Activase Region on Chloroplast Ribulose-1,5-bisphosphate Carboxylase/Oxygenase

NONCONSERVATIVE SUBSTITUTION IN THE LARGE SUBUNIT ALTERS SPECIES SPECIFICITY OF PROTEIN INTERACTION*

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In the active form of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), a carbamate at lysine 201 binds Mg$^{2+}$, which then interacts with the carboxylation transition state. Rubisco activase facilitates this spontaneous carbamylation/metal-binding process by removing phosphorylated inhibitors from the Rubisco active site. Activase from Solanaceae plants (e.g. tobacco) fails to activate Rubisco from non-Solanaceae plants (e.g. spinach and Chlamydomonas reinhardtii), and non-Solanaceae activase fails to activate Solanaceae Rubisco. Directed mutagenesis and chloroplast transformation previously showed that a proline 89 to arginine substitution on the surface of the large subunit of Chlamydomonas Rubisco switched its specificity from non-Solanaceae to Solanaceae activase specificity. To define the size and function of this putative activase binding region, substitutions were created at positions flanking residue 89. As in the past, these substitutions changed the identities of Chlamydomonas residues to those of tobacco. Whereas an aspartate 86 to arginine substitution had little effect, aspartate 94 to lysine Rubisco was only partially activated by spinach activase but now fully activated by tobacco activase. In an attempt to eliminate the activase/Rubisco interaction, proline 89 was changed to alanine, which is not present in either non-Solanaceae or Solanaceae Rubisco. This substitution also caused reversal of activase specificity, indicating that amino acid identity alone does not determine the specificity of the interaction.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (Rubisco) catalyzes carboxylation of RuBP in the first step of the Calvin cycle of photosynthesis (reviewed in Refs. 1 and 2). To be active, Lys-201 on the Rubisco large subunit must be carbamylated and coordinated with Mg$^{2+}$ prior to binding RuBP (reviewed in Refs. 2 and 3). Thus, Rubisco is similar to urease, which also uses a carbamate as a ligand for metal binding (5). However, whereas urease requires a set of proteins for the addition of Ni$^{2+}$ to its active site (6), Rubisco appears to require only one. This protein, Rubisco activase, removes inhibitory sugar phosphates, including RuBP, from the nonactivated active site, thereby facilitating subsequent, spontaneous carbamylation and Mg$^{2+}$ binding (reviewed in Refs. 3 and 4). In some cases, fully activated (metal-bound) Rubisco may bind inhibitory sugar phosphates that mimic the carboxylation transition state (7, 8). These molecules are also removed by Rubisco activase to restore a functional active site (8, 9). Like one of the urease activation proteins, UreG (5), Rubisco activase hydrolyzes nucleoside triphosphate during the activation process (10, 11). However, except for a P-loop motif, there is little homology between these two proteins.

Rubisco activase from plants in the family Solanaceae (e.g. tobacco) fails to activate Rubisco from plants outside the family (e.g. spinach and the green alga Chlamydomonas reinhardtii), and activase from non-Solanaceae plants fails to activate Solanaceae Rubisco (12). Comparison of sequences and x-ray crystal structures revealed that there are seven residues clustered on the surface of the Rubisco large subunit that differ in charge between the Solanaceae and non-Solanaceae enzymes (residues 86, 89, 94, 95, 356, 466, and 468) (3, 13). Because the large subunit is coded by a chloroplast gene (rbcL) in plants and green algae, and because chloroplast transformation has been difficult to achieve in land plants with respect to this gene (14, 15), directed mutagenesis and chloroplast transformation were used to investigate the significance of this region in Chlamydomonas Rubisco (13, 16). Two of the most conserved residues of non-Solanaceae Rubisco were switched to those characteristic of Solanaceae Rubisco. A K356Q substitution had no detectable effect on Rubisco function or activation properties. However, a P89R mutant enzyme could no longer be activated by non-Solanaceae Rubisco activase but, instead, could be activated by Solanaceae activase (13). Thus, it is likely that this surface region plays a role in the interaction between Rubisco and Rubisco activase.

Because of its important role in plant productivity, a deeper understanding of the interaction between Rubisco and its activation protein may present new ideas for engineering an in-

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† The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; Tricine, N-[2-hydroxy-1,1-bis[(hydroxymethyl)ethyl]glycine; bis-Tris, 1,3-bis[(tris(hydroxymethyl)methylamino)propane.

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crease in enzyme activity (reviewed in Refs. 3 and 4). Furthermore, species specificity between Rubisco and Rubisco activase may prove to be a stumbling block for the transfer of a catalytically improved enzyme from one species into the chloroplast of a different, agronomically important species (reviewed in Ref. 2). In the present study, additional substitutions were made to define the nature of the large-subunit region that interacts with activase. One substitution changes the specificity of the interaction by introducing a residue at position 89 that differs from either of the residues characteristic of Solanaceae and non-Solanaceae Rubisco.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—C. reinhardtii 2137 mt⁻ is the wild-type strain (17). Mutant 18-7G mt⁻ was used as the host for transformation (18). It lacks photosynthesis and requires acetate for growth because of an rbcL nonsense mutation that terminates large-subunit translation after residue 65 (18, 19). Photosynthesis-competent mutant P89R was created by directed mutagenesis and chloroplast transformation in a previous study (13). All strains are maintained in darkness at 25 °C with 10 mM acetate medium containing 1.5% Bacto agar (17). For biochemical analysis, cells were grown in 50–250 ml of liquid acetate medium on a rotary shaker in darkness.

**Directed Mutagenesis and Chloroplast Transformation**—A 2670-base pair HpaI DNA fragment (bases 742 to 1928), containing the entire rbcL gene (bases 1–1428) (20), was cloned into HpaI-digested pUC19 (21) and propagated in Escherichia coli XL1-Blue (Stratagene). Site-directed mutagenesis was performed with a kit from Amersham Pharmacia Biotech (22). To produce the D96R substitution, the rbcL gene sequence GAT (bases 256–258) was changed to CGT, which eliminated an RV restriction site. To create the P89A substitution, CCA (bases 256–258) was changed to GCT, which eliminated a BsrI restriction site. The mutations were confirmed by restriction enzyme digestion and DNA sequencing. The resulting rbcL mutant plasmids, named pLS-D86R, pLS-P89A, and pLS-D94K, were transformed into the chloroplast by microprojectile bombardment (23, 24), and photosynthesis-competent colonies were recovered in all cases. Following previous methods (13, 24, 25), rbcL genes were completely sequenced to ensure that only the expected mutations were present. The rbcL mutant strains created by directed mutagenesis and chloroplast transformation were named D86R, P89A, and D94K.

**Sucrose Gradients, Electrophoresis, and Immunoblotting**—Total soluble cell proteins were extracted from dark-grown cells by sonication (26) and quantified (27). Cell extract was fractionated on sucrose gradients (26) or subjected to SDS-polyacrylamide gel electrophoresis with a 7.5–15% polyacrylamide gradient in the running gel (28). Proteins were transferred from the gel to nitrocellulose, probed with rabbit anti-tobacco Rubisco immunoglobulin G (0.5 μg/ml), and detected via enhanced chemiluminescence (Amersham Pharmacia Biotech) as described previously (13).

**Large Scale Preparation of Rubisco**—Rubisco was purified from wild-type and mutant cells in a carboxylation buffer (50 mM Tricine, pH 8.0, 10 mM MgCl₂, 10 mM NaHCO₃, 10 mM dithiothreitol, 1 mM EDTA) as described previously (13). The non-carbamylated, RuBP-inhibited enzyme was prepared in 50 mM Tricine, pH 8.0, 2 mM RuBP, and 0.1 mM EDTA (13). The amount of protein was determined by assuming an extinction coefficient of 1.64 absorbance units for 1 mg/ml at 280 nm. Enzyme was stored in liquid N₂.

Rubisco Activase Purification and Assay—Leaves from spinach and tobacco plants were powdered in liquid N₂, and Rubisco activase was purified as described previously (12). The purified enzyme was stored in bis-Tris, pH 7.0, and 0.2 mM ATP under liquid N₂. Rubisco activase activity was measured by following the increase in carboxylase activity of the RuBP-inhibited Rubisco enzyme with time in spectrophotometric assays that couple the production of phosphoglycerate to the oxidation of NADH (13, 29).

**RESULTS**

**Reciprocal and Phenotypes of the D86R and D94K Mutants**—It was previously shown that a P89R substitution in the Chlamydomonas large subunit reversed the specificities of the interactions with non-Solanaceae (spinach) and Solanaceae (tobacco) Rubisco activases (13). Of those residues that differ between non-Solanaceae and Solanaceae Rubisco in the region surrounding large-subunit residue 89 (13), residues 86 and 94 flank residue 89 and may cause substantial differences in conformation (Fig. 1). His-86 interacts with Glu-88 in spinach (30), but Arg-86 interacts with Glu-88 in tobacco (31). Glu-94 interacts with Asn-95 in spinach, but Lys-94 interacts with Glu-93 in tobacco (Fig. 1). Like many of the other non-Solanaceae enzymes, Chlamydomonas Rubisco contains Asp at positions 86 and 94. Therefore, to gain further insight into the interaction between Rubisco and Rubisco activase, we decided to create D86R and D94K large-subunit substitutions that would change the identities of the Chlamydomonas residues to those of tobacco. In addition, because most Rubisco enzymes contain either Pro (non-Solanaceae) or Arg (Solanaceae) at residue 89, we reasoned that it might be possible to eliminate the interaction between Rubisco and Rubisco activase (without substan-
typically altering Rubisco structural stability) by creating a P89A substitution.

D86R, D94K, and P89A mutant strains were recovered by transforming the chloroplast of the 18-7G rbcL nonsense mutant with directed mutant rbcL genes and selecting for photosynthetic competence on minimal medium in the light. Because transformant colonies were recovered at frequencies comparable with the transformation frequency with wild-type rbcL (13, 23–25), it was apparent that none of the amino acid substitutions substantially affected Rubisco holoenzyme structure, activation, or function in vivo. When compared with wild type in spot tests (17), only the D94K mutant exhibited slightly reduced growth on minimal medium in the light (80 photons/m²/s). Growth at 18 or 35 °C or under 15-min light (80 photons/m²/s) failed to exacerbate the phenotype of D94K or reduce the growth of the D86R, P89A, or P89R mutant strains relative to that of wild type.

Sucrose-gradient fractionation and Western analysis (Fig. 2) revealed that the D86R, D94K, and P89A mutants contain about half as much Rubisco as wild-type and mutant P89R, presumably because of increased holoenzyme instability/protellysis in vivo (24, 25). However, when fully carbamylated, all of the purified mutant enzymes had near normal RuBP carboxylase specific activities. As found previously for P89R (13), the large subunits of the D86R and D94K enzymes displayed increased mobility during SDS-polyacrylamide gel electrophoresis (Fig. 2). Because this is not the case for the P89A large subunit (Fig. 2, lane 5), it is likely that the introduction of positive charge, rather than an alteration in conformation (32), is responsible for the altered migration.

**Rubisco Activase Assays—Non-carbamylated, RuBP-inhibited Rubisco was prepared from the mutant strains. The rates at which these enzymes became carbamylated were then determined in the presence of either spinach or tobacco Rubisco activase (Fig. 3). Mutant D86R Rubisco was indistinguishable from the wild-type enzyme with regard to activation properties. Both enzymes were activated by spinach activase, but activation by tobacco activase was not much greater than the spontaneous rate in the absence of activase (Fig. 3, compare panels A and B). In contrast, mutant D94K Rubisco was activated only slightly above the spontaneous rate by spinach activase, but at a near normal rate by tobacco activase (Fig. 3C). This reversal of specificity is similar to that observed for P89R Rubisco in a previous study (13). However, when mutant P89A Rubisco was analyzed, it was also found to be activated by tobacco activase but not at all by spinach activase (Fig. 3D). This was an unexpected result because tobacco Rubisco contains Arg at this position.

**DISCUSSION**

A large-subunit P89R substitution, shown previously (13), or a D94K substitution, in the present study (Fig. 3), alters the specificity of the interaction between Chlamydomonas Rubisco and land-plant Rubisco activase. Whereas the wild-type Chlamydomonas enzyme can be activated by spinach (non-Solanaceae) activase but not tobacco (Solanaceae) activase, both of the mutant enzymes can be activated by tobacco activase but not spinach activase (Ref. 13; Fig. 3). These results would seem to indicate that either of at least two residues (Arg-89 or Lys-94) on the surface of the Rubisco large subunit is sufficient for recognition by tobacco activase, but both of at least two residues (Pro-89 and Asp-94) are necessary for recognition by spinach activase. However, a large-subunit P89A substitution also blocks Rubisco activation by spinach activase and permits activation by tobacco activase (Fig. 3). Perhaps it is the absence of Pro rather than the presence of Arg at residue 89 that is required for activation by tobacco activase.

Inspection of the spinach and tobacco Rubisco crystal structures (30, 31) (Fig. 1) reveals that Lys-94 in tobacco forms an ionic bond with Glu-93, but the side chain of Glu-94 in spinach is either exposed to solvent or may form a hydrogen bond with Asn-85. By replacing Asp-94 in Chlamydomonas Rubisco with Lys, an ionic interaction may be formed with the otherwise solvent-exposed Glu-93, mimicking the structure of tobacco Rubisco. Arg-89 in tobacco might be able to form an ionic interaction with Asp-95 in vivo, preventing an ionic interaction between Asp-95 and Lys-94. Replacing Pro-89 in Chlamydomonas Rubisco with Arg would not mimic this potential interaction because Chlamydomonas contains Asn-85 and Asp-94 (Fig. 1). Instead, by introducing a solvent-exposed Arg or small, aliphatic Ala, the conformational constraints imposed by Pro-89 would be eliminated. Thus, we propose that either of at least two residues (Pro-89 and Asp-94) are responsible for the specificity of activase for Rubisco. It may be necessary to compare mutant enzyme crystal structures to address this possibility (33). However, now that it has recently become possible to engineer tobacco Rubisco in vivo (14, 15), it would be interest-
ing to see whether an R89A substitution would reverse activase specificity, presumably by promoting the formation of an ionic bond between Lys-94 and Asp-95 and mimicking the loop structure of spinach Rubisco.

It is of interest to note that β-strands C and D pack against α-helix B in the N-terminal domain of the Rubisco large subunit (30). Whereas the loop between β-strands C and D interacts with Rubisco activase, as evidenced by the effect of P89R, P89A, and D94K substitutions on Rubisco activase specificity (13, Fig. 3), the last residue of α-helix B, Glu-60, forms an ionic bond with Lys-334 in the C-terminal domain of a neighboring large subunit (30, 31). Lys-334 resides at the apex of α/β-barrel loop 6, which determines the specificity of Rubisco for carboxylation and oxygenation (34–36). Because loop 6 traps substrate and intermediates in the active site (30, 31), it is tempting to consider whether an interaction with Rubisco activase might produce subtle changes in the structure of the N-terminal domain that are responsible for the removal of phosphorylated inhibitors from the active site. Although a site of recognition between Rubisco and Rubisco activase is not necessarily the site at which activase facilitates activation, it may be interesting to examine the activation properties of N-terminal domain mutant enzymes that have alterations in CO₂/O₂ specificity (23, 37).

The D94K, P89R, and P89A mutant strains grow under photoautotrophic conditions even though their mutant Rubisco lacks the specificity of spinach and tobacco activases or because it recognizes a large-subunit conformation that is not affected by the D94K, P89R, and P89A substitutions. It has not yet been possible to purify sufficient quantities of Chlamydomonas activase to resolve these questions. Nonetheless, now that we have a better understanding of the large-subunit region that interacts with activase (Figs. 1 and 3), it may be possible to engineer single or multiple substitutions that would eliminate the interaction between Rubisco and Rubisco activase. Such mutants may be useful for identifying amino acid substitutions in activase that restore the interaction with Rubisco (16), thereby defining a complementing binding site on Rubisco activase.

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