Mutagenesis at Two Distinct Phosphate-Binding Sites Unravels Their Differential Roles in Regulation of Rubisco Activation and Catalysis

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Orthophosphate (P(i)) has two antagonistic effects on ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), stimulation of activation and inhibition of catalysis by competition with the substrate RuBP. The enzyme binds P(i) at three distinct sites, two within the catalytic site (where 1P and 5P of ribulose 1,5-bisphosphate [RuBP] bind), and the third at the latch site (a positively charged pocket involved in active-site closure during catalysis). We examined the role of the latch and 5P sites in regulation of Rubisco activation and catalysis by introducing specific mutations in the enzyme of the cyanobacterium Synechocystis sp. strain PCC 6803. Whereas mutations at both sites abolished the P(i)-stimulated Rubisco activation, substitution of residues at the 5P site, but not at the latch site, affected the Pi inhibition of Rubisco catalysis. Although some of these mutations substantially reduced the catalytic turnover of Rubisco and increased the K_m(RuBP), they had little to moderate effect on the rate of photosynthesis and no effect on photoautotrophic growth. These findings suggest that in cyanobacteria, Rubisco does not limit photosynthesis to the extent previously estimated. These results indicate that both the latch and 5P sites participate in regulation of Rubisco activation, whereas Pi binding only at the 5P site inhibits catalysis in a competitive manner.

Activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC4.1.1.39) is essential for catalysis and occurs by carbamylation of Lys-201 and binding of Mg^{2+} by the carbamion (14). Several effectors, mostly anions such as orthophosphate (P(i)), sugar-phosphates, sulfate, and NADPH, modulate the activation process via a mechanism that is still controversial (2, 3, 5, 11, 12, 16, 18, 20, 21, 26). It is accepted that these compounds evoke activation by elevating the carbamylation level, presumably by slowing the rate of enzyme deactivation (3, 12, 18). Nonetheless, evidence was brought that in addition to stimulation of Rubisco carbamylation, Pi also enhances the activation of the enzyme without a parallel increase in the carbamylation level (16, 21). Whereas McCurry et al. (18) reported monophasic, hyperbolic Pi concentration-dependent activation of Rubisco, nonmonotonous biphasic kinetics was obtained in other, more recent studies (2, 16), indicating that P(i) stimulates enzyme activation via a mechanism that involves multiple interacting sites (28).

Paradoxically, in addition to the stimulatory effect on activation, the effectors also inhibit the catalytic activity of the enzyme by competing with the substrate ribulose 1,5-bisphosphate (RuBP) (3, 16). Based on the observation of McCurry et al. (18) and the ability of the transition state analog carboxyarabinitol-bisphosphate to prevent 6-phosphogluconate from binding, Badger and Lorimer (3) suggested that inhibition of activity and stimulation of activation are both induced by effector binding to the catalytic site. Although this would imply that RuBP competitively inhibits the stimulation of Rubisco activation by P(i), we have previously found that saturating RuBP concentrations decreased only slightly in a noncompetitive manner the stimulatory effect on activation of the cyanobacterial enzyme (16). This unexpected difference raised the question of whether binding of P(i) to the catalytic site stimulates the activation of Rubisco.

X-ray analysis of a tobacco Rubisco in complex with P(i) revealed that the anion binds at three sites: two sites are those occupied by 1P and 5P of RuBP, and the third site is a positively charged pocket on the surface of the enzyme (Fig. 1) (7). As implied from the X-ray data, the last site is involved in structural transitions of the enzyme during catalysis. Binding of RuBP leads to closure of the catalytic site by movement of loop 6 across the substrate. The C-tail, which is randomly oriented in the open state, pushes against loop 6 and binds tightly via strong ionic interactions with a positively charged site on the surface of the enzyme. This charged site is formed by Arg-134 and His-310, which are surrounded by Arg-41, Lys-305, and Arg-312 (Fig. 1) (7, 27). For that reason, this site was named ‘the latch’ (Fig. 1). As P(i) competes with Asp-473 of the C-tail on binding to this site, it may prevent enzyme closure and hence, inhibit catalysis (Fig. 1B and C) (7).

In this study we used a mutant of the cyanobacterium Synechocystis sp. strain PCC 6803, Syn6803Δarcb, which enables site-directed mutagenesis of Rubisco and analysis of the effects in vitro on the isolated enzyme as well as in vivo on the photosynthetic performance and growth of the cells (1). We introduced mutations at two Pi binding sites (5P and the latch) in order to examine their role in stimulation of activation and inhibition of Rubisco activity.

MATERIALS AND METHODS

Growth conditions and photosynthesis assays. Synechocystis sp. strain PCC 6803 wild type and mutants were grown on BG-11 medium as was described (17). Photosynthesis rate was determined using a Clark-type O2 electrode (Rank Brothers, United Kingdom) according to Marcus et al. (16). Rubisco was partially purified by two steps of ammonium sulfate fractionation followed by dial-
FIG. 1. A, Diagram of the three P_i-binding sites on Rubisco. The three-dimensional structure of a large and small subunit protomer of a nonactivated tobacco enzyme in complex with P_i (PDB code 1EJ7) (7). The three P_i ions are in red, residues directly associated with P_i are in light green, and residues that contribute indirectly to P_i binding are in dark green; activator Lys is in magenta. Moving elements (C-tail and loop 6) that participate in closure of the catalytic site are in blue. Thr-65 of the paired large subunit, which also participates in P_i binding, is not labeled. B, Space-filled model of P_i bound to the latch site of tobacco Rubisco, a top view. Basic residues, blue; acidic residues, red; neutral residues, green; P_i, yellow; water molecules, white. C, Space-filled model of the latch site in Synechococcus Rubisco in the closed state (PDB code 1RBL). The last four residues of the C-tail (Met-472, Asp-473, Lys-474, and Leu-475) are shown as balls and sticks. Note that Asp-473 occupies the region where P_i binds. Colors are as in B. The three models were drawn using RasMol.
properties of both types of enzyme preparations were identical. Examination of enzyme integrity using native and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by immunochromatographic analysis (for details, see reference 17) did not reveal any dissociation or degradation products (Fig. 2). Since the yield of purified enzyme decreased substantially with further purification steps, all experiments were performed using the ammonium sulfate preparation.

The molar concentration of Rubisco catalytic sites was determined by binding of [14C]CPBP (carboxy-pentitol-bisphosphate) as described in detail (16). Briefly, the enzyme was incubated for 10 min with 15 μM [14C]CPBP (66.6 × 10^7 Bq μmol⁻¹) at 30°C. [14C]CPBP-bound Rubisco was separated from the free ligand by gel filtration using a 10-cm Sephadex G-100 column. The reliability of measuring catalytic site concentration by CPBP in mutants substituted at the Rubisco binding site (H298A and H327Q) was validated by comparing the ratio of CPBP data of the wild type and the mutants to the ratio obtained following denaturation of enzyme bands separated on a native polyacrylamide gel electrophoresis (Fig. 2A). The similar ratio obtained, irrespective of the mutation, indicated that determination of the catalytic site number by CPBP binding was reliable. Determination of carbamylation level, enzyme activation, and assays have been described previously (16).

**Mutagenesis and genetic analysis.** Plasmid pSynR4.0, a pBlueScript derivative containing the entire rbc operon and flanking sequences from Synechocystis sp. strain PCC 6803 (1), was used for mutagenesis. Mutations were introduced by PCR using three oligonucleotide primers (Table 1). 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 (1) was the DNA template, and Pwo DNA polymerase (Roche) was used for amplification. The resulting DNA fragment was used in a second PCR step with a complementary primer encompassing a restriction site. For mutagenesis of Arg-134 we used a 488-bp XbaI-NsiI fragment of Synechocystis PCC 6803 (1). All other mutations were carried out using an 516-bp NsiI -NruI fragment (Table 1). The final PCR product was cleaved by the appropriate enzymes and inserted back into the pSynR4.0 vector. The final construct was used to transform the Syn6803/H9262 recipient (1). Transformed cells were inoculated onto solid BG-11 medium at ambient CO₂ levels. Air-grown colonies appeared within 14 to 21 days. The mutations were verified in the cyanobacterial genome by DNA sequencing.

**RESULTS AND DISCUSSION**

The role of amino acid residues in the phosphate binding sites of Rubisco of the cyanobacterium Synechocystis sp. strain PCC 6803 was examined using site-directed mutagenesis. Either P₅ or 5P of RuBP interacts with the 5P binding site via hydrogen bonds with Arg-295 and His-298 in the open state of the enzyme, whereas 5P of RuBP interacts with Arg-295 and His-327 in its closed state (6, 7). The highly conserved His-298 and His-327 were substituted by Ala and Gln, respectively (mutants H298A and H327Q, respectively). Arg-134 and His-310 of the latch site, which directly bind P₅ and Asp-473 as well as Lys-305 at the other side of this domain (Fig. 1) (7), were

**TABLE 1. Oligonucleotide primers used for mutagenesis**

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Mutation or cloning site</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutagenesis</td>
<td>R134A</td>
<td>GAAAGAGAAATATCTTCTAAAGCGAGGGCCCGCAGAGGCC</td>
</tr>
<tr>
<td></td>
<td>R134E</td>
<td>GAAAGAGAAATATCTTCTAAATTGAGGGCCCGCAGAGGCC</td>
</tr>
<tr>
<td></td>
<td>H298A</td>
<td>CGGTCACACTACGCCGCCCATTTGCCCATG</td>
</tr>
<tr>
<td></td>
<td>K305A</td>
<td>GTTGACCACGTCAGAAACACCGGATC</td>
</tr>
<tr>
<td></td>
<td>K305E</td>
<td>GTTGACCACGTCAGAAACACCGGG</td>
</tr>
<tr>
<td></td>
<td>H310A</td>
<td>CCAACGGGATCCTTCCGGTTTGG</td>
</tr>
<tr>
<td></td>
<td>H310D</td>
<td>CCAACGGGATCCTTCCGGTGG</td>
</tr>
<tr>
<td></td>
<td>H327Q</td>
<td>GGTGACCACCTTCCGGTACCGGT</td>
</tr>
<tr>
<td>Subcloning</td>
<td>Xbal upstream</td>
<td>CTGTTCACCAAGAAAGATCCC</td>
</tr>
<tr>
<td></td>
<td>NsiI upstream</td>
<td>CACCCCATCATCATGGATAC</td>
</tr>
<tr>
<td></td>
<td>NsiI downstream</td>
<td>CCGGGGTTAGAGAAGTC</td>
</tr>
<tr>
<td></td>
<td>NruI downstream</td>
<td>CCAGTAAAGCGTATACCTTGCG</td>
</tr>
</tbody>
</table>

a Substituted nucleotides are in bold, and altered codons are underlined.
substituted by neutral or negatively charged residues (mutations R134A/E, K305A/E, and H310A/D). In addition, a double mutant, K305A/H310A (latch site) as well as mutations R134A/E, K305A/E, and H310A/D. In addition, a double mutant, K305A/H310A, was constructed. All these mutants, including H327Q (at the catalytic site), grew photoautotrophically at ambient CO2 levels. PCR analysis and the inability of the mutants to grow on chloramphenicol indicated that the mutations segregated fully among the identical Synechocystis chromosomes (data not shown).

Identification of the Pi binding site involved in inhibition of Rubisco activity. To identify the Pi binding site that competitively inhibits the catalytic activity of Rubisco, we determined carboxylase activity at various Pi and RuBP concentrations of enzyme mutants R134A and K305E (latch site) as well as H327Q and H298A (5P site). Dixon analysis (25) of the data revealed that RuBP carboxylation by all four mutant enzymes was competitively inhibited by Pi. The inhibition constant of Pi, \( K_{i}^{(\text{Pi})} \), was not affected by any of the mutations at the latch site or by H298A at the 5P site (Table 2), although mutations K305E and H298A hindered the interaction with Pi, as was evident from the inability of Pi to stimulate enzyme activation (Fig. 3A and B). In contrast, mutation H327Q reduced the \( K_{i}^{(\text{Pi})} \) 10-fold (Table 2). This suggests that inhibition of the catalytic activity of Rubisco occurs when Pi and RuBP compete for binding to Arg-295 and His-327 of the 5P subsite during catalysis but not to the Arg-295 and His-298 5P subsites at the open state.

An alternative interpretation of the competitive inhibition of Rubisco activity by Pi, with respect to RuBP, was raised by Duff et al. (7). They have suggested that competition between Pi and the C-terminal Asp-473 on binding to the latch site inhibits the closure of the catalytic site and consequently the catalytic activity of the enzyme. If this hypothesis is correct, mutagenesis of residues in the latch site that differentially affect the binding of Pi and Asp-473 should influence the inhibitory effect of Pi. However, the inhibitory effect of Pi remained competitive with respect to RuBP (data not shown) and the \( K_{i}^{(\text{Pi})} \) was not changed by mutations R134A and K305E (Table 2). This implies that competition between Pi and the C-terminal Asp-473 on binding to the latch site does not prevent the closure of the catalytic site. It is noteworthy that although Pi may also compete with RuBP on binding to the 1P site, probing of this site by mutagenesis seems more difficult and has not been reported thus far.

Analysis of the Pi-binding sites involved in stimulation of Rubisco activation. All Rubisco mutants modified at the Pi-binding sites (Fig. 1) were activated in vitro by CO2 and Mg2++. Moreover, their activation in vivo was evident from the ability of the mutant cells to grow photoautotrophically. Interestingly, the activation of R134A mutant enzyme, determined by carboxylase activity after activation with 285 \( \mu \text{M} \) CO2 and 20 \( \text{mM} \) MgCl2, was apparently higher than that of the wild type and the other mutants.

**Table 2. Kinetic properties of Rubisco mutants**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Site</th>
<th>( K_{m}^{(\text{RuBP})} ) (( \mu \text{M} ))</th>
<th>( K_{m}^{(\text{CO2})} ) (( \mu \text{M} ))</th>
<th>( K_{i}^{(\text{Pi})} ) (( \text{mM} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Latch</td>
<td>545</td>
<td>140</td>
<td>181</td>
</tr>
<tr>
<td>R134A</td>
<td>Latch</td>
<td>178</td>
<td>212</td>
<td>161</td>
</tr>
<tr>
<td>K305E</td>
<td>Latch</td>
<td>185</td>
<td>242</td>
<td>190</td>
</tr>
<tr>
<td>K305A</td>
<td>Latch</td>
<td>177</td>
<td>199</td>
<td>185</td>
</tr>
<tr>
<td>H310D</td>
<td>Latch</td>
<td>185</td>
<td>387</td>
<td>NM</td>
</tr>
<tr>
<td>H310A</td>
<td>Latch</td>
<td>540</td>
<td>179</td>
<td>NM</td>
</tr>
<tr>
<td>H298A</td>
<td>5P</td>
<td>43</td>
<td>267</td>
<td>NM</td>
</tr>
<tr>
<td>H327Q</td>
<td>5P</td>
<td>52</td>
<td>490</td>
<td>175</td>
</tr>
</tbody>
</table>

\( ^{a} \text{Enzyme was activated for 30 min by 280} \mu \text{M CO2 and 10 mM MgCl}_2\text{ at 30°C. Aliquots of 10}\mu\text{L (1.25 to 1.5} \mu \text{M activated Rubisco) were added to 290-}\mu\text{l reaction mixtures containing either various concentrations of }^{14}\text{C CO}_2\text{ and 0.5 mM RuBP or various concentrations of RuBP and Pi and 660} \mu \text{M CO}_2\text{.} \)

\( ^{b} \text{Apparent } K_{i}^{(\text{CO2})} \text{ was determined at 21% } \text{O}_2. \text{NM, not measured.} \)

\( ^{c} \text{Activation was defined as the ratio between the activity of the enzyme} \)

\( ^{d} \text{with RuBP on binding to the 1P site, probing of this site by} \text{mutagenesis seems more difficult and has not been reported thus far.} \)

**FIG. 3. Effect of Pi concentration on the kinetics of Rubisco activation (A and B).** Rubisco from *Synechocystis* sp. strain PCC 6803 (wild type) and mutants R134A, H298A and H327Q (A) and K305E, H310A/D, and K305A/H310A (B) was activated for 30 min at 30°C with 1 mM dithiothreitol, 70 \( \mu \text{M} \text{ CO}_2\text{, 10 mM MgCl}_2\text{, and various concentrations of potassium phosphate (phosphate was added 5 min after the other substances). Aliquots of 12} \mu \text{L} \text{ activated enzyme were added to 288} \mu \text{l reaction mixture containing 0.5 mM RuBP, 70} \mu \text{M}^{14}\text{C CO}_2\text{ (11.3 Bq nmol}^{-1}\text{), 10 mM MgCl}_2\text{, 1 mM dithiothreitol, and 7.5 Wilbur-Anderson units carbonic anhydrase in 50 mM HEPES buffer, pH 8.0. The reaction was terminated after 2 min with 200} \mu \text{l} \text{ 6 N acetic acid.} \)

Activation was defined as the ratio between the activity of the enzyme activated at the indicated Pi concentration and that in the absence of Pi during activation.
Since the wild-type and R134A enzymes were fully carbamylated (data not shown), the apparent higher activation ability of the R134A enzyme could be the result of a lower activation level at ambient CO2 concentrations, presumably due to a higher deactivation rate. This assumption was examined by comparing the kinetics of deactivation of the wild-type and R134A enzymes. Following activation (in the presence of 285 μM CO2 and 20 mM MgCl2), enzyme deactivation was monitored by carboxylase activity following 10-fold dilution with HEPES buffer, pH 8.0, with no change in the concentration of MgCl2 and CO2 or in the absence of these substances (final concentrations were 28 μM CO2 and 1 mM MgCl2) and incubated at 18°C and at the indicated time. Rubisco activity was determined as described for Fig. 2.

The effect of the various mutations at the P1-binding sites on stimulation of enzyme activation by phosphate was examined. The kinetics of P1-stimulated activation in the presence of suboptimal CO2 and Mg2+ concentrations is biphasic, i.e., two distinct concentration-dependent phases separated from each other by an intermediary plateau (Fig. 3A and B), a phenomenon previously reported by Anwaruzzaman et al. (2) and Marcus and Gurevitz (16). Under these conditions P1 has two effects on Rubisco activation: it raises the carbamylation level, but also elevates the activity of the carbamylated enzyme (16, 21). Whereas neutralization of a single positive charge at the latch site (substitution K305A or H310A) had no effect, the double mutation K305A/H310A had a clear inhibitory effect, especially at low P1 concentrations (Fig. 3B).

The lack of effect upon single-charge elimination could be due to the fact that the latch site is composed of five positively charged residues (see Fig. 1). The effect of the double mutation could be due to perturbation and, hence, reduced affinity of P1 for the latch site. Substitution of Arg-134, which is also involved in P1 binding (mutant R134A; Fig. 1), apparently elevated the activation level (Fig. 3A). We found that this unprecedented effect resulted from a higher decarbamylation rate at suboptimal CO2 concentrations (Fig. 4), leading to an extremely low level of activation at the onset of the experiment. Charge inversion of Lys-305 and His-310 (mutants K305E and H310D, respectively) abolished the P1 effect on activation (Fig. 3A and B), probably due to P1 exclusion from this site. These results suggest that although these residues bind P1, they are not involved in its stimulatory effect.

Inhibition of P1-stimulated Rubisco activation was obtained upon H327Q and H298A substitutions at the two subsites of the 5P domain made of Arg-295 with His-298 or Arg-295 with His-327, in the open and closed states of the catalytic site, respectively (Fig. 3A). This may be explained in a number of ways. Interaction of P1 with one of these subsites initiates the stimulatory effect, yet substitution at the other subsite hinders this effect. Assuming that Arg-295, which is shared by both subsites, induces the stimulation of Rubisco activation upon P1 binding, substitution of either His-298 or His-327, involved in these subsites, hinders this interaction. One of these subsites is the receptor site for P1, whereas the other subsite transduces the P1 effect to the target site. As the stimulation of activation occurs in general at the open state of the enzyme, it is likely that P1 associates with His-298, while the effect on the target site, which is most likely Lys-201, is mediated by the adjacent His-327 (Fig. 1). Despite the crystallographic data suggesting that P1 interacts with His-298 (7), perhaps P1 also interacts with His-327 in the open state of the catalytic site, which stimulates the activation.

All the above results indicate that at least two P1 ions are involved in P1 stimulation of activation by interaction with the 5P site and with the latch site.

**Photosynthetic performance of the mutants.** Substitution of Arg-134, Lys-305, and His-310 at the latch site had only a marginal effect on the rate of photosynthesis of the mutant cells. The substitutions that had the most effect on the rate of CO2 fixation (20% reduction) at saturating light intensity and CO2 concentration were R134A (Fig. 5) and R134E (not shown). Yet, at limiting light intensity (Fig. 5) or CO2 concentration, the rate of photosynthesis was unaffected in either of these mutants (data not shown). Unlike the mutations at the latch site, substitutions H298A and H327Q at the RuBP binding site reduced the maximal rate of photosynthesis by 40% and 70%, respectively (Fig. 5).

Despite the minor effect on the rate of photosynthesis, in most instances the mutations at the latch site reduced up to two-thirds of the catalytic turnover of the enzyme and increased the R5(Ka) (Table 2). On the other hand, these mutations did not significantly alter the affinity of Rubisco for CO2 (Table 2). Whereas similar findings were obtained upon truncation of the last eight residues of the large subunit of Rubisco from *Synechococcus* sp. (9), substitution of the C-tail Asp-473 in *Chlamydomonas* Rubisco reduced the affinity of the enzyme for CO2 (24). These results suggest that the mutations at the latch site or at the C-terminal region affect the K_{cat} and
was only 8% that of the wild type (Table 2) and the $P_i$-stimulated activation was abolished as well (Fig. 3A), a threefold elevation in Rubisco content was observed (Fig. 6). Consequently, Rubisco activity per cell in mutants H327Q, H298A, and R134A was similar (Fig. 6), mutation H327Q prominently decreased 10-fold (Table 2), and stimulation of enzyme activity 60% with a minor effect on the carbon assimilation rate (17).

Even though cyanobacteria concentrate CO$_2$ at the carboxylation site (13, 15), thereby compensating for the low affinity of Rubisco for CO$_2$ (Table 2), the carboxylation rate cannot rise above that dictated by all other kinetic properties of the enzyme. Therefore, the CO$_2$ concentrating mechanism cannot compensate for a substantial loss in the catalytic turnover and $K_m$(RuBP) of a mutated enzyme. For that reason, the lack of effect of the mutant enzymes on the CO$_2$-limited rate of photosynthesis implies that, unlike in higher plants (8, 22, 23), the constraint imposed by the cyanobacterial Rubisco on the photosynthetic flux is low, presumably due to its higher catalytic turnover (4) and the ability to regulate its concentration in the cell (Fig. 6). Since the most pronounced effect on the photosynthetic rate was observed at saturating CO$_2$ and light intensities, we suggest that RuBP recycling is the bottleneck of photosynthesis in cyanobacteria (Fig. 5) (29).

Although Rubisco activity per cell in mutants H327Q, H298A, and R134A was similar (Fig. 6), mutation H327Q prominently decreased 84%, the $K_m$(RuBP) increased 3.5-fold, stimulation of enzyme activity 60% with a minor effect on the carbon assimilation rate (17).

Moreover, we were able to evaluate how these changes affect the photosynthetic and growth abilities of the mutant cells. We found that the cyanobacterial mutants K305E, H310D, H327Q, and H298A partially compensated for the impaired kinetic properties of Rubisco by increasing its cellular content. For example, in mutant H327Q, in which the $K_m$ of the enzyme declined 84%, the $K_m$(RuBP) increased 3.5-fold, $K_m$(Pi) decreased 10-fold (Table 2), and stimulation of enzyme activation by $P_i$ was abolished (Fig. 3), and the cellular Rubisco content doubled compared to that in the wild type (Fig. 6). Similarly, in mutant H298A, in which the $K_m$ of the enzyme was only 8% of that of the wild type, and the $K_m$(RuBP) increased 90% (Table 2). This suggests that His-298 in the cyanobacterial enzyme is important for RuBP binding and hence for catalysis, which is in accordance with the findings of Duff et al. (7).

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$K_m$(RuBP) of Rubisco by hindering the mechanism of the catalytic site closure.

It is noteworthy that His-291 and His-321 of the dimeric *Rhodospirillum rubrum* enzyme, which are the equivalents of His-298 and His-327, respectively, in the hexadecameric enzyme, were mutagenized and the enzymes were expressed in *Escherichia coli* (10, 19). Whereas substitution of His-291 in the *R. rubrum* enzyme had a moderate effect and therefore this residue was considered nonessential (19), the $K_m$(RuBP) of the cyanobacterial mutant enzyme, H298A, was only 8% of that of the wild type, and the $K_m$(RuBP) increased 90% (Table 2). This suggests that His-298 in the cyanobacterial enzyme is important for RuBP binding and hence for catalysis, which is in accordance with the findings of Duff et al. (7).

$K_m$(RuBP) of Rubisco by hindering the mechanism of the catalytic site closure.

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inhibited the rate of photosynthesis, whereas R134A had little effect and H298A had a moderate effect (Fig. 5). This difference could result from the elevated P_i inhibitory effect of mutation H327Q (Table 2). The lack of effect of mutations K305E and H310D on the photosynthesis rate (Fig. 5), despite the elimination of P_i-stimulated activation (Fig. 3A and B), was due to a high activation level of Rubisco, most likely achieved by the CO_2 concentrating mechanism (13, 15).

Conclusions. Our mutational analysis at the P_i binding sites of Rubisco indicates that binding of P_i to Arg-295 and His-327 during catalysis competitively inhibits carboxylation. P_i binding to the latch site or to His-298 of the 5P site had no such effect (2). P_i stimulates Rubisco activation via at least two distinct sites, the 5P site, which involves His-298, His-327, and presumably Arg-295, and the latch site, in which the residues that bind P_i differ from those mediating the P_i stimulatory effect (3). Mutations R134A and K305E at the latch site decrease catalytic turnover and increase the K_m (RuBP) of Rubisco, most likely due to obstruction of the catalytic-site closure (4). Arg-134 at the latch site is distant from the activation site and yet has a stabilizing effect on the carbamate, as indicated by the accelerated rate of Rubisco deactivation upon its substitution to Ala (5). Although the limitation imposed by Rubisco on the photosynthetic flux is lower than that observed in higher plants, Synechocystis cells partially compensate for the inferior kinetic properties of Rubisco mutants by elevating the enzyme content per cell.

This study not only provides further molecular details on P_i effectors by active and inactive forms of ribulose 1,5-bisphosphate carboxylase. J. Biol. Chem. 22:3410–3418.


