Specificity for Activase Is Changed by a Pro-89 to Arg Substitution in the Large Subunit of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase*

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Tobacco activase does not markedly facilitate the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) from non-Solanaceae species, including the green alga Chlamydomonas reinhardtii. To examine the basis of this specificity, we focused on two exposed residues in the large subunit of Rubisco that are unique to the Solanaceae proteins. By employing in vitro mutagenesis and chloroplast transformation, P89R and K356Q substitutions were separately made in the Chlamydomonas enzyme to change these residues to those present in tobacco. Both mutants were indistinguishable from the wild type when grown with minimal medium in the light and contained wild-type levels of holoenzyme. Purified Rubisco was assessed for facilitated activation by spinach and tobacco activase. Both wild-type and K356Q Rubisco were similar in that spinach activase was much more effective than tobacco activase. In contrast, P89R Rubisco was not activated by spinach activase but was well activated by tobacco activase. Thus, the relative specificities of the spinach and tobacco activases for Chlamydomonas Rubisco were switched by changing a single residue at position 89. This result provides evidence for a site on the Rubisco holoenzyme that interacts directly with Rubisco activase.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (Rubisco) catalyzes the addition of atmospheric CO₂ to RuBP in plant chloroplasts and photosynthetic prokaryotes (1, 2). Before catalysis, a Mg²⁺ metal-stabilized carbamate must form on a lysine residue in the α/β barrel active site (3). Carbamylation is spontaneous (and reversible), but it cannot occur if the active site is occupied by a sugar phosphate (4). The rapid increase in RuBP during the onset of illumination actually inhibits carboxylation because RuBP binds tightly to the decarbamylated form of Rubisco that predominates in darkness (4, 5). In addition, misprotonation of RuBP can produce sugar phosphate inhibitors that bind to both decarbamylated and carbamylated sites (6, 7).

Plants overcome this limitation to rapid and maximal activation via the action of another chloroplast protein, Rubisco activase (8, 9). Activase couples the energy of ATP hydrolysis to the release of inhibitory sugar phosphates bound to both carbamylated and decarbamylated Rubisco active sites. High activase activity, along with high concentrations of RuBP, allows rapid and complete carbamylation to occur via a quasi-equilibrium state (10, 11) and reverses inhibition by misprotonated sugar phosphates (12). Activase is essential for photosynthesis in higher plants. A mutant of Arabidopsis thaliana that lacks activase requires elevated CO₂ for survival and growth (13).

Evidence for a direct interaction between Rubisco and activase is lacking. It is known that activases from Solanaceae species (e.g. tobacco) cannot activate non-Solanaceae Rubisco (e.g. spinach) and vice versa (14). Because of this specificity, one might expect that primary structure comparisons would identify residues involved in the interaction between Rubisco and Rubisco activase. Whereas few protein sequences exist for activase (9), sequences abound for Rubisco (15). A number of crystal structures also exist for the eukaryotic Rubisco holoenzyme (16–20). By comparing primary structures of Solanaceae and non-Solanaceae Rubisco, six residues on the surface of the chloroplast-encoded large (catalytic) subunit were tentatively identified as being phylogenetically distinct (21). These residues cluster in equatorial regions between the nuclear-encoded small subunits, and several are close to the carboxyl terminus of the large subunit. The carboxyl terminus is an attractive target for Rubisco activase interaction because it and α/β barrel loop 6 close over and sequester sugar phosphate in the catalytic site (19, 20).

Because the Rubisco large subunit is coded by a chloroplast gene, it remains difficult to engineer this gene in higher plants (1). In contrast, directed mutagenesis and chloroplast transformation can be routinely applied in the green alga Chlamydomonas reinhardtii (22, 23). Because Chlamydomonas Rubisco can be activated by spinach Rubisco activase (24) but not by tobacco activase (14), two large subunit residues were separately changed to those in tobacco Rubisco (Lys-356 to Gln and Pro-89 to Arg). Species specificity of the K356Q mutant Rubisco was unchanged. However, P89R caused a reversal in Rubisco/activase specificity. The P89R mutant enzyme can no longer be activated by spinach activase, but it can now be activated by tobacco activase.

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§ The abbreviations used are: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate; mt, mating type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; [α-35S]dATP, deoxyadenosine 5’-[α-35S]thiotriphosphate; bis-Tris, 1,3-bis[tris(hydroxymethyl)methylamino]propane.
FIG. 1. Space-filling spinach Rubisco quaternary structure (8RUC) highlighting residues that differ between Solanacea and non-Solanacea species. The seven candidate residues are colored, and one group is identified with arrows along with the two mobile elements (light blue, loop 6; light green, carboxyl terminus) observed in Rubisco crystals. Other structural elements are coded as follows: white, gray, large subunits; blue/orange, small subunits. Pos. position. Spin., spinach; C. rein., C. reinhardtii; Tob., tobacco; Pet., petunia; Tom., tomato. The image was generated using the program RASMOL (R. Sayle).

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Wild-type C. reinhardtii 2137 mt+ (25), rbcL mutant 18-7G mt+ (26), and rbcL insertion mutant 25B1 mt+ (27) were used as hosts for chloroplast gene transformation. The 18-7G strain contains a Trp-66 to amber (UAG) mutation within its rbcL gene (28). The 25B1 strain contains a 480-base pair yeast DNA insertion at a PsI site within the 3‘-region of rbcL (27). Each of these mutations eliminates Rubisco holoenzyme and produces a photosynthesis-deficient, acetate-requiring phenotype. All strains were maintained in darkness at 25 °C with 10 mM acetate medium containing 1.5% Bacto B. subtilis-deficient, acetate-requiring phenotype. All strains were maintained in darkness at 25 °C with 10 mM acetate medium containing 1.5% Bacto

Directed Mutagenesis—A 2961-base pair HaeIII DNA fragment (base 175 to 2776) containing the entire rbcL gene (bases 1–1428) (29) was cloned into HinCII-digested pBluescript SK- (Stratagene). Site-directed mutagenesis was performed as described previously (23) by employing a kit from Pharmacia Biotech Inc. (30). To produce the P89R mutation, the rbcL gene sequence CCA (bases 265–267) was changed to CGT. This mutation abolished a BsrI site and expedited the initial screening of transformants. To create the K356Q mutation, AAA (bases 1066–1068) was changed to CAA. All mutations were confirmed by performing DNA sequencing with Sequenase (U. S. Biochemical Corp.). 

Chloroplast Transformation—The mutant rbcL plasmids were transformed into the chloroplast by employing a helium-driven device for microprojectile bombardment (31) as described previously (22, 23). Because the phenotypes conferred by the mutant rbcL genes could not be predicted, transformants were either selected based upon photosynthetic ability in either 18-7G mt+ (for the P89R mutation) or 25B1 mt+ (for the K356Q mutation), or they were recovered by screening for a photosynthesis-deficient, acetate-requiring phenotype in 2137 mt+ (22, 23). Successive rounds of single-colony isolation were performed to ensure homoplasy of the mutant genes (22, 23). Total DNA was then purified from potential transformants (32), and a 1917-base pair fragment (bases 1–1756) containing the entire rbcL gene (bases 1–1428) was amplified by the polymerase chain reaction (33). A Sau3A subfragment (bases 33 to 1706) containing the complete rbcL gene was cloned into pUC19 (34) and transformed into Escherichia coli strain SDM (U. S. Biochemical Corp.). At least five independent E. coli transformants were screened for the mutant rbcL genes by plasmid isolation, restriction enzyme analysis, and DNA sequencing (22, 23). The expected mutations were found in all cases. A representative gene from each mutant was then sequenced in its entirety to confirm that only the intended mutation was present. The rbcL mutant strains created by directed mutagenesis and transformation were named pLS-P89R and pLS-K356Q, respectively.

Sucrose Gradients, Electrophoresis, and Immunoblotting—Total soluble cell proteins were extracted from dark-grown cells by sonication (35) and quantified by a dye binding method (36). Cell extract was fractionated on sucrose gradients to quantify the amount of Rubisco holoenzyme (35). SDS-polyacrylamide gel electrophoresis was performed (37) by employing a 7.5–15% polyacrylamide gradient in the running gel. Proteins were transferred from the gel to nitrocellulose (38) by using a Trans-Blot apparatus (Bio-Rad). The membranes were then probed with rabbit anti-tobacco Rubisco immunoglobulin G (5 μg/ml). Goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad) was used as the secondary antibody, and complexes were detected by enhanced chemiluminescence (Amersham Corp.).

Large Scale Purification of Rubisco—Wild-type and mutant cells were grown in 16-liter batch cultures with acetate medium, collected by centrifugation, and resuspended in 160 ml of carbamylation buffer (50 mM Tricine, pH 8.0, 10 mM MgCl2, 10 mM NaHCO3, 10 mM dithiothreitol, 1 mM EDTA) containing 10 μg leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. The cells were broken with a French press, and Rubisco was differentially precipitated with 55% ammonium sulfate. The Rubisco was resuspended in 40 ml of carbamylation buffer and reprecipitated with 15% (w/v) polyethylene glycol 10,000. Rubisco was resuspended in 20 ml of carbamylation buffer and fractionated on a 20-ml Q-Sepharose column with a chloride gradient. The Rubisco fractions were concentrated and desalted by Centricon (30 kDa) ultrafiltration.

The RubBP-inhibited form of Rubisco was prepared in carbamylation buffer by adding 15 mM EDTA for 15 min followed by elution through a Sephadex G25–150 desalting column equilibrated with 50 mM Tricine, pH 8.0, 0.1 mM EDTA. RubBP (2 mM) was then added, and the enzyme was stored in liquid N2. Protein amount was assayed spectrophotometrically at 280 nm assuming an extinction coefficient of 1.64 absorbance units for 1 mg/ml.

Rubisco Activase Purification—Leaves from spinach and tobacco plants were powdered in liquid N2, and activase was purified as described previously (14). The purified enzyme was stored in bis-Tris, pH 7.0, and 0.2 mM ATP under liquid N2.

Activase Specificity Assay—The ability of RubBP-inhibited Rubisco to be activated by Rubisco activase was assessed by a spectrophotometric assay at 25 °C that couples the product of RuBP carboxylation, 3-phosphoglycerate, to the oxidation of NADH (10, 39). Reaction mixtures contained 50 mM Tricine, pH 8.0. 12 mM MgCl2, 10 mM NaHCO3, 10 mM dithiothreitol, 2 mM ATP, 2 mM RubBP, 10 mM phosphocreatine, 750 μM NADH, and coupling enzymes (60 IU/ml 3-phosphoglycerate kinase, 300 IU/ml triose-phosphate isomerase, and 30 IU/ml each of creatine phosphokinase, glyceraldehyde-3-phosphate dehydrogenase, glyceral-3-phosphate dehydrogenase, and carbonic anhydrase). Activase was added to the reaction mixture 2 min before initiating the reactions with 50 μg/ml RubBP-inhibited Rubisco. The average absorbance of NADH (370–380 nm) was monitored with an HP 8452A (Hewlett-Packard Co.) diode array spectrophotometer, and data were analyzed by moving linear regressions of 1-min duration. Observed increases in the rates of carboxylation are ascribed to an increase in the extent of activation (39). Maximal rates of carboxylation were measured with fully activated Rubisco (no preincubation with RubBP). Carboxylation rates were calculated by dividing the observed carboxylation rate by the maximal carboxylation rate and adjusted to a site ratio assuming 1 mg of Rubisco contains 14.28 nmol of active sites (4).
RESULTS

Mutants and Phenotypes—Sequences of Rubisco large subunits from three non-Solanaceae (spinach, pea, Chlamydomonas) and three Solanaceae (tobacco, petunia, tomato) species were compared. Any residue that was identical in the sequences of one or more members of both groups was not considered further. The resulting list of 12 residues (Fig. 1) was reduced to 7 by eliminating replacements that were apparently conservative (residues 19, 353, and 443) and residues that did not exist in the shorter non-Solanaceae large subunit (residues 476 and 477). As shown in Fig. 1, three residues (89, 356, and 468) displayed a clear and consistent distinction between Solanaceae and non-Solanaceae large subunit sequences. We decided to focus on those two residues that are farthest from the carboxyl terminus (residues 89 and 356). By employing directed mutagenesis and chloroplast transformation, each of these Chlamydomonas residues was changed to the corresponding residue commonly found in Solanaceae Rubisco. To reduce the chance of recombination with wild-type sequence regions, mutant P89R was recovered by transforming the 18-7G rbcL nonsense mutant, and mutant K356Q was recovered by transforming the 25B1 rbcL insertion mutant. In both cases, transformants were recovered by selecting for photosynthetic ability on minimal medium in the light. Thus, it was readily apparent that the mutant Rubisco enzymes must maintain a substantial level of activity in vivo.

Both mutants are indistinguishable from wild type when grown with minimal medium in the light, and both were found to have wild-type levels of Rubisco holoenzyme when cell extracts were fractionated on sucrose gradients (data not shown). Western analysis confirmed the normal levels of Rubisco in both mutants (Fig. 2) but also revealed that the P89R large subunit has increased mobility on an SDS-polyacrylamide gel (Fig. 2, lane 2). Considering that normal levels of holoenzyme are present, it seems likely that this apparent molecular weight alteration arises from a difference in the way that SDS binds to P89R Rubisco. A change of proline to a positively charged arginine might also change the structure of the SDS-protein complex and increase its mobility within the gel matrix (40). Nonetheless, the apparent size difference for the P89R protein was inherited in a uniparental pattern (data not shown), indicating that it arises from the mutation in the P89R rbcL gene. Furthermore, extensive DNA sequencing identified only the expected mutation in this gene.

Biochemical Analysis—Large scale preparations of Rubisco were obtained from wild type and both mutants. Preliminary studies indicated that the mutant enzymes had wild-type carboxylase activities. The purified, non-carbamylated Rubisco enzymes were then inhibited by RuBP and assessed for facilitated activation by spinach and tobacco activases (Fig. 3). Both wild-type and K356Q Rubisco were similar in that spinach activase was much more effective than tobacco activase. In contrast, P89R Rubisco was little affected by spinach activase but was well activated by tobacco activase (Fig. 3). The response of the three Rubisco enzymes to increases in activase concentration was linear over the range examined (Fig. 4) and allowed a determination of the relative specific carbamylation rates. With spinach activase, the specific carbamylation rate with wild-type enzyme was 1.22 nmol of CO2/nmol site-min/mg of activase, whereas activation of the K356Q enzyme had a somewhat lower value of 0.83. Most significantly,
FIG. 5. Stereodiagrams of the structural differences at the surfaces of spinach (8RUC) and tobacco (4RUB) Rubisco. Regions are compared that are characteristic of Solanaceae and non-Solanaceae Rubisco (Fig. 1). Amino acid residues are colored as indicated in the schematic (right). Peptide backbones are colored dark gray (331–340) or light gray (86–95, 356–360, and 466–475). Residue positions from 473 to 477 are not specified in 4RUB and are not shown.

The P89R mutant was unresponsive to spinach activase (Fig. 4). The pattern shifted when tobacco activase was used. Both the wild-type and K356Q enzymes were activated at a low rate (0.21 nmol of CO₂/nmol site-min-mg of activase, but the P89R enzyme was activated at a higher rate (0.72 nmol of CO₂/nmol site-min-mg of activase).

**DISCUSSION**

We have demonstrated that the relative specificities of the spinach and tobacco activases for *Chlamydomonas* Rubisco can be switched by changing a single residue, Pro-89 to Arg. This result also provides evidence for a site on the Rubisco holoenzyme that interacts directly with Rubisco activase.

In the absence of an activase crystal structure, the molecular basis for the activase specificity reversal caused by the P89R large subunit mutation is difficult to discern. However, it is possible to compare the x-ray structures of tobacco and spinach Rubisco (17), the latter of which may be more like *Chlamydomonas* Rubisco activase. The pattern shifted when tobacco activase was used. Both the wild-type and K356Q enzymes were activated at a low rate (0.21 nmol of CO₂/nmol site-min-mg of activase, but the P89R enzyme was activated at a higher rate (0.72 nmol of CO₂/nmol site-min-mg of activase).

**Lys-94 and Asp-95 in tobacco Rubisco** (Fig. 1). Glu-94 is solvent-accessible in the spinach structure, whereas Lys-94 in tobacco Rubisco points in the opposite direction and interacts with Glu-93 and Gln-96 (Fig. 5). Both Asn-95 (spinach) and Asp-95 (tobacco) interact with Trp-97. If the negative charge of Rubisco residue 94 is important for an interaction with spinach and *Chlamydomonas* activase, perhaps a similar interaction occurs with Asp-95 in tobacco Rubisco by reorientation of the residue upon activase binding. The effect of amino acid substitutions at these residues in the *Chlamydomonas* enzyme may answer this question.

Replacement of Lys-356 with Gln had little effect on the ability of *Chlamydomonas* Rubisco to be activated by spinach activase and did not enable activation by tobacco activase (Figs. 3 and 4). Thus, the difference at this residue in the spinach and tobacco Rubisco enzymes (Figs. 1 and 5) does not appear to play a role in activase specificity. Perhaps the creation of a less conservative substitution would be necessary to determine whether this region is involved in activase recognition.

The close proximity of residue 89 to the carboxyl terminus and loop 6 (Fig. 5), both flexible elements that close off the active site during tight binding of various sugar bisphosphates (16–20), prompts future consideration of these areas in molecular mechanisms for the influence of activase on sugar phosphate binding in the Rubisco active site. Whereas the ability of tobacco activase to activate the P89R *Chlamydomonas* enzyme indicates that other structural regions are not critical for determining activase specificity, this result does not eliminate the possibility that other structural interactions are necessary for Rubisco activation. To investigate the possible roles of the Rubisco carboxyl-terminal and loop 6 regions in activation will require the creation and study of amino acid substitutions, specifically in these regions. The loop 6 residues that are clearly solvent-accessible (Glu-338, Arg-339, and Asp-340) (Fig. 5) may interact with activase, thereby providing a means for the release of sugar phosphate from its interaction with loop 6 Lys-334 (16–20).

The *Chlamydomonas* P89R Rubisco is not activated by spinach activase (Figs. 3 and 4), but the mutant strain has a wild-type phenotype. Although we have not yet been able to adequately purify *Chlamydomonas* activase for in vitro studies, it seems likely that the mutant Rubisco can still be sufficiently activated by *Chlamydomonas* activase. Nonetheless, as addi-
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Mutations of this type might then allow compensatory substitutions in Rubisco activase to be recovered via genetic selection and studied (32, 41).

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