Rubisco mutagenesis provides new insight into limitations on photosynthesis and growth in *Synechocystis* PCC6803

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Abstract

Orthophosphate (Pi) stimulates the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) while paradoxically inhibiting its catalysis. Of three Pi-binding sites, the roles of the 5P- and latch sites have been documented, whereas that of the 1P-site remained unclear. Conserved residues at the 1P-site of Rubisco from the cyanobacterium *Synechocystis* PCC6803 were substituted and the kinetic properties of the enzyme derivatives and effects on cell photosynthesis and growth were examined. While Pi-stimulated Rubisco activation diminished for enzyme mutants T65A/S and G404A, inhibition of catalysis by Pi remained unchanged. Together with previous studies, the results suggest that all three Pi-binding sites are involved in stimulation of Rubisco activation, whereas only the 5P-site is involved in inhibition of catalysis. While all the mutations reduced the catalytic turnover of Rubisco ($K_{\text{cat}}$) between 6- and 20-fold, the photosynthesis and growth rates under saturating irradiance and inorganic carbon (Ci) concentrations were only reduced 40–50% (in the T65A/S mutants) or not at all (G404A mutant). Analysis of the mutant cells revealed a 3-fold increase in Rubisco content that partially compensated for the reduced $K_{\text{cat}}$ so that the carboxylation rate per chlorophyll was one-third of that in the wild type. Correlation between the kinetic properties of Rubisco and the photosynthetic rate ($P_{\text{max}}$) under saturating irradiance and Ci concentrations indicate that a >60% reduction in $K_{\text{cat}}$ can be tolerated before $P_{\text{max}}$ in *Synechocystis* PCC6803 is affected. These results indicate that the limitation of Rubisco activity on the rate of photosynthesis in *Synechocystis* is low. Determination of Calvin cycle metabolites revealed that unlike in higher plants, cyanobacterial photosynthesis is constrained by phosphoglycerate reduction probably due to limitation of ATP or NADPH.

Key words: Orthophosphate (Pi), photosynthesis, rate-limiting factor, Rubisco (ribulose-bisphosphate carboxylase/oxygenase), *Synechocystis* PCC6803 (cyanobacteria).

Introduction

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), the main enzyme in nature that assimilates inorganic carbon (Ci) into organic compounds, catalyses the primary reactions of photosynthesis and photorespiration by carboxylation or oxygenation of ribulose-1,5-bisphosphate (RuBP), respectively. Both reactions initiate upon carbamylation of Lys201 at the enzyme large subunit and subsequent stabilization of the carbamate by a magnesium ion, which turns the enzyme catalytically active. Binding of RuBP stimulates closure of the catalytic site and facilitates the conversion of the pentose phosphate into its enediol form. This intermediate reacts either with CO$_2$ (carboxylation) to form two molecules of 3-phosphoglycerate (PGA), or with O$_2$ (oxygenation) to form one molecule of PGA and another molecule of 2-phosphoglycolate that enters the photorespiratory pathway. The products of both reactions are liberated as the catalytic site opens (Taylor and Andersson, 1996; Cleland *et al.*, 1998; Duff *et al.*, 2000).

Despite its central role in photosynthetic metabolism, Rubisco catalysis is considered slow and inefficient, as the catalytic turnover and affinity for CO$_2$ are low (120–720 carboxylations per catalytic site min$^{-1}$ and 10–300 $\mu$M,
respectively). Moreover, the carboxylation reaction is competitively inhibited by O₂, and side reactions of the enzyme generate products inhibitory to activation and activity (reviewed by Kellogg and Juliano, 1997). On the basis of biochemical constraints and considering free energy differences in Calvin cycle reactions (Bassham and Krause, 1969; Farquhar et al., 1980; Dietz and Heber, 1984), as well as variations in the rate of photosynthesis, resulting from differential expression of Rubisco using antisense technology (Stitt et al., 1991; Furbank et al., 1996), Rubisco activity has been considered the main limiting factor of photosynthesis under saturating irradiance and limiting CO₂ concentrations.

Rubisco activity is tightly regulated by activation-deactivation and accessibility of substrates, as well as stimulatory and inhibitory effectors (Woodrow and Berry, 1988; Kellogg and Juliano, 1997). Orthophosphate (Pi) is a key regulatory effector of the photosynthetic machinery (Heber et al., 1986; Woodrow and Berry, 1988) and it affects Rubisco in an antagonistic manner. On the one hand, it stimulates Rubisco activation and, on the other hand, it inhibits enzyme activity by competition with RuBP (Heldt et al., 1978; Tabita and Colletti, 1979; McCurry et al., 1981; Parry et al., 1985; Anwaruzzaman et al., 1995; Marcus and Gurevitz, 2000). The crystal structure of Rubisco from Nicotiana tabacum in complex with Pi reveals three Pi-binding sites: a positively charged pocket at the enzyme surface named the ‘latch’ site that also interacts with the C-terminus of the enzyme large subunit during catalysis to close the catalytic site, and two sites that bind the 1P and 5P of RuBP (Duff et al., 2000). Substitution of residues at the 5P- and latch sites of Rubisco from the cyanobacterium Synechocystis PCC6803 revealed that they play a role in stimulation of Rubisco activation, whereas only the 5P-site is involved in inhibition of the catalytic activity by Pi. These experiments, together with the biphasic kinetics of the Pi-stimulated activation of the enzyme (Anwaruzzaman et al., 1995), led to the suggestion that stimulation of Rubisco activation occurs via a multisite mechanism and that the inhibitory site only partially overlaps with the stimulatory site at the 5P-binding site (Marcus et al., 2005). However, the possibility that the 1P-binding site is involved in regulation of Rubisco activity by Pi has not been examined. In the closed state of the enzyme, phosphate at the 1P-binding site forms hydrogen bonds with the backbone amides of Gly381, Gly403, and Gly404, and with the side chain of Thr65 (Fig. 1). As the catalytic site opens, phosphate interaction with Thr65 is replaced by an interaction with Trp66 (Duff et al., 2000).

Using a mutant of the cyanobacterium Synechocystis PCC6803, Syn6803Δrbc (Amichay et al., 1993), which enables site-directed mutagenesis of Rubisco and analysis of the effects in its natural photoautotrophic environment (Marcus et al., 2003, 2005), the effect of the substitutions at the 1P-binding site on catalysis and regulation of enzyme activation by Pi were examined. Surprisingly, although some of these substitutions substantially decreased the catalytic turnover of Rubisco, the photosynthetic and growth rates of the mutant cells were only slightly affected. Therefore, the content and kinetic properties of Rubisco in these mutant cells were examined and a search for an additional rate-limiting factor of carbon fixation in Synechocystis in comparison with plants was conducted.

Materials and methods

Growth conditions and photosynthesis assays

Synechocystis PCC6803 was grown on BG-11 medium as was previously described (Marcus et al., 2003). Nuphar lutea plants, collected in the basin of the Yarkon River in the coastal plain of Israel, were grown in an open pond. Net photosynthetic rates were determined using a Clark-type O₂ electrode (Rank Brothers, UK). Relative rates of gross photosynthesis of N. lutea leaves were measured under saturating irradiance (2000 μmol photons m⁻² s⁻¹) and ambient CO₂ concentration using a modulated fluorometer (Diving-PAM, Walz, Germany) as was previously described (Snir et al., 2006). Rubisco was partially purified from cell extracts by two steps of ammonium sulphate fractionation followed by dialysis (cut-off 12000 Da). Enzyme integrity was evaluated electrophoretically on non-denaturing and denaturing polyacrylamide gels followed by immunochromical analysis (Marcus et al., 2003, 2005). Neither dissociation nor proteolytic products of Rubisco were observed in these extracts (data not shown). The molar concentration of Rubisco catalytic sites was determined by incubating the enzyme with 15 μM [¹⁴C]CPBP (carboxypeptidase b bisphosphate; 66.6×10⁷ Bq mol⁻¹) for 10 min at 30 °C. [¹¹C]CPBP was synthesized as previously described (Marcus and Gurevitz, 2000). [¹¹C]CPBP-bound Rubisco was separated from the free ligand by gel filtration using Sephadex G-100. The ratio of CPBP binding to the wild-type and mutant Rubisco was compared with the densitometric ratio of bands of the

Fig. 1. Diagram of RuBP interactions at the 1P- and 5P-binding sites. In the closed state of Rubisco, phosphate forms hydrogen bonds with Gly381, Gly403, Gly404, and Thr65 at the 1P-binding site (dashed lines), whereas in the open state of the enzyme the interaction with Trp66 replaces the interaction with Thr65 (not shown). Phosphate also forms hydrogen bonds with Arg295 and His327 at the 5P-binding site in the closed state of the enzyme (dashed lines). In the open state of the enzyme, the interaction of phosphate with His327 is replaced by interaction with His298 (not shown). The diagram is based on the crystal structure of non-activated spinach Rubisco in complex with RuBP (PDB code 1RCX), and was produced using RasMol software. The carbon, nitrogen, oxygen, and phosphate atoms are coloured in grey, blue, red, and orange, respectively.
wild-type and mutant enzymes separated on a native polyacrylamide gel. The similarity of the two ratios, irrespective of the mutant analysed, indicated that determination of the catalytic site number by $[^{14}]$CPBP binding was reliable. The activation and carboxylase assays of Rubisco were performed as described (Marcus and Gurevitz, 2000; Marcus et al., 2005).

Electron microscopy

*Synechocystis* cells were fixed in 2.5% glutaraldehyde and then in OsO$_4$, and dehydrated by increasing concentrations of ethanol. The fixed samples were embedded in Glycid ether and stained with uranyl acetate and lead citrate.

Mutagenesis and genetic analysis

Plasmid pSynR4.0, a pBluescript derivative containing the entire *rbc* operon and flanking sequences from *Synechocystis* PCC6803 (Amichay et al., 1993), was used for mutagenesis via PCR using three oligonucleotide primers (Supplementary Table S1 available at *JXB* online). A primer designed with a mutation was reacted with either an upstream or a downstream primer encompassing the restriction sites used for subcloning. Plasmid pSynR4.0 (Amichay et al., 1993) was the DNA template and Taq DNA polymerase was used for amplification. The resulting DNA fragment was used in a second PCR step with a complementary primer encompassing a restriction site. A 580 bp *HpaII* fragment was used for mutagenesis of Thr65 and a 516 bp *NotI–NruI* fragment for mutagenesis of Gly404 (Supplementary Table S1). The final PCR product was cleaved by the appropriate enzymes and ligated into the pSynR4.0 vector that was used to transform the Syn6803 recipient (Amichay et al., 1993). Transformed cells were inoculated onto solid BG-11 medium at ambient CO$_2$ concentrations. Air-grown colonies appeared within 14–21 d. PCR analysis and DNA sequencing verified the full segregation of the introduced mutations in the few copies of the cyanobacterial genome.

Determination of the concentrations of Calvin cycle metabolites

*Synechocystis* cells (30–50 g chlorophyll ml$^{-1}$) were incubated in a transparent O$_2$ electrode chamber under the indicated illumination and Ci concentration at 30°C until the rate of photosynthesis was immediately stopped all metabolic activities. The acid was 0.35 M perchloric acid that denatured the cell proteins and was collected from the Swiss Protein Database using the PSI-Blast algorithm (Altschul et al., 1997) were calculated by a Bayesian method using ConSurf software (Landau et al., 2005). The structural model was drawn using RasMol software.

Bioinformatic analysis

The evolutionary conservation scores of glycine residues in 400 homologues of Rubisco from *Synechococcus* (PDB code 1RBL) that were collected from the Swiss Protein Database using the PSI-Blast algorithm (Altschul et al., 1997) were calculated by a Bayesian method using ConSurf software (Landau et al., 2005). The structural model was drawn using RasMol software.

**Results**

**Effects of substitutions at the 1P-binding site on regulation of Rubisco activation by Pi and its catalytic properties**

To examine the role of the 1P-binding site in regulation of Rubisco activation and catalysis conserved residues involved in RuBP and Pi binding were substituted (Fig. 1). Whereas no cyanobacterial mutants were recovered for W66A, G381A, and G403A, presumably because of the low activity or instability of the modified enzymes, G404A and T65A/S mutant cells grew photoautotrophically at a rate similar to that of the wild type (data not shown). The main effects of these substitutions were a drastic (85–95%) reduction in the *K*$_{cat}$ of Rubisco and a decrease in the *K*_m(RuBP) of mutants G404A and T65S, whereas the apparent *K*_m(CO$_2$) at ambient O$_2$ concentration for G404A and T65A/S was similar to that of the wild type (Table 1). To analyse the role of the 1P-binding site in Rubisco regulation by Pi, the kinetics of RuBP carboxylation catalysed by the mutant enzymes at various RuBP and Pi concentrations under saturating CO$_2$ concentration were compared with those of the unmodified enzyme (Table 1). Dixon analysis (Segel, 1975) of the data for RuBP revealed that Pi competitively inhibited the carboxylation reaction of the wild-type and mutant enzymes, with no significant alteration in *K*$_i$(Pi) (Table 1). However, while Pi-stimulated activation of the wild-type and T65S enzyme derivatives was similar under suboptimal concentrations of CO$_2$ and in the presence of MgCl$_2$ (0.07 mM and 5 mM, respectively), this stimulatory effect was abolished for mutants T65A and G404A (Fig. 2). The difference in Pi effect between T65A and T65S might be attributed to the ability of serine to maintain the hydrogen bond with Pi via the hydroxyl side chain (Morell et al., 1994). Notably, although the stimulation of enzyme activation by Pi was abolished for T65A and

| Strain   | *K*$_{cat}$ (min$^{-1}$) | *K*$_{m}$(RuBP) (μM) | *K*$_{m}$(CO$_2$)a (μM) | *K*$_i$(Pi) (mM) |
|----------|------------------------|----------------------|------------------------|-----------------|
| Wild type | 857±1.63               | 146±2.24             | 268±4.8                | 8.3±1.1         |
| T65A     | 40±5.6                 | 193±29               | 283±5.7                | 9.0±2.2         |
| T65S     | 132±17                 | 47.3±7.6             | 258±5.1                | NMd             |
| G404A    | 71.7±13                | 46.3±8.1             | 296±5.6                | 8.35±0.9        |
| Wild type$^c$ | 545±95                | 140±11               | 181±4.3                | 5.8±0.4         |
| H327Q$^c$ | 92±15                 | 490±12               | 175±5.7                | 0.67±0.07       |

* a Apparent *K*$_m$(CO$_2$), determined at 21% O$_2$.
* b NM, not measured.
* c Results taken from Marcus et al. (2005).

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MgCl₂, and various concentrations of potassium phosphate added in the absence of Pi was 40, 5.5, 2.56, and 22.5 min in the presence or absence of Pi, respectively. Rubisco activity in the absence of Pi was defined as the ratio of Rubisco activity activated in the presence and absence of Pi, respectively. Rubisco activity in the absence of Pi was 40, 5.5, 2.56, and 22.5 min for the wild type and G404A, T65A, and T65S Rubisco mutants, respectively.

G404A, the activation by CO₂ and Mg²⁺ was not impaired for either mutant enzyme (data not shown).

**Effects of substitutions at the 1P-site on photosynthesis**: Despite the marked reductions in $K_\text{cat}$ for the Rubisco mutants (6- to 20-fold), the photosynthesis rates of the cells were reduced only 40-50% (T65A/S mutants) or barely at all (G404A mutant) under saturating irradiance and varying Ci concentrations (Fig. 3). In an attempt to explain the difference between these mutant cells, the concentration of Rubisco per chlorophyll, their carboxylation capacity, and the concentrations of four Calvin cycle metabolites (RuBP, PGA, GA₃P, and DHAP) were determined.

Rubisco content, determined by binding of radiolabelled [¹⁴C]CPBP, increased 2.6-, 3.5-, and 4.6-fold, respectively, in G404A, T65S, and T65A mutant cells grown under 20 μmol photons m⁻² s⁻¹ at ambient CO₂ concentration (Fig. 4). Electron micrographs of these mutants revealed an increase in the content of carboxysomes (Fig. 5), which encapsulate Rubisco in cyanobacteria (Codd and Marsden, 1984; Marcus et al., 1992). As a result, the carboxylation capacity per chlorophyll at saturating substrate concentrations reached 25, 49, and 30% of that of the wild-type in mutants G404A, T65S, and T65A, respectively (Fig. 4).

Since *Synechocystis* cells are routinely grown in the laboratory under low irradiance (20 μmol photons m⁻² s⁻¹), it may be assumed that under these conditions the photosynthesis rate is limited to a lesser extent, as was shown for tobacco plants (Stitt et al., 1991). Therefore, the effects of varying irradiance during growth on Rubisco content and cell photosynthesis were examined. It was found that although Rubisco content doubled in the wild-type cells and did not change in the mutant cells grown under 100 μmol photons m⁻² s⁻¹, the photosynthesis rate was not affected by the elevated irradiance (data not shown). These results led to examination of the correlation between the carboxylation capacity and the kinetic properties, $K_m$(RuBP) and $K_\text{cat}$, of Rubisco variants (Marcus et al., 2003, 2005; Figs 6, 7) and the photosynthesis rate in the cells under saturating irradiance and Ci concentration ($P_{\text{max}}$).

**Analysis of correlations between Rubisco kinetic properties and the rate of photosynthesis**

In light of the inverse relations between $K_\text{cat}$ and $K_m$(CO₂) of Rubisco from various species (Savir et al., 2010), the relationships between $K_\text{cat}$ and $K_m$(RuBP) of Rubisco were examined in 11 *Synechocystis* mutants of the present work (Table 1) as well as of previous studies (Marcus et al., 2003, 2005). An optimum curve was obtained (Fig. 6A) in which the maximal $K_\text{cat}$ values pertain to $K_m$(RuBP) values of 140–170 μM, which were also determined in the wild-type enzyme. At a higher or lower affinity for RuBP the catalytic turnover was lower. The optimum curve implies that there is no simple relations between the correlation of $P_{\text{max}}$ and Rubisco $K_\text{cat}$ and the correlation of $P_{\text{max}}$ and Rubisco $K_m$(RuBP) if the relations between $K_\text{cat}$ and $K_m$(RuBP) were reciprocal.

Correlation between $K_m$(RuBP) and the $P_{\text{max}}$ in these 11 Rubisco mutants revealed that fluctuations in $K_m$(RuBP) in the range of 50–250 μM had little effect on the $P_{\text{max}}$. An exception to this was the $K_m$(RuBP) value for the H327Q Rubisco mutant (490 μM), which could be one of the reasons for its low $P_{\text{max}}$ (Fig. 6B). As additional factors (e.g. Rubisco content and $K_\text{cat}$; Figs 4, 5, 6C; Table 1) could be affected by the substitutions and prominently affect the rate of photosynthesis, the variance in this correlation analysis was high. Since the RuBP concentration limits the $P_{\text{max}}$ (Farquhar et al., 1980), the $P_{\text{max}}$ should increase as $K_m$(RuBP) decreases. However, the decrease in $K_m$(RuBP)
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Fig. 4. Rubisco activity, content, and catalytic turnover in Synechocystis wild type and mutants. The cells, grown on BG-11 medium at ambient CO2 under continuous illumination (20 μmol m⁻² s⁻¹), were spun down and resuspended in 50 mM HEPES buffer, pH 8.0, containing 285 μM CO2, 10 mM MgCl2, 1 mM dithiothreitol, and a protease inhibitor cocktail (P2714, Sigma). The cells were broken under 24000 lb inch⁻² using a French press. The crude extract was centrifuged and the supernatant containing the enzyme was activated by incubation for 30 min at 30 °C. The content of Rubisco catalytic sites was determined by incubation of 200 μl of activated enzyme with 15 μM [¹⁴C]CPBP (185 Bq nmol⁻¹). The CPBP-bound enzyme was separated from the free ligand using a 10 cm Sephadex-G100 column. Rubisco activity was determined as described in Table 1 in the presence of 1 mM RuBP and 640 μM ¹⁴C12CO₂ (1.3 Bq nmol⁻¹) in the reaction mixture. Each assay was performed in triplicate. Standard deviations were <10%.

of Rubisco mutants G404A, T65S, and C172A (Fig. 6B) was not reflected by an increase in P max probably due to the marked decrease in Kcat, which reduced the carboxylation rate (Fig. 6C; Table 1; Marcus et al., 2003). Consequently, the net effect of these alterations in Kcat and Km(RuBP) on P max was modest. This relation between Kcat and Km(RuBP) would prevent further elevation in Rubisco affinity for RuBP because it may severely decrease the Kcat. Correlation between Rubisco Kcat and P max of the cells (Fig. 6C) revealed that a decrease in Kcat from 700 min⁻¹ to 200 min⁻¹ essentially did not change the rate of photosynthesis, although a further decrease in Kcat resulted in a prominent reduction in P max. As Rubisco Kcat decreased while its content increased in the mutants modified at the 1P-binding site (Fig. 4), the correlations between the carboxylation capacity (carboxylation per chlorophyll under saturating RuBP and CO2 concentrations) and P max were analysed in extracts of nine Rubisco variants (Marcus et al., 2003, 2005; Figs 3, 4), and compared with similar correlations in higher plants. Whereas the light-saturated rate of photosynthesis at ambient CO2 concentration in the C3 plant N. lutea (Fig. 7) or in the C4 plant Flaveria bidentis (Furbank et al., 1996) decreased along with a reduced carboxylation capacity, up to an ~66% reduction in the carboxylation capacity of Synechocystis hardly affected the rate of photosynthesis (Fig. 7). Under these conditions, the control coefficient [a quantitative measure of the limitation imposed by a single factor over the flux through the pathway, whose absolute value varies between 0 for a non-limiting to 1 for a factor that totally limits the flux (Kacser and Burns, 1973)] of Rubisco on photosynthesis was zero. However, any further decrease in carboxylation capacity resulted in a steep decline in the rate of Synechocystis photosynthesis (Fig. 7), raising the control coefficient of the enzyme on photosynthesis to 0.6. By interpolation, it seems that the minimal carboxylation capacity required to support Synechocystis phototrophic growth is ~20% that of the wild type (Figs 6, 7).

Analysis of Calvin cycle metabolites

As the limitation imposed by Rubisco on the rate of photosynthesis is low (Figs 6, 7), the possibility was considered that another reaction in the Calvin cycle limits the rate of photosynthesis in Synechocystis. The concentration ratio of products to substrates for a limiting reaction generally differs from that calculated at equilibrium. Hence, large deviations from the equilibrium concentrations of the products and substrates of a given reaction could suggest a limiting reaction (Bassham and Krause, 1969). Therefore, the concentrations of four Calvin cycle metabolites (RuBP, PGA, GA3P, and DHAP) were determined, the products and substrates of the reactions catalysed by Rubisco, phosphoglycerate-kinase, glyceraldehyde-3-phosphate dehydrogenase, and triose-phosphate isomerase, respectively) were determined, their product to substrate ratios were calculated, and how they would be affected by varying irradiance and CO2 concentrations was examined.

Under limiting irradiance, the RuBP concentration was high, whereas the concentrations of PGA, GA3P, and DHAP were low (Fig. 8). As the irradiance increased, the RuBP concentration decreased and the concentrations of PGA, GA3P, and DHAP increased. Consequently, the product to substrate concentration ratios of PGA to RuBP and that of GA3P to PGA increased with higher irradiance (Fig. 8). The concentration of these metabolites was also determined for mutant G404A and for a formerly studied mutant, H327Q (Marcus et al., 2005). While Rubisco Kcat and content were similar in the two mutants, Km(RuBP) was different (46 μM and 490 μM, respectively; Table 1 and Fig. 4; Marcus et al., 2005). As substitution H327Q increased the competitive inhibition of carboxylation by Pi, one would expect an elevated Km(RuBP) in vivo, depending on the intracellular Pi concentration (Marcus et al., 2005). Indeed, the RuBP concentration in mutant H327Q was higher than that in the wild type at either irradiance, whereas in mutant G404A it was lower under limiting irradiance and equal to that of the wild type under saturating irradiance. In both mutants, the PGA concentration was lower than that of the wild type. Consequently, the highest PGA to RuBP concentration ratio was determined for the wild type, whereas the lowest was determined for mutant H327Q. The GA3P concentration in both mutants
was lower than that of the wild type, and the DHAP concentration was higher in mutant G404A compared with the wild type and with mutant H327Q. The GA3P to PGA concentration ratio was similar in the wild type and in both mutants (Fig. 8).

The differences in concentrations of RuBP, PGA, GA3P, and DHAP in mutants G404A and H327Q apparently resulted from the large difference in Rubisco affinity for RuBP. The higher affinity for RuBP probably enabled mutant G404A to utilize low RuBP concentrations that exist in these cells at saturating irradiance and Ci concentrations and maintain their wild-type-like rate of photosynthesis (Fig. 3).

Discussion
Role of the 1P-binding site in Rubisco regulation by Pi

Mutational analysis highlighted the role of the 1P-binding site in stimulation of Rubisco activation. As shown in the crystal structure of the Rubisco–Pi complex (Fig. 1), phosphate interacts with Thr65, Trp66, Gly381, Gly403, and Gly404 (Duff et al., 2000). While substitution of Trp66, Gly381, and Gly403 with alanine appeared lethal to cell viability, analysis of mutants T65A/S and G404A demonstrates how the substitutions differentially effect the $K_{\text{in}}$(RuBP) and $K_{\text{cat}}$ of the enzyme as well as its activation by Pi, but not the competitive Pi inhibition of its activity (Table 1, Fig. 2). These findings along with previous analyses of the 5P- and the latch-binding sites of Pi (Marcus et al., 2005) imply that all three Pi-binding sites play a role in stimulation of Rubisco activation, whereas only the 5P-site is involved in Pi inhibition of Rubisco activity. Whereas at the 5P- and latch-binding sites, phosphate forms hydrogen and electrostatic interactions with the side chains of positively charged residues (histidine, arginine, and lysine), at the 1P-binding site it interacts only via hydrogen bonds with the backbone amides of Gly381, Gly403, and Gly404, and with the side chain hydroxyl of Thr65 (Fig. 1; Taylor and Andersson, 1996; Duff et al., 2000). Although the interaction of phosphate with the backbone amides of glycine residues (Fig. 1) seems non-specific, Gly381, Gly403, and Gly404 are highly conserved, and substitutions of Gly381 and Gly403 with alanine were lethal. Evidently, even a change as minor as the change introduced to Gly404 (e.g. addition of methyl upon substitution with alanine) dramatically alters the kinetic properties of the enzyme (Table 1). Gly381 belongs to loop 7 between the $\beta$7 strand and the $\alpha$7 helix, and Gly403 is located in loop 8 between the $\beta$8 strand and the $\alpha$P helix. However, Gly404 resides at the edge of the $\alpha$P helix (the numbering of helices and strands follows Knight et al., 1990) and is slightly distant from RuBP, which may explain the lack of lethality of the corresponding mutant cells. Interestingly, this structure resembles a ‘P-loop’—a common motif that has been found in the binding sites of nucleotide phosphates. This element consists of a glycine-rich sequence that connects a $\beta$-sheet...
with an α-helix (Rossman et al., 1974; Kinoshita et al., 1999). Comparison of 400 distinct rbcL sequences (see the Materials and methods) reveals that two-thirds of the glycine residues in the large subunit of Rubisco appear at loops and most of them are conserved (data not shown). Thus, the substitution of glycine may affect the loop structure or dynamics during catalysis. Alternatively, the side chain of alanine might sterically hinder RuBP binding.

The limiting factors for photosynthesis in Synechocystis

Since the discovery of the Calvin cycle in the middle of the 20th century, extensive physiological, metabolic, and genetic studies have shown that Rubisco activity is the main limiting factor for photosynthesis under saturating irradiance and limiting Ci concentrations (Farquhar et al., 1980; Dietz and Heber, 1984; Stitt et al., 1991; Snir et al., 2006) and C4 (Furbank et al., 1996) plants, as well as in CO2-concentrating green algae (Bassham and Krause, 1969). In contrast, in the G404A Synechocystis PCC6803 mutant, the 90% decrease in Rubisco $K_{cat}$ hardly affected its photosynthesis and growth rates (Figs 3–6; Table 1). Similar to plants that respond to reduced Rubisco activity by elevating its activation status (Stitt et al. 1991), it was found that Synechocystis PCC6803 cells increased their Rubisco content by as much as 4.6-fold (Fig. 4). Despite this increase, the carboxylation capacity was not returned to the wild-type level. In light of the minor effect of substitution G404A on cell growth and especially on $P_{max}$ (Fig. 3), Rubisco activity does not seem to be the limiting factor for cyanobacterial photosynthesis under saturating irradiance and limiting Ci concentrations. In contrast to the G404A mutant, the rate of photosynthesis in the T65A/S mutants was modestly impeded under saturating Ci and irradiance (Fig. 3), suggesting that limitations such as RuBP regeneration, rather than Rubisco activity, were limiting photosynthesis.

To examine the extent of Rubisco limitation on the rate of photosynthesis, $P_{max}$ was correlated with the carboxylation capacity of the cells, and with $K_{cat}$ as well as with $K_{m}(RuBP)$ of Rubisco, and the concentration ratio of PGA to RuBP, the product and substrate of the carboxylation reaction, was determined. On the one hand, the correlation between $P_{max}$ and carboxylation capacity revealed that unlike in C3 and C4 higher plants (Fig 7; Stitt et al., 1991; Furbank et al., 1996), the rate of photosynthesis in Synechocystis PCC6803 is insensitive to large variations in Rubisco $K_{cat}$ and $K_{m}(RuBP)$ and in the carboxylation capacity of the cells (Figs 6, 7). Thus, the limitation imposed by Rubisco on the rate of photosynthesis is low.
These results are in accordance with the findings of Daniell et al. (1989), who showed that overexpression of Rubisco did not influence the growth rate of the cyanobacterium *Synechococcus*, but are in conflict with those of Iwaki et al. (2006), who claimed that expression of foreign type 1 Rubisco stimulated photosynthesis in *Synechococcus*. On the other hand, the PGA to RuBP concentration ratio (Fig. 8) was affected by the irradiance and Ci concentration, and by mutations in the enzyme that reduced the carboxylation capacity. These effects demonstrated that the carboxylation reaction of RuBP is reduced to GA3P using NADPH and ATP produced by the light reactions in two subsequent steps catalysed by phosphoglycerate kinase (PGK) and glyceraldehyde phosphate dehydrogenase (GAPDH; Equation 1). Triose-phosphate isomerase converts GA3P to DHAP. The concentrations of triose-phosphates (GA3P and DHAP) increased in *Synechocystis* with elevation in irradiance, but they differed from the concentrations at equilibrium (Fig. 8), whereas in higher plants these metabolites are in equilibrium (Bassham and Krause, 1969). This could result from accumulation of phosphoglycolate or Calvin cycle metabolites (e.g. RuBP or PGA) that inhibit the activity of triose-phosphate isomerase (Leegood, 1990).

The equilibrium equation of PGA reduction implies that the GA3P to PGA concentration ratio equals the product of the mass–action ratio for ATP production and for NADPH production multiplied by the equilibrium constant (Keq) as described in Equation 2 (Arnon et al., 1958; Heber et al., 1986).

\[
\frac{[\text{GA3P}]}{[\text{PGA}]} = \frac{\text{Keq} \times \frac{[\text{NADPH}]}{[\text{NADP}^+ \times [\text{H}^+]]}}{\text{ATP}/(\text{ADP} \times [\text{Pi}])}
\]

The GA3P to PGA concentration ratio increased with irradiance in *Synechocystis* cells up to 500 μmol photons m\(^{-2}\) s\(^{-1}\) (Fig. 8), whereas in higher plants it is constant or decreases (Heber et al., 1986). Assuming that the cytoplasmic pH is constant in the illuminated cells, photosynthesis in cyanobacteria, unlike in higher plants, is limited by ATP, NADPH, or by both under limiting irradiance. In contrast to higher plants, cyanobacteria concentrate CO\(_2\) at the carboxylation site by uptake of CO\(_2\) and HCO\(_3^-\) driven by ATP, ion gradients, and photosynthetic electron transport (Kaplan et al., 1987; Marcus et al., 1992; Badger et al., 2006). The excess energy required for Ci transport may decrease the pools of ATP and NADPH, and hence PGA reduction is limited. Conversely, competition between PGA reduction and Ci transport on limited resources of ATP and NADPH may limit the Ci uptake and, as a result, also the rate of photosynthesis. Indeed, it has been shown that high CO\(_2\)-grown *Anabaena variabilis* cells are limited by Ci uptake. However, elevation of the Ci uptake capacity in air-grown cells lifted this limitation (Kaplan et al., 1980). As the GA3P to PGA concentration ratio is stabilized at high irradiance (Fig. 8), it remains unclear whether under saturating irradiance ATP or NADPH limit PGA reduction or Ci uptake and consequently the rate of photosynthesis.
Conclusions

Mutagenic analysis of the phosphate-binding sites in *Synechocystis* Rubisco revealed that: (i) all three phosphate-binding sites play a role in Pi-induced stimulation of Rubisco activation, whereas only the 5P-binding site is involved in Pi inhibition of enzyme activity; (ii) under saturating irradiance and limiting Ci concentrations, Rubisco is not the main rate-limiting factor for cyanobacterial photosynthesis, as up to 90% reduction in its $K_{cat}$ or two-thirds of the carboxylation capacity of the cells hardly affected the carbon assimilation and growth rates; and (iii) PGA reduction limits the light-limited rate of photosynthesis due to ATP or NADPH limitation as revealed by the analysis of Calvin cycle metabolites.

Supplementary data

Supplementary data are available at *JXB* online.

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