Tsukamoto et al., 2013). As stated by the authors, this may suggest that, due to their critical role, particular enzymes should be subjected to extensive reduction in the cell. A similar process may account for the low level of oxidized forms of AGP detected in *Synechocystis* cells, even in the dark.

Moreover, the analysis of AGP mutant proteins in which the cysteines residues were changed to serine has revealed that none of them were essential for its enzyme activity, although the C45S presented about half of the WT specific

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activity. Furthermore, all mutant proteins were still able to form oxidized species (Figure 5A). Although a crystal structure of cyanobacterial AGP is not available, location of the cysteines in *Synechocystis* AGP can be mapped by homology to the *Solanum tuberosum* enzyme (Jin et al., 2005). This enzyme consists of an N-terminal catalytic domain and a C-terminal β-helix domain and *Synechocystis* Cys315, Cys320 and Cys337 are located in the C-terminal β-helix domain, while Cys45 is located in the N-terminal domain. Therefore, the 50 % reduction in specific activity observed in the AGP_{C45S} could be a consequence of an environment perturbation of the active site in the catalytic domain (Figure 4). This result is in agreement with Cys45 conservation in all AGP sequences suggesting an important role in AGP function (Figure S3). On the other hand, Cys315, Cys320 and Cys337 are located close enough in a way that any two of them could be able to form an intramolecular disulfide and that explains why disulfides are still observed in any of these single mutants (Figure 5). Although these cysteines are not conserved in all cyanobacteria, most of the sequences contain at least a pair of cysteines in this region, suggesting that a disulfide could be formed (Figure S3). In plants, the cysteine responsible for the intermolecular disulfide and AGP activity redox regulation is Cys81 in APS1, which is not present in *Synechocystis* ((Fu et al., 1998; Hadrich et al., 2012); highlighted in green in Figure S3). It is interesting to note that *Synechocystis* AGP activity is also redox sensitive despite the lack of APS1 region containing Cys81 (Figure S3). Similarly to cyanobacteria, *Chlamydomonas* AGP small and large subunits also lack a conserved Cys81 (Figure S3) but their sequences present Cys homologues to Cys320 and Cys337 and to Cys315 and Cys337, respectively. However, *Chlamydomonas* AGP has not been identified as Trx target (Lemaire et al., 2004) and only the large subunit was identified as putative glutathionylated protein (Zaffagnini et al., 2012). This highlights the importance of modulating the carbon storage in photosynthetic organisms via the redox state of the photosynthetic electron transport chain through the Trx system. Surprisingly, analysis of the glycogen content of the AGP mutant strains, as well as strain expressing the wild-type AGP version (the WTc strain), indicated a strong reduction on the total amount of glycogen. Besides that,

this glycogen was consumed during dark periods and accumulated in the light like in the WT (Figure 4C). These mutants were generated in the ΔAGP strain, which carried a complete deletion of the *glgC* gene. Our results suggest that our ΔAGP strain could carry a secondary mutation, although the phenotype of our mutant was identical to previously published phenotypes for other AGP mutants, i.e. non-bleaching after nitrogen starvation or lack of glycogen (Figure 4, 6 and data not shown; (Carrieri et al., 2012; Grundel et al., 2012; Miao et al., 2003)). In fact, similar phenotypes have been recently reported in another complemented *glgC* mutant strain (Carrieri et al., 2012), which suggest that this mutation is required for full segregation of the *glgC* inactivation. This secondary mutation affects glycogen metabolism and accumulation because all these strains accumulated less glycogen than the WT. We have checked that this mutation is not a consequence of the antibiotic cassette introduced down-stream to the *glgC* gene, because WT strain transformation with pGLGC-Km, the plasmid used to generate WTc in the ΔAGP background, rendered a strain indistinguishable from the WT (data not shown). Furthermore, this mutation is not clone specific either, as different clones behaved in the same way when we re-introduced the wild-type AGP version (data not shown). Further work is under way in our lab to elucidate the gene(s) mutated, and its effect in glycogen metabolism.

Finally, we have also analyzed the effects of the mutation of different thioredoxins genes in the accumulation of carbohydrates during nitrogen starvation, a condition that has been shown to induce glycogen accumulation (Ball and Morell, 2003; Grundel et al., 2012; Suzuki et al., 2010). Under this condition most of the carbohydrates accumulated corresponded to glycogen as reflected by the lack of carbohydrates accumulation in ΔAGP. None of the thioredoxin mutant strains tested are affected in carbohydrate accumulation. Unfortunately, a complete *trxA* mutant could not be tested since this is an essential gene in *Synechocystis* (Navarro and Florencio, 1996). Instead a mutant that expresses the *trxA* gene under *gifB* promoter was used. This promoter is induced by NH_4, not induced in nitrate and repressed during nitrogen deprivation (Garcia-Dominguez et al., 2000). The STXAc strain has less TrxA than the WT strain under all conditions (Figure

6C), but despite this fact, it showed similar growth and carbohydrate accumulation to the WT (Figure 6A and data not shown). These suggest that even the reduced amounts of TrxA present in the STXAc strain are enough to support all the essential functions of TrxA. The lack of phenotype of these thioredoxin mutants is in agreement with the data which showed that TrxA is the most abundant thioredoxin in *Synechocystis* (Florencio et al., 2006), while TrxB and TrxQ has been proposed to be expressed only under specific stress conditions (Perez-Perez et al., 2009a; Perez-Perez et al., 2009b). Therefore, TrxA will probably be in charge of regulating most of the light modulated processes in *Synechocystis*.

Methods.

Strains and culture conditions.

Synechocystis cells were grown photoautotrophically on BG11C (Rippka et al., 1979) at 30º C under continuous illumination (50 µE m^{-2} s^{-1}) and bubbled with a stream of 1% (v/v) CO_2 in air. Nitrogen source removal was carried out by washing cell cultures with BG110C (BG11C medium lacking NaNO$_3$). 10 mM NH$_4$Cl and 20 mM N-tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (TES) buffer pH 7.5 were used for ammonium supplementation of cultures. For plate cultures, medium were supplemented with 1% (wt/vol) agar. Kanamycin, chloramphenicol and spectinomycin were added to a final concentration of 50 µg mL^{-1}, 20 µg mL^{-1} and 5 µg mL^{-1}, respectively. Strains used in this work are described in Table 1. A description of the plasmids used to construct new strains is included in Supplementary methods. Primers used in this work and plasmids constructed to generate *Synechocystis* strains are described in Table S1.

Expression and purification of recombinant proteins

Using *Synechocystis* DNA as template, full coding sequences from *glgC* (*slr1176*), *glgA1* (*sll0945*) and *glgA2* (*sll1393*) were amplified and unique restriction sites *Bam*HI/*Sal*I added by PCR using the following pairs of primers: glgC-BamHI F/glgC-SalI R, glgA1-BamHI F/glgA1-SalI R and glgA2-BamHI F/glgA2-SalI R. The amplified products were cloned BamHI/SalI into pET45b vector and correct sequence confirmed by DNA sequencing. For 6xHis-tagged recombinant proteins expression, the plasmids were transformed into *E. coli* BL21. Under standard protocol for expression induction with IPTG, all three recombinant proteins were insoluble, however culture incubation for 48h at 25ºC, 100 rpm without IPTG induction allows synthesis of enough soluble recombinant protein to be purified. Cultures were harvested by centrifugation, resuspended in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1 mM PMSF and disrupted (20 kHz, 75 W) on ice for 3 min (in 30-s periods) in a Branson sonifyer. Lysates were then centrifuged at 20000 *g* for 30

min for clarification. Supernatants were supplemented with imidazole to a final concentration of 25 mM and loaded onto a Ni-NTA agarose (Invitrogen) column for affinity chromatography purification. After extensive washing with 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 mM imidazole, bound recombinant proteins were eluted with increasing concentrations of imidazole in 50 mM Tris-HCl pH 8.0, 500 mM NaCl. The purified proteins were then desalted using PD-10 columns (GE Healthcare) pre-equilibrated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 20% glycerol and concentrated in a 30K-molecular-weight cutoff Amicon Ultra centrifugal filter unit (Millipore). Wild type TrxA, and the modified TrxA35, in which Cys35 has been substituted by Ser, were expressed and purified as previously described (Lindahl and Florencio, 2003).

Antiboby production, Western blotting and preparation of crude extracts from Synechocystis cells.

Anti-AGP, anti-GlgA1 and anti-GlgA2 antisera were obtained according to standard immunization protocols by injecting purified AGP, GlgA1 and GlgA2 proteins in rabbits. For analysis of AGP, GlgA1 and GlgA2 in *Synechocystis* cells, crude extracts were prepared using glass beads and minibead-beater. Cells were resuspended in 50 mM Hepes-NaOH pH 8.0, 150 mM KCl and subjected to 2 cycles of 1' vortexing separated by 5' on ice. Cell extracts were recovered from the beads and samples were clarified by two sequential centrifugations: 5' at 5000 *g* to eliminate cells debris and 15' at 15000 *g* to remove membranes. Protein concentration in cell-free extracts or purified protein preparations were determined by the method of Bradford, using ovalbumin as a standard and the specified amounts of proteins were separated on SDS PAGE under reducing or non-reducing conditions. Gel were transferred, blocked in PBS containing 0.1% tween 20 and 5% of skimmed milk and incubated with antibodies at a 1:3000 dilution for TrxA (Navarro et al., 2000) and 1:5000 for GlgA1, GlgA2 and AGP.

Gel filtration

Soluble extract from *Synechocystis* wild type strain was prepared as indicated above and 100 μg in a volume of 200 μl was loaded onto a Superdex 200 column (GE Healthcare) at a flow rate of 0.5 ml/min. 0.5-ml fractions were collected and the elution profile of AGP was analyzed by western blot. The column was calibrated with known standards (Bio-Rad).

Glycogen synthase activity determination.

Glycogen synthase activity was assayed using glycogen as primer and purified GlgA1 and GlgA2. 2.5 micrograms of the proteins samples were incubated at 30°C for 30 min in 100 μL of the following buffer: 100 mM Tricine pH 8.0, 25 mM K--acetate, 5 mM EDTA, 0.5 mg mL^{-1} BSA, 1 mM ADP-glucose and 1 mg mL^{-1} glycogen. The reaction was stopped by boiling for 10' and samples were centrifuged 20' at 15000 *g*. The amount of ADP production and ADP-glucose consumption was determined by HPLC fitted with a Partisil-10-SAX column as described in (Munoz et al., 2005).

AGP activity determination.

AGP activity was assayed in the ADP-glucose synthesis direction as described in (Fusari et al., 2006). Reaction mixtures contained 50 mM HEPES pH 8, 10 mM MgCl$_2$, 2 mM ATP, 1 mM glucose 1-phosphate, 2 mM 3-phosphoglycerate and 1 U yeast inorganic pyrophosphatase. Assays were initiated by the addition of the purified recombinant AGP or 0.5 μg *Synechocystis* soluble extracts in a total volume of 50 μl. After 30 min incubation at 30ºC, reactions were stopped by the addition of Malachite green reagent and read at 650 nm. Activity was calculated by subtracting the amount of phosphate formed in the absence of glucose1-phosphate in the same samples (this background phosphate was equivalent to the amount released in ΔAGP extracts assayed in the same conditions and therefore is produced by other(s) enzyme(s)). Samples were assayed in triplicate for each experiment.

Carbohydrates and glycogen content determinations.

Total carbohydrates were determined using the phenol–sulfuric acid method as described in (Dubois et al., 1951). Total protein content was determined by the method of Lowry as described in (Markwell et al., 1978). Glycogen was determined using the method described in (Xu et al., 2013) with some modifications. Cells were disrupted in 300 µl of 30 mM Na-acetate pH 5.2 using a beadbeater and glass beads. Two aliquots of 100 µl of broken cells suspension were boiled for 20 min and one of them was treated with 10 U of amyloglucosidase (from *Asperguillus niger*; from Sigma) overnight while the other was not treated. Glucose released was determined in both aliquots using the glucose oxidase/peroxidase method (Sigma catalog #GAGO-20). A calibration curve was prepared with commercial glycogen. Glycogen content was determined by substracting glucose present in the samples that were not treated with amyloglucosidase.

Acknowledgments.

Table 1. Strains used in this work.

Synechocystis strains	Genotype	Mutated ORF(s)	Source or Study
WT	*Synechocystis* sp. PCC 6803	None	
ΔAGP	ΔglgC::C.C1	slr1176	This study.
WTc	glgC$_{WT}$ ::C.K1	None	This study.
C45S	glgC$_{C45S}$ ::C.K1	slr1176	This study
C315S	glgC$_{C315S}$ ::C.K1	slr1176	This study.
C320S	glgC$_{C320S}$ ::C.K1	slr1176	This study.
C337S	glgC$_{C337S}$ ::C.K1	slr1176	This study.
ΔglgA1	ΔglgA1::C.K1	sll0945	This study.
ΔglgA2	ΔglgA1::SpΩ	sll1393	This study.
STXB	*slr1139*::SpΩ	slr1139	(Perez-Perez et al., 2009a)
STXQ	*slr1139*::SpΩ; *slr0233*::C.K1	slr0233	(Perez-Perez et al., 2009a)

STXBQ	*slr0233*::C.K1	slr1139; slr0233	(Perez-Perez et al., 2009a)
STXC	*sll1057*::C.K1	sll1057	This study.
STXAc	*slr0623::* SpΩ *slr0796::PgifB:trxA*::*C.K1*	slr0623; slr0796	This study.

Figure Legends.

Figure 1. Glycogen synthases are not redox regulated.

A. Glycogen synthase activity in untreated, oxidized and reduced conditions. Glycogen synthase activity of purified GlgA1 or GlgA2 was measured *in vitro* by measuring ADP formation as described in the Methods section. Proteins were not treated (control), oxidized in the presence of 10 μM of $CuCl_2$ or oxidized with copper and subsequently reduced with 5 mM DTT. Activity was normalized to untreated sample. Data represents average of three independent experiments and error bars represent SE.

B. Western blot analysis of GlgA1 under the same conditions used for activity measurements in A. Proteins were separated on a 7% acrylamide non-reducing gel and proteins detected using GlgA1 specific antibodies.

C. Western blot analysis of GlgA2 under the same conditions used for activity measurements in A. Proteins were separated on a 7% acrylamide non-reducing gel and proteins detected using GlgA2 specific antibodies

Figure 2. AGP is able to interact with TrxA.

A. AGP interacts with TrxAC35S in *Synechocystis* extracts. Soluble extracts from WT and ΔAGP strains were oxidized with 25 μM $CuCl_2$ for 30 min and incubated with 50 μM TrxAC35S for 1h to allow the formation of mixed disulfides. Proteins

were resolved in 7% reducing (left panel) or non-reducing acrylamide gels (right panel) and probed with AGP antibodies.

B. AGP interacts with TrxAC35S *in vitro*. Purified AGP was incubated with a 4 fold excess of TrxAC35S for 1 h to allow mixed disulfide to be formed, proteins were resolved in 12% reducing (left panel) or non-reducing (right panel) acrylamide gels and probed with TrxA antibodies.

C. AGP is a tetramer in *Synechocystis* cell extracts. Extracts from light grown cells (top panel) or cells maintained in the dark for 8 h (bottom panel) were separated on a Superdex 200 column and fractions were probed for the presence of AGP by western blot on 10% acrylamide gels. The column was previously calibrated with molecular mass protein standards which are indicated by arrows.

Figure 3. AGP activity is redox regulated.

A. AGP activity is redox regulated *in vitro*. AGP activity was measured using 1 µg of purified protein as described in the Methods section. Protein was not treated (control), oxidized in the presence of 10 µM of $CuCl_2$, oxidized with copper and subsequently reduced with 5 mM DTT or oxidized with copper and reduced with 100 µM of TrxA. Activity was normalized to the untreated sample. Data represents average of three independent experiments and error bars represent SE.

B. Analysis of the oligomerization state of purified recombinant AGP. 2 µg of AGP were oxidized with 10 µg of $CuCl_2$ and incubated with 2 mM DTT or increasing amounts of reduced TrxA. Proteins were separated on a 7% acrylamide non-reducing gel and Coomassie stained.

C. AGP activity was measured in soluble extracts (0.5 µg total proteins) as described in the Methods section. Extracts were not treated (control), oxidized in the presence of 25 µM of $CuCl_2$ and subsequently reduced with 5 mM DTT or oxidized with copper and reduced with 100 µM of TrxA. Activity was normalized to

untreated sample. Data represents average of three independent experiments and error bars represent SE.

D. Western blot analysis of AGP oligomerization state under the same conditions used for activity measurements in C. Extracts were separated on a 7% acrylamide non-reducing gel and probed with AGP antibodies.

Figure 4. All conserved cysteines are not essential for AGP activity.

A. AGP specific activity of *Synechocystis* extracts. AGP activity was measured from extracts prepared from WT, WTc, C45S, C315S, C320S and C337S strains. Data represents average of three independent experiments and error bars represent SE.

B. Glycogen content in WT cells grown in long day conditions. Glycogen was determined in late exponential phase (10-15 µg chl mL^{-1}) in WT (white circles) and ΔAGP cells (black circles) grown in a 16 h-light/8-h dark photoperiod for at least 3 cycles.

C. Glycogen content in WT, WTc, C45S, C315S, C320S and C337S strains. Glycogen was determined in late exponential phase cells (10-15 µg chl mL^{-1}), grown in a 16 h-light/8-h dark photoperiod for at least 3 cycles, at the end of the day (white bars) or at the end of the night (black bars).

Figure 5. All conserved cysteines are involved in oxidoreduction of AGP.

A. AGP redox state in cysteine mutants. Soluble extracts from WT, C45S, C315S, C320S and C337S strains were oxidized in the presence of 25 µM of CuCl$_2$, 5 µg of total proteins were separated in 7% non-reducing (top panel) or reducing acrylamide gels (bottom panel) and the redox state of AGP was analyzed by western blot.

B. AGP redox state in dark treated cells. Soluble extracts from WT or ΔAGP mutant were prepared from cells grown in the light or incubated in the dark for the indicated times. 30 μg of total proteins were separated in 7% non-reducing (top panel) or reducing acrylamide gels (bottom panel) and the redox state of AGP was analyzed by western blot.

Figure 6. Analysis of the carbohydrate accumulation during nitrogen starvation in thioredoxin mutant strains.

A. Total carbohydrates content were determined during a nitrogen starvation time course in WT, STXB, STRXQ, STXC, STXBQ, STXAc and ΔAGP mutant strains. Exponential cultures growing in BG11C were washed twice with BG110C and resuspended in the same media at a 4 μg chl mL^{-1}. After 24 h of nitrogen starvation, 10 mM NH$_4^+$ was added and cultivation was continued for another 24 h. Total carbohydrate contents were normalized to total protein contents.

B. Bleaching of WT, STXB, STRXQ, STXC, STXBQ, STXAc and ΔAGP strains during the nitrogen starvation time course. Photograph of the different strains used in A after 24 h of nitrogen starvation.

C. Western blot analysis of TrxA levels in WT and STXAc strains during the nitrogen starvation time course. Samples were collected at the beginning of the experiment (0h), 24 h after nitrogen removal (24 h) and 24 h after ammonium addition (48h).

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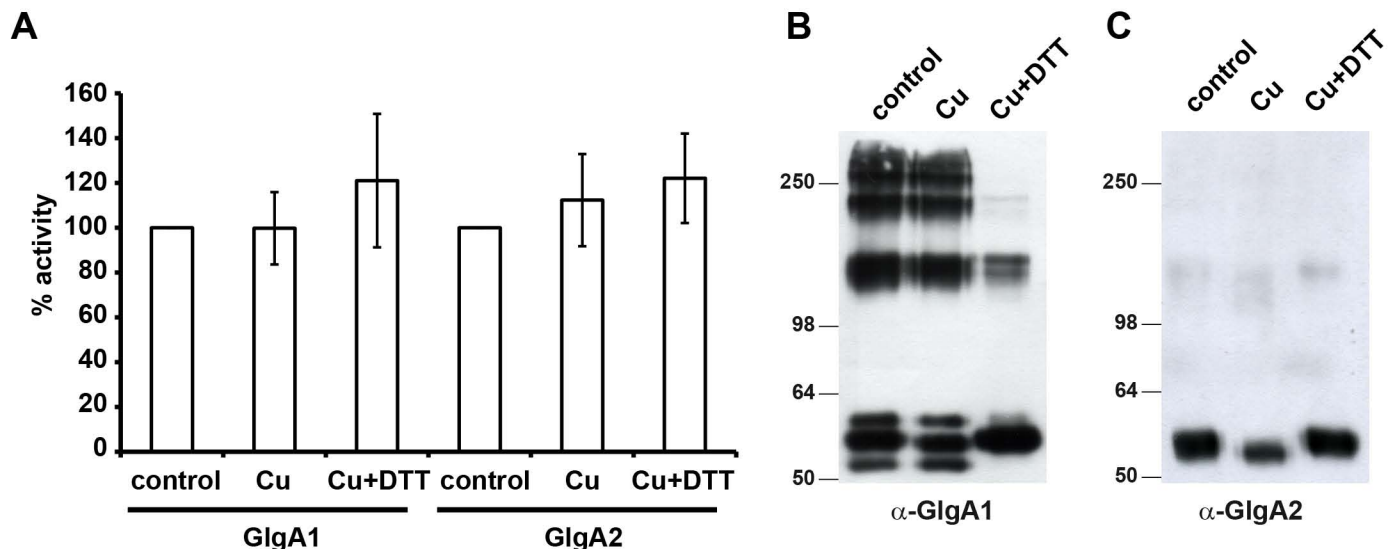
Figure 1.

Figure 1. Glycogen synthases are not redox regulated.

A. Glycogen synthase activity in untreated, oxidized and reduced conditions. Glycogen synthase activity of purified GlgA1 or GlgA2 was determined *in vitro* by measuring ADP formation as described in the Methods section. Proteins were not treated (control), oxidized in the presence of 10 μM of CuCl$_2$ or oxidized with copper and subsequently reduced with 5 mM DTT. Activity was normalized to untreated sample. Data represents average of three independent experiments and error bars represent SE.

B. Western blot analysis of GlgA1 under the same conditions used for activity measurements in A. Proteins were separated on a 7% acrylamide non-reducing gel and detected using GlgA1 specific antibodies.

C. Western blot analysis of GlgA2 under the same conditions used for activity measurements in A. Proteins were separated on a 7% acrylamide non-reducing gel and detected using GlgA2 specific antibodies
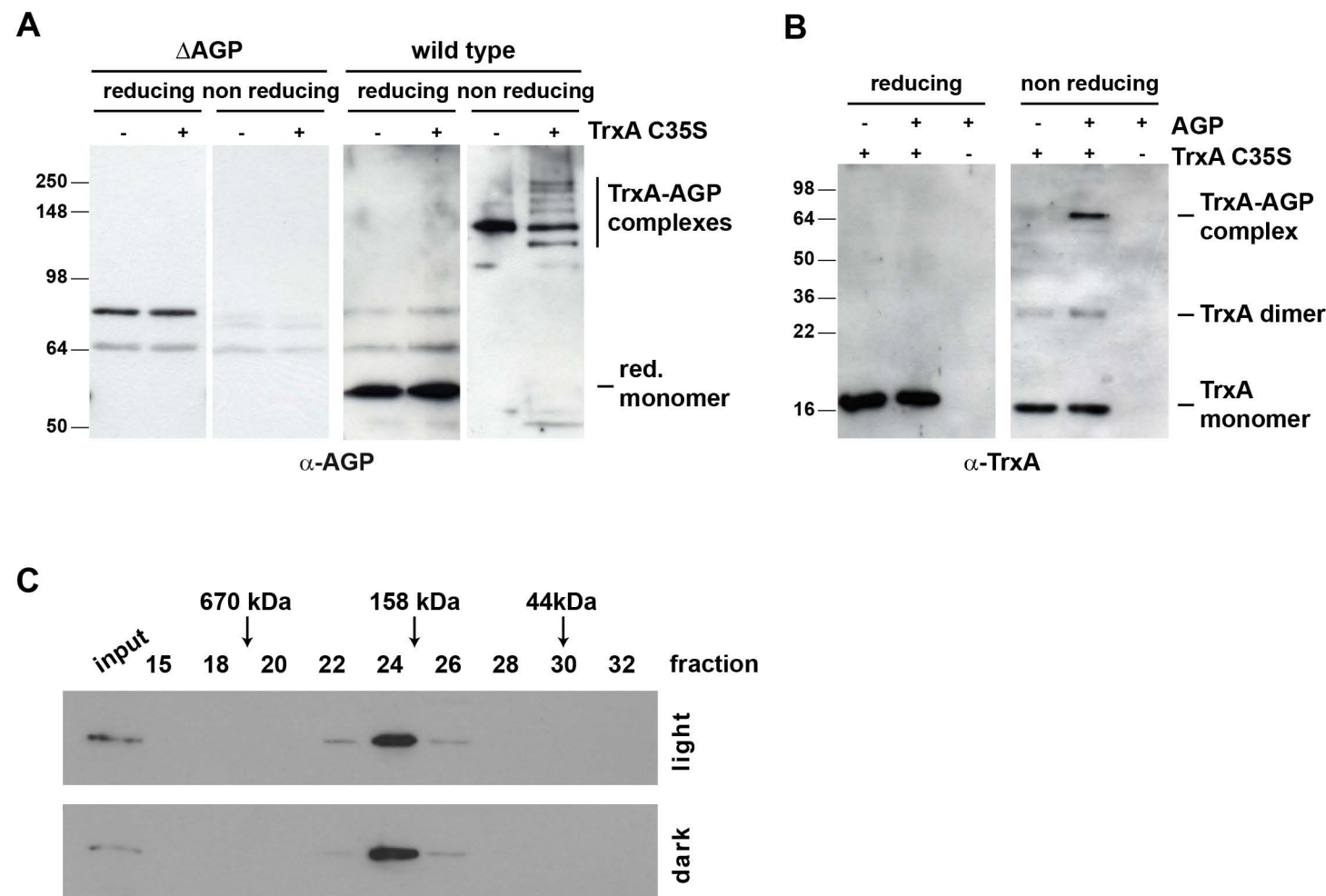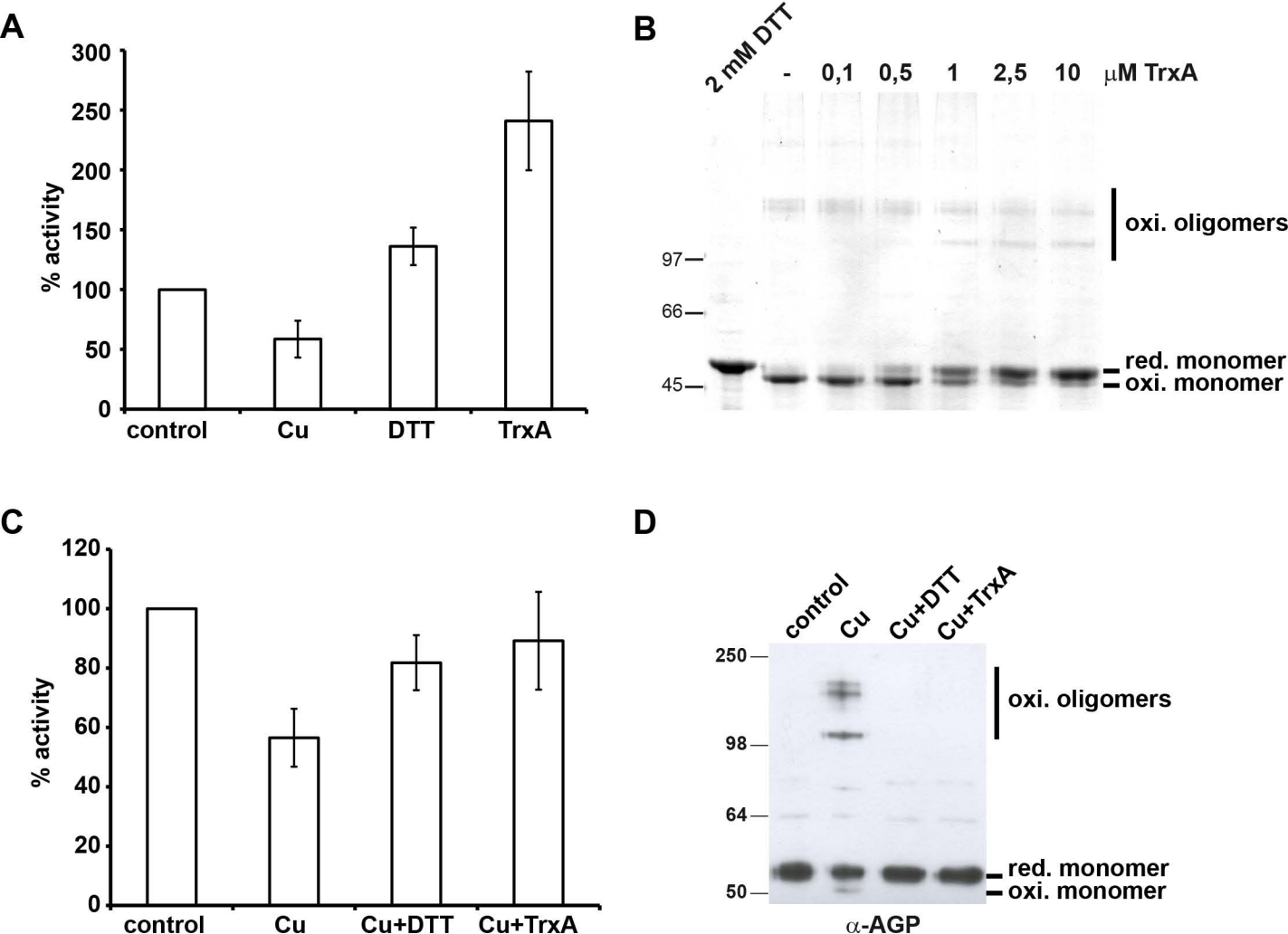
Figure 2.

Figure 2. AGP is able to interact with TrxA.

A. AGP interacts with TrxAC35S in *Synechocystis* extracts. Soluble extracts from WT and ΔAGP strains were oxidized with 25 µM $CuCl_2$ for 30 min and incubated with 50 µM TrxAC35S for 1h to allow the formation of mixed disulfides. Proteins were resolved in 7% reducing (left panel) or non-reducing acrylamide gels (right panel) and probed with AGP antibodies.

B. AGP interacts with TrxAC35S *in vitro*. Purified AGP was incubated with a 4 fold excess of TrxAC35S for 1 h to allow mixed disulfide to be formed, proteins were resolved in 12% reducing (left panel) or non-reducing (right panel) acrylamide gels and probed with TrxA antibodies.

C. AGP is a tetramer in *Synechocystis* extracts. Extracts from light grown cells (top panel) or cells maintained in the dark for 8 h (bottom panel) were separated on a Superdex 200 column and fractions were probed for the presence of AGP by western blot on 10% acrylamide gels. The column was previously calibrated with molecular mass protein standards which are indicated by arrows.

Figure 3.

Figure 3. AGP activity is redox regulated.

A. AGP activity is redox regulated *in vitro*. AGP activity was measured using 1 µg of purified protein as described in the Methods section. Protein was not treated (control), oxidized in the presence of 10 µM of $CuCl_2$, oxidized with copper and subsequently reduced with 5 mM DTT or oxidized with copper and reduced with 100 µM of TrxA. Activity was normalized to the untreated sample. Data represents average of three independent experiments and error bars represent SE.

B. Analysis of the oligomerization state of purified recombinant AGP. 2 µg of AGP were oxidized with 10 µg of $CuCl_2$ and incubated with 2 mM DTT or increasing amounts of reduced TrxA. Proteins were separated on a 7% acrylamide non-reducing gel and Coomassie stained.

C. AGP activity was measured in soluble extracts (0.5 µg total proteins) as described in the Methods section. Extracts were not treated (control), oxidized in the presence of 25 µM of $CuCl_2$ and subsequently reduced with 5 mM DTT or oxidized with copper and reduced with 100 µM of TrxA. Activity was normalized to untreated sample. Data represents average of three independent experiments and error bars represent SE.

D. Western blot analysis of AGP oligomerization state under the same conditions used for activity measurements in C. Extracts were separated on a 7% acrylamide non-reducing gel and probed with AGP antibodies.
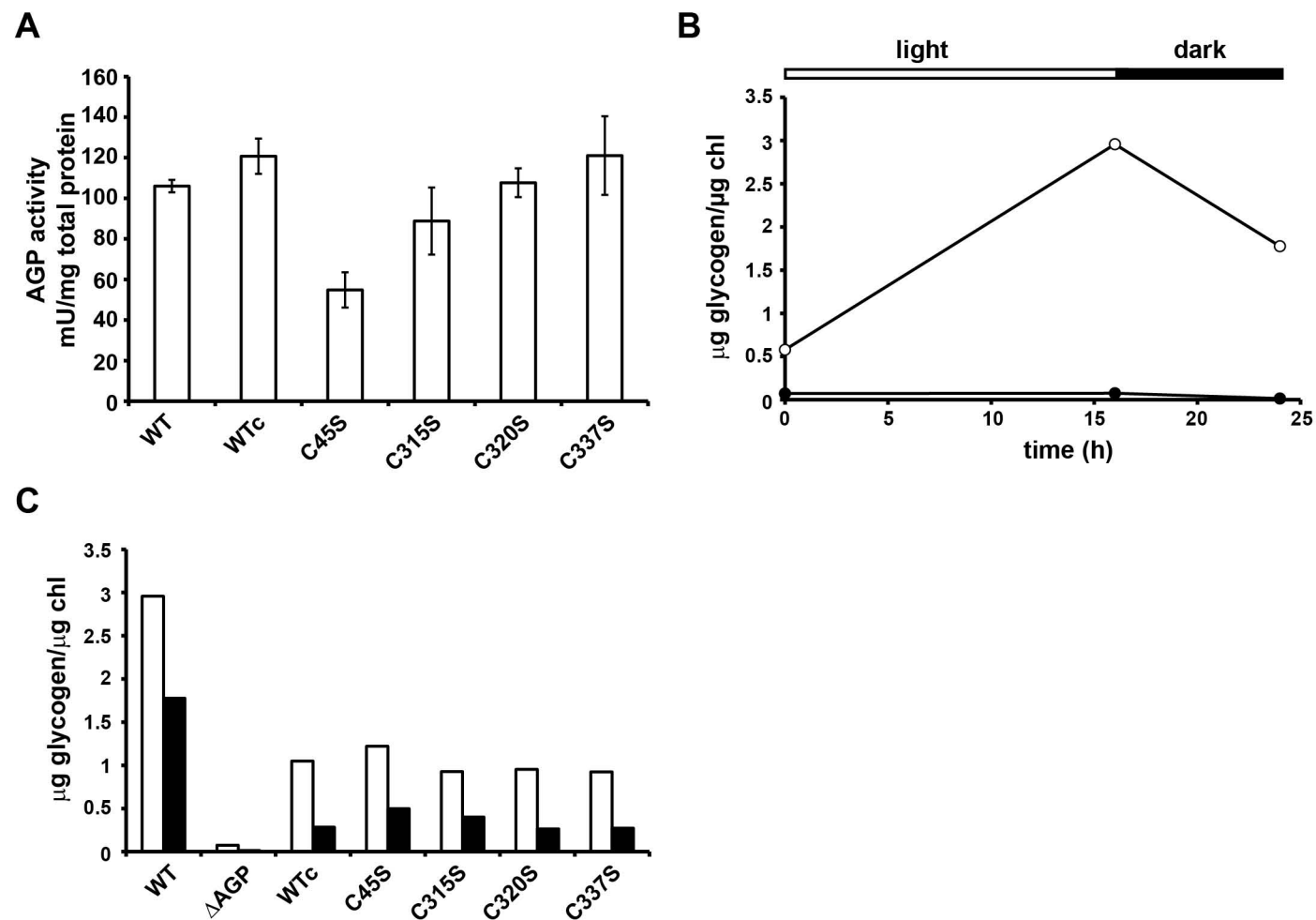
Figure 4.

Figure 4. All conserved cysteines are not essential for AGP activity.

A. AGP specific activity of *Synechocystis* extracts. AGP activity was measured from extracts prepared from WT, WTc, C45S, C315S, C320S and C337S strains. Data represents average of three independent experiments and error bars represent SE.

B. Glycogen content in WT cells grown in long day conditions. Glycogen was determined in late exponential phase (10-15 µg chl mL-1) in WT (white circles) and ΔAGPcells (black circles) grown in a 16 h-light/8-h dark photoperiod for at least 3 cycles.

C. Glycogen content in WT, WTc, C45S, C315S, C320S and C337S strains. Glycogen was determined in late exponential phase cells (10-15 µg chl mL-1), grown in a 16 h-light/8-h dark photoperiod for at least 3 cycles, at the end of the day (white bars) or at the end of the night (black bars).
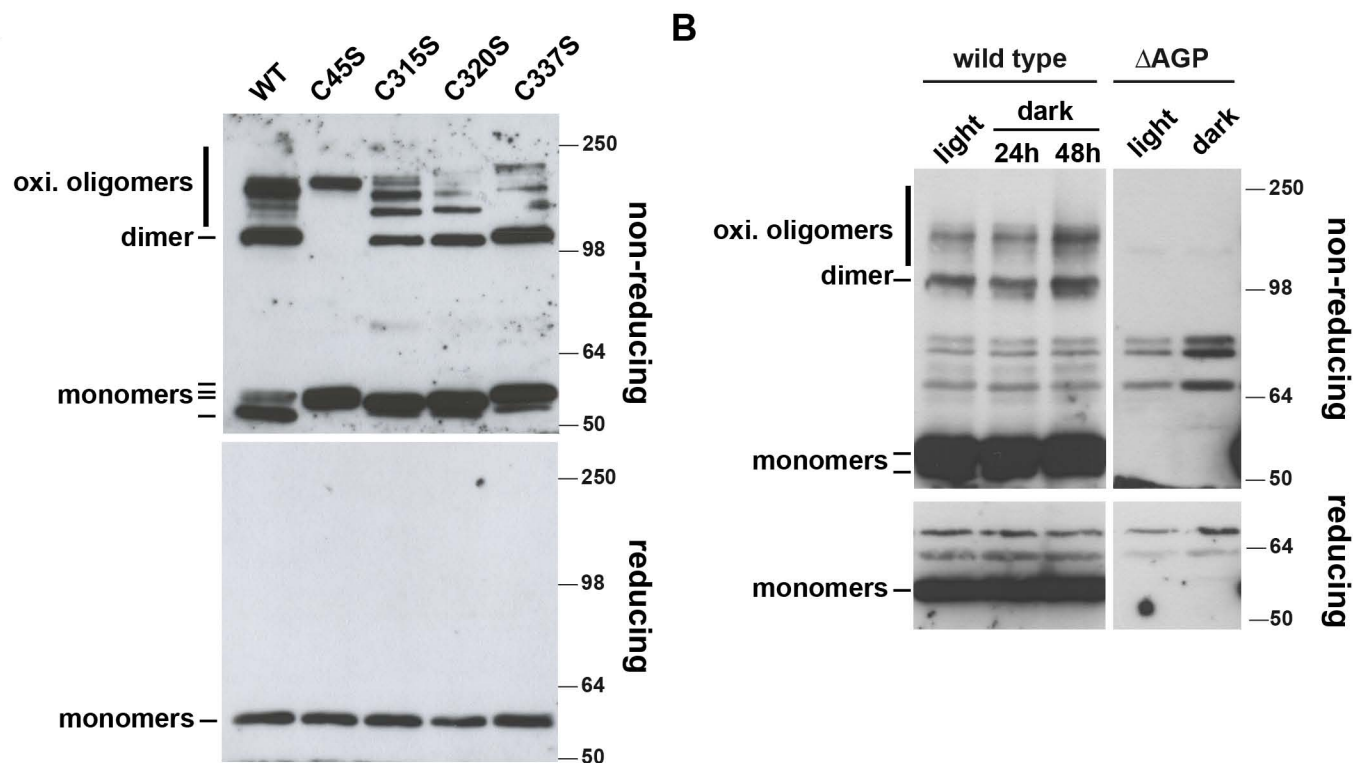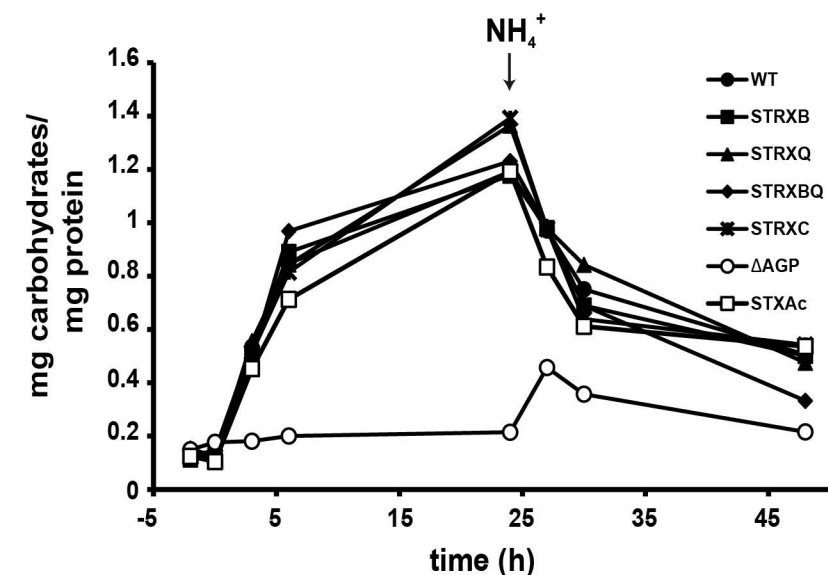
Figure 5.

Figure 5. All conserved cysteines are involved in oxidoreduction of AGP.

A. AGP redox state in cysteine mutants. Soluble extracts from WT, C45S, C315S, C320S and C337S strains were oxidized in the presence of 25 µM of CuCl$_2$, 5 µg of total proteins were separated in 7% non-reducing (top panel) or reducing acrylamide gels (bottom panel) and the redox state of AGP was analyzed by western blot.
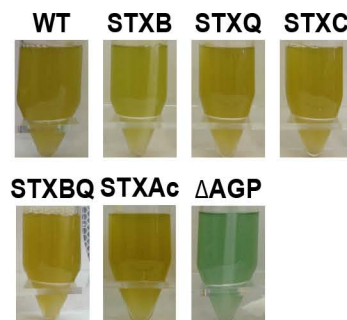
B. AGP redox state in dark treated cells. Soluble extracts from WT or ΔAGP mutant were prepared from cells grown in the light or incubated in the dark for the indicated times. 30 µg of total proteins were separated in 7% non-reducing (top panel) or reducing acrylamide gels (bottom panel) and the redox state of AGP was analyzed by western blot.
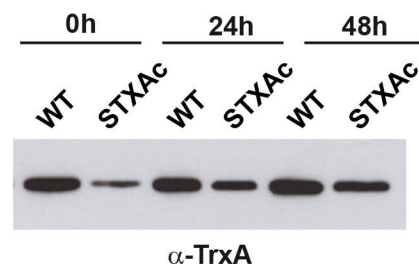
Figure 6.

Figure 6. Analysis of the carbohydrate accumulation during nitrogen starvation in thioredoxin mutant strains.

A. Total carbohydrates content were determined during a nitrogen starvation time course in WT, STXB, STRXQ, STXC, STXBQ, STXAc and ΔAGP mutant strains. Exponential cultures growing in BG11C were washed twice with BG110C and resuspended in the same media at a 4 μg chl mL-1. After 24 h of nitrogen starvation, 10 mM NH4+ was added and cultivation was continued for another 24 h. Total carbohydrate contents were normalized to total protein contents.

B. Bleaching of WT, STXB, STRXQ, STXC, STXBQ, STXAc and ΔAGP strains during the nitrogen starvation time course. Photograph of the different strains used in A after 24h of nitrogen starvation.

C. Western blot analysis of TrxA levels in WT and STXAc strains during the nitrogen starvation time course. Samples were collected at the beginning of the experiment (0h), 24h after nitrogen removal (24h) and 24h after ammonium addition (48h).