Rubisco activase is a AAA+ protein, a superfamily with members that use a "Sensor 2" domain for substrate recognition. To determine if the analogous domain of activase is involved in recognition of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39), two chimeric activases were constructed, interchanging a Sensor 2-containing region between activases from spinach and tobacco. Spinach chimeric activase was a poor activator of both spinach and tobacco Rubisco. In contrast, tobacco chimeric activase activated spinach Rubisco far better than tobacco Rubisco, similar to spinach activase. A point mutation, K311D, in the Sensor 2 domain of the tobacco chimeric activase abolished its ability to better activate spinach Rubisco. The opposite mutation, D311K, in wild type tobacco activase produced an enzyme that activated both spinach and tobacco Rubisco, while a second mutation, D311K/L314V, shifted the activation preference toward spinach Rubisco. The involvement of these two residues in substrate selectivity was confirmed by introducing the analogous single and double mutations in cotton activase. The ability of the two tobacco activase mutants to activate wild type and mutant *Chlamydomonas* Rubiscos, was also examined. Tobacco D311K activase readily activated wild type and P89R, but not D94K Rubisco, while the tobacco L314V activase only activated D94K Rubisco. The tobacco activase double mutant, D311K/L314V, activated wild type *Chlamydomonas* Rubisco better than either the P89R or D94K Rubisco mutants, mimicking activation by spinach activase. The results identify a substrate recognition region in activase in which two residues may directly interact with two residues in Rubisco.

Rubisco (ribulose 1,5-bisphosphate carboxylase/oxygenase) activase is a chloroplast protein that activates and maintains Rubisco in an active state by facilitating removal of various sugar phosphates that either block substrate binding or prevent carbamylation (1, 2). Plants lacking activase or having a very low level of activase cannot survive at atmospheric CO2 level (3, 4) and those expressing reduced levels exhibit reduced rates of photosynthesis and growth (5, 6). The activation process requires ATP hydrolysis (7), but this activity, as measured in vitro, appears to be related to self-association which occurs even in the absence of Rubisco (8). The activase is subjected to redox regulation in Arabidopsis and probably other plant species (9, 10). The proposed mechanism of action includes a binding step between activase and Rubisco, which is consistent with the kinetics of the activation process (11, 12).

Although chemical cross-linking (13) and co-immunoprecipitation of Rubisco and activase have been reported (14, 15), most of the details of the binding process are still largely unknown. However some insight into the process has been obtained by exploiting the peculiar specificity of Rubisco activase from plants in the family Solanaceae. Activase from plants in this family, which includes tobacco, tomato, potato and petunia, was shown to be a poor activator of Rubisco from a wide range of plants outside the Solanaceae family, including spinach, barley, Arabidopsis and maize, and vice-versa. In contrast, activase and Rubisco from distant families of non-solanaceous higher plants and...
even the green alga, *Chlamydomonas reinhardtii*, were capable of interacting with each other (16).

Several approaches have been used to examine the species selectivity between Rubisco and activase. In one approach, residues of *Chlamydomonas* Rubisco were targeted for modification by site-directed mutagenesis based on a comparison of the amino acid sequences and crystal structures of tobacco and spinach Rubiscos. Two separate point mutations, P89R and D94K, of the *Chlamydomonas* Rubisco large subunit greatly improved its activation by tobacco activase, but severely impaired its activation by spinach activase (17, 18). These results indicated an involvement of the N-terminus of the large subunit of Rubisco in the interaction with activase. In another approach, Rubisco activation was examined with chimeric activase proteins that were constructed by interchanging DNA fragments from regions of tobacco and spinach activase cDNA (19). The results showed that the C-terminal domain of activase was a major determinant of Rubisco specificity, whereas the N-terminal domain had little or no involvement in selectivity.

Recently, Rubisco activase was identified as a member of a superset of the AAA protein family (ATPases associated with diverse cellular activities) (20) called the AAA family (21) based on sequence and structural homologies. In certain members of this family of proteins, including the chaperone components of all ATP-dependent proteases (22), a specific C-terminal domain, the “Sensor 2” domain, has been shown to be involved in protein-substrate recognition (23, 24). Here we demonstrate that a C-terminal region of activase, which contains the Sensor 2 domain, is responsible for differences in Rubisco substrate recognition between activases from solanaceous and non-solanaceous plant species. Two critical amino acid residues in this region were identified and their individual roles were characterized.

**Materials and Methods**

**Materials** — RuBP was synthesized and purified as described previously (25). Wild type Rubiscos from spinach/tobacco were isolated as previously reported (16). Wild type and mutant Rubiscos from *Chlamydomonas* were obtained as previously reported (12, 13). cDNA’s of spinach (26), tobacco (27) and cotton (28) activase were used as starting materials. Site-directed mutagenesis primers were obtained from Integrated DNA Technologies, Inc. A QuickChange site-directed mutagenesis kit was purchased from Stratagene, Inc. All other reagents were of the highest purity readily available.

**Chimeric protein construction** — cDNA clones of spinach and tobacco activase were used as the starting material. A region (including the Sensor-2 domain) close to the C-terminus and encompassing residues 267 to 334 was the targeted region for replacement. Site-directed mutagenesis was performed to create a *PstI* site at the 5’ end of this region in the tobacco cDNA and an *XhoI* site at the 3’-end of this region in the spinach cDNA. The small fragments obtained by digestion with *PstI/XhoI* were ligated into the corresponding large fragment to create cDNA’s encoding tobacco chimeric activase 267-334 (T<sub>t</sub>) and spinach chimeric activase 267-334 (S<sub>t</sub>). The numbering of amino acids in all activases presented here is based on the mature spinach activase sequence. The ligation products were introduced into the expression vector (pET23d) in *Escherichia coli* strain DH5α. DNA sequencing was performed to verify the constructions.

**Site-directed mutagenesis** — Site-directed mutagenesis was used to create the desired enzyme digestion sites and mutations on activase following protocols provided in the QuickChange kit. Four mutants were generated from the tobacco chimeric activase (T<sub>t</sub>) construct, T<sub>t</sub>H269A, T<sub>t</sub>302S/S303G, T<sub>t</sub>N308S and T<sub>t</sub>K311D. Another six mutants were created in the native cDNAs: K311D and K311A in spinach activase (T<sub>s</sub>K311D and T<sub>s</sub>K311A); D311K and D311A in tobacco activase (T<sub>d</sub>D311K and T<sub>d</sub>D311A); L314V and D311K/L314V in tobacco activase (T<sub>d</sub>314V and T<sub>d</sub>D311K/L314V). All mutations were confirmed by DNA sequencing.

**Protein expression and purification** — All verified constructions/mutations were introduced into BL21(DE3) pLysS cells (Novagen). The procedures for expression and purification were the same as previously reported (9, 19, 27).

**ATPase and Rubisco activation assays** — ATP hydrolysis was measured by coupling ADP production to NADH oxidation using a diode array spectrophotometer as reported previously (2). Activase K<sub>cat</sub> was calculated assuming a mass of 42 KDa. Rubisco activation by activase was also
assayed spectrophotometrically as previously reported (29). The fraction of sites activated was calculated by dividing the observed carboxylation rates by the maximal carboxylation rate, which is based on the observation that activity ratios are directly correlated with the extent of carbamylation (30). The carbamylation rates of *Chlamydomonas* Rubiscos were calculated from the fraction of sites active in carboxylation, assuming that 1mg of Rubisco contains 14.3 nmol active sites (17).

*Cotton activase* — Procedures for the purification and assay of cotton Rubisco and the β-form of recombinant cotton activase have been described in detail previously (28, 31, 32). Point mutations were introduced into the cotton activase cDNA using the Gene Tailor Kit according to the manufacturer’s instructions (Invitrogen Life Systems, Carlsbad, CA).

**RESULTS**

*Chimeric proteins* — The location of eleven well established domains within the AAA⁺ module of activase is shown in Fig. 1. A DNA fragment encoding residues 267 to 334 of the mature spinach activase, spanning the Box VII’ and Sensor 2 domains, was interchanged between tobacco and spinach activase by recombinant DNA technology and targeted for mutagenesis. Alignment of the amino acid sequences that comprise this region showed that most of the differences among species exist in the C-terminal section that includes the Sensor 2 domain. Several residues that are quite divergent between the Solanaceae and non-solanaceous species and that were later selected for mutagenesis are highlighted.

Both the tobacco chimeric activase containing spinach residues 267 to 334 (Tₛ) and the spinach chimeric activase containing tobacco residues 267 to 334 (Sᵀ) were expressed in an active form. The ATPase activity of the Tₛ was comparable to wild type tobacco activase, whereas the activity of Sᵀ was consistently lower in several separate preparations (Table 1).

Assays of Rubisco activation confirmed (16) that wild type spinach activase is a poor activator of tobacco Rubisco and vice versa (Table 1). The activation activities of the chimeras, Tₛ and Sᵀ, differed markedly from the wild type enzymes. The Tₛ enzyme activated spinach Rubisco better than tobacco Rubisco despite the fact that a majority of its sequence was derived from tobacco. This result indicates that the region from residues 267 to 334 was probably responsible for proper activase - Rubisco recognition between these two species. Unfortunately, the spinach chimeric protein Sᵀ was a very inefficient activator of both tobacco and spinach Rubisco. The low ATPase activity of this protein combined with its minimal activation activity with both Rubiscos suggests that the overall structure of the spinach activase was disrupted by the substituted tobacco residues.

*Mutations in chimeric activases* — Because the only differences between the chimeric protein, Tₛ, and wild type tobacco activase are 17 different amino acids in the replaced region, one or more residues in the Box VII’ and/or Sensor 2 domains must be responsible for the observed change in specificity. Five residues of the spinach insert, H269, N302/S303, N308, K311, were selected for mutation back to the corresponding tobacco amino acid residue(s) to create the mutated chimeric proteins Tₛ^H269A, Tₛ^N302S/S303G, Tₛ^K311D, and Tₛ^K311D. With the possible exception of Tₛ^K311D, the ATPase activity of these proteins were comparable to the non-mutated chimera, Tₛ, indicating that these mutations did not cause drastic changes to the structure of activase (Table 2).

The activation preferences of the mutated chimeras, Tₛ^H269A, Tₛ^N302S/S303G and Tₛ^K311D were similar to the non-mutated chimera, exhibiting greater activation activity towards spinach compared with tobacco Rubisco. In contrast, the Tₛ^K311D mutant chimera exhibited reduced activity toward spinach Rubisco and enhanced activity towards tobacco Rubisco. This result indicates that a lysine at position 311 is required for Tₛ to better activate spinach Rubisco.

*Mutations in wild type activases* — To explore the role of residue 311 in activase specificity, this residue was mutated in tobacco and spinach activase. Both TⁿD₃₁¹A and TⁿD₃₁¹K had increased ATPase activity compared with wild type tobacco activase, while ATPase activity in the SⁿK₃₁¹A and SⁿK₃₁¹D mutants was less than the spinach wild type (Tables 1 and 3).

Compared with wild type tobacco activase (Table 1), the TⁿD₃₁¹K mutant exhibited reduced
activity toward tobacco Rubisco and enhanced activity toward spinach Rubisco. The T<sup>D311A</sup> mutant did not activate spinach Rubisco and its capacity to activate tobacco Rubisco was decreased by more than 50%. The analogous mutations in spinach activase, S<sup>K311D</sup> and S<sup>K311A</sup>, produced proteins that were unable to activate either spinach or tobacco Rubisco. Thus a Lys residue at 311 is required for both activases to interact with spinach Rubisco, while an Asp residue at 311 in tobacco activase allows for the better activation of tobacco Rubisco.

Residue 314, which is in close proximity to 311, is a Leu in activase from the Solanaceae and a Val in non-solanaceous species. Therefore, this residue was mutated in wild type tobacco activase and the T<sup>D311K</sup> mutant to produce the single mutant, T<sup>L314V</sup> and the double mutant, T<sup>D311K/L314V</sup>. Compared to the wild type (Table 1), the ATPase activity of both mutants was reduced by about 50% (Table 3). In activation assays, the T<sup>L314V</sup> mutant exhibited the same preference for tobacco over spinach Rubisco as the wild type tobacco activase (Table 3). However the double mutant T<sup>D311K/L314V</sup> maintained the ability to activate spinach Rubisco (compared to T<sup>D311K</sup>) and reduced the ability to activate tobacco Rubisco. Thus these two residues appear to work in combination to determine the species specificity of activase.

**Mutations in cotton activase** — Because mutation of K311 in spinach activase was deleterious for Rubisco activation, an activase from another non-solanaceous species was selected to perform the reciprocal experiments with residues 311 and 314 that were conducted with tobacco activase. K311 and V314, as well as the intervening residue, K312, were mutated in cotton and the mutant proteins were analyzed. Cotton activase was an inefficient activator of tobacco Rubisco and vice-versa (Table 4). The K311D and, to a lesser extent, the V314L mutations decreased the ability of cotton activase to activate cotton Rubisco, but enhanced the enzyme’s ability to activate tobacco Rubisco (Table 4). An even greater effect on altering specificity towards tobacco was observed with the double mutant, C<sup>K311D/V314L</sup>. Measurement of ATPase activity showed that the reduced activity towards cotton Rubisco was not caused by a general disruption in activase structure because the ATPase activity was higher in the mutants compared with wild type. Residue 312, which is an Arg in activase from some non-solanaceous plants (Fig. 1), was also mutated to examine the effect on Rubisco activation and ATPase activity. The cotton mutant, C<sup>K312R</sup>, exhibited reduced ATPase activity, but its selectivity for cotton Rubisco was unchanged from wild type.

**Activation of Chlamydomonas Rubiscos by tobacco activase mutants** — Chlamydomonas Rubisco is more efficiently activated by spinach activase than tobacco activase (17). However, this preference can be changed by introducing specific mutations into the large subunit of Chlamydomonas Rubisco. The mutant Rubisco proteins, either P89R or D94K, were more efficiently activated by tobacco compared with spinach activase (17, 18). To determine if these mutations in the large subunit of Rubisco could be complemented by mutations in tobacco activase that change its specificity (Table 3), activation of the Chlamydomonas Rubisco mutants was examined in assays containing various concentrations of tobacco mutant activase (Fig. 2). As was observed previously (17), the rates of Rubisco carboxylation were directly proportional to the concentration of activase in the assay. The mutant tobacco activase, T<sup>D311K</sup>, was a much more efficient activator of wild type Chlamydomonas Rubisco compared with wild type tobacco activase. This preference was lost with the D94K mutant of Rubisco, but was retained with the P89R mutant. In contrast, the tobacco activase mutant, T<sup>L314V</sup>, was a much more efficient activator of the D94K mutant of Rubisco compared with wild type tobacco activase, but was a poor activator of the P89R mutant. Unlike wild type tobacco activase, the tobacco activase double mutant, T<sup>D311K/L314V</sup>, activated wild type Chlamydomonas Rubisco much better than it activated either of the Rubisco mutants.

**DISCUSSION**

In this report, we demonstrated that the selectivity of Rubisco activase for Rubisco can be altered by interchanging amino acids in the region spanning the Box VII’ and Sensor 2 domains. Two of the amino acids in this region, 311 and 314, were shown to play a critical role in substrate (i.e., Rubisco) preference. Furthermore, mutation of
each of these residues complemented a different one of the specificity-changing mutations in the *Chlamydomonas* Rubisco large subunit. These results indicate that there is a direct interaction between a domain in the N-terminus of the large subunit of Rubisco and the Sensor 2 domain near the C-terminus of activase and provide new and detailed information about the nature of this interaction. The data suggest that the interaction involves specific residue pairs, Rubisco-94 with activase-311 and Rubisco-89 with activase-314.

Even though both residues, 311 and 314, influenced the recognition of Rubisco by activase, residue 311 appeared to play the dominant role. Mutating Asp 311 to Lys was sufficient to allow tobacco activase to activate spinach Rubisco, whereas changing Leu 314 to Val caused almost no change in specificity. The reciprocal mutations in activase from the non-solanaceous species, cotton, had the corresponding effect; K311D improved activation of tobacco Rubisco and reduced activation of cotton Rubisco, while V314L had much less of an effect. Changing both residues was even more effective in switching the selectivity of non-solanaceous activase towards Solanaceae and away from non-solanaceous Rubisco and vice-versa.

Residues in proximity to 89 and 94 of the large subunit of Rubisco and ones near activase residues 311 and 314 are almost certain to participate in interactions between Rubisco and activase, but these residues probably do not determine specificity because they are conserved. Without a 3-D structure for activase and particularly a structure for the binary complex between Rubisco and activase, it is difficult to identify all of the residues involved in their interaction. However, it is interesting that, within the region of the large subunit of Rubisco from 89 to 94, there is a negatively charged Glu-93 that is conserved in Rubisco and a potentially complementary and positively charged Lys or Arg at position 312 in activase. This pair represents a potential target of interest for future mutagenesis experiments.

Changes in the position of the N-terminus of Rubisco that occurs as the molecule assumes the closed and open conformations (33) provide some insight into possible activase-Rubisco interactions (Fig. 3). During the transitions from the closed to the open conformation, the N-terminus of Rubisco moves away from the catalytic active site, making the site accessible to solvent. The results presented here suggest that the interaction of activase with Rubisco may occur through electrostatic and other forces, involving the region 89 to 94 of Rubisco and the region 311 to 314 of activase and specifically the pairing of residues Rubisco-94 to activase-311, Rubisco-89 to activase-314 and possibly Rubisco-93 to activase-312. Once bound to Rubisco, ATP hydrolysis could promote movement of the C-terminal domain of activase, as occurs for other AAA⁺ ATPases (reviewed in 34), which may alter the position of the region from 89 to 94 and subsequently the entire N-terminal domain of the large subunit of Rubisco. It is likely that additional interactions between Rubisco and activase are required to complete the transition from closed to open conformation, because residues in the extreme N- and C-termini of Rubisco activase have been shown to be involved in Rubisco activation (27, 29). Because these residues are conserved among all activases, they are probably not involved in determining the observed specificity in the interaction.

Measurements of ATPase activity in the various activase mutants highlight the complex relationship between Rubisco activation and ATPase hydrolysis. Although ATP hydrolysis is absolutely required for Rubisco activation (7), the two activities are not strictly coupled since ATP hydrolysis can proceed both in the absence of Rubisco and in directed and truncated mutants of activase that no longer activate Rubisco (27, 29). Also, since the specific activities of ATPase and Rubisco activation change upon self-association of the enzyme (35, 36), mutations that affect activase aggregation would have an effect on activase activities that would be dependent on the concentration of activase protein. Thus, it is likely that mutations in activase that affect both ATPase hydrolysis and Rubisco activation, for example the ST chimera, probably cause global changes in the overall structure of the protein, whereas the structural integrity of the enzyme is largely preserved in mutants like C[K312R] that only affect one of these activities.

The Sensor 2 domain is a key structural feature of the AAA⁺ module. Among many AAA⁺ proteins, there is a critical Arg residue in this domain (Fig. 1) which interacts with the bound nucleotide and is required for ATPase activity (reviewed in 37). The identification of activase-
Rubisco interaction sites in the same region provides a direct way to couple ATP hydrolysis with the “opening” of Rubisco and is consistent with the proposed role(s) of this domain in other AAA⁺ proteins. The role of the conserved Arg is being investigated and preliminary data suggest that it is required for ATP hydrolysis (unpublished data).

REFERENCES


FOOTNOTES

1 The abbreviations used are: AAA+, super family of ATPases associated with diverse cellular activities; ATPase, ATP hydrolysis; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, d-ribulose-1,5-bisphosphate.

2 Mention of a trademark, proprietary product of vendor does not constitute a guarantee or warranty of the product by the US Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

ACKNOWLEDGMENTS

We thank Dr. R. J. Spreitzer (University of Nebraska) for providing the Chlamydomonas strains used in this work.

FIGURE LEGENDS

Fig. 1. Schematic representation of the domains within the AAA+ module of activase and a comparison of amino acids 267-334 in activase from selected Solanaceae and non-solanaceous plants. Alignment was performed in ClustalW from SDSC Biology Workbench (workbench.sdsc.edu) and the residues selected for mutagenesis are enclosed by rectangles. The conserved Arg in Sensor 2 that is discussed in the text is marked with “●”.

Table 1. ATPase activity and Rubisco activation specificity of tobacco activase, spinach activase, tobacco chimeric activase (T5) and spinach chimeric activase (S5). Rubisco activation, which was assayed with 5 µg/ml of either tobacco or spinach Rubisco and 80 µg/ml Rubisco activase, was measured by the fraction of Rubisco sites activated after 5 min. The specific activities of fully activated spinach and tobacco Rubisco were 2.30 and 2.21 U mg protein\(^{-1}\), respectively.

Table 2. ATPase activity and Rubisco activation specificity of tobacco chimeric activase (T5) with mutations H269A (T5\(^{H269A}\)), N302S/S303G (T5\(^{N302S/S303G}\)), N308K (T5\(^{N308K}\)) and K311D (T5\(^{K311D}\)). Assay conditions and Rubisco specific activities were the same as in Table 1.

Table 3. ATPase activity and Rubisco activation specificity of activase with mutations K311A (S\(^{K311A}\)) and K311D (S\(^{K311D}\)) in spinach activase and D311A (T\(^{D311A}\)), D311K (T\(^{D311K}\)), L314V (T\(^{L314V}\)) and D311K/L314V (T\(^{D311K/L314V}\)) in tobacco activase. Assay conditions and Rubisco specific activities were the same as in Table 1.

Table 4. ATPase and Rubisco activation specificity of cotton activase, cotton activase with mutations K311D (C\(^{K311D}\)), V314L (C\(^{V314L}\)), K311D/V314L (C\(^{K311D/V314L}\)), K312R (C\(^{K312R}\)) and tobacco activase. Rubisco activation, which was assayed with 500 µg/ml of either tobacco or spinach Rubisco and 500 µg/ml Rubisco activase, was measured by the fraction of Rubisco sites activated after 6 min. The specific activities of fully activated cotton and tobacco Rubisco were 1.68 and 1.31 U mg protein\(^{-1}\), respectively.

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Fig. 2. Carbamylation rate of *Chlamydomonas* Rubisco from wild type, mutant P89R and mutant D94K with increasing concentrations of wild type tobacco activase (A), and mutant D311K (B), L314V (C) and double mutant D311K/L314V (D). Carbamylation rates were calculated as described in Methods with the following measured fully activated Rubisco specific activities: 3.10 (wild type), 2.30 (P89R) and 3.01 (D94K) U mg protein⁻¹, respectively.

Fig. 3. Key differences between “open” (orange, 1RXO) and “closed” (blue, 1RCX) structures of spinach Rubisco LSU dimer. The backbones for residues 55-100, 331-340 (loop 6) and 465-475 (C-terminus) are compared. The C-terminus of the “open” structure is disordered and thus not shown. The side chains of residues interacting with activase (Pro89, Glu93 and Glu94) are depicted and numbered; RuBP, shown in spacefill, interacts with both LSUs. The alignment was performed according to Duff et al. (33) and Portis (38).
<table>
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<th>ATPase Activity (min⁻¹)</th>
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NA, not applicable; ND, not determined
Figure 2

**A**

**B**

**C**

**D**

Carbamylation Rate (nmol CO₂ nmol site⁻¹ min⁻¹) vs. Activase (µg ml⁻¹) for different conditions:

- **A**: T + Wild Type, T + P89R, T + D94K
- **B**: D371K + Wild Type, D371K + P89R, D371K + D94K
- **C**: T₁-L316V + Wild Type, T₁-L316V + P89R, T₁-L316V + D94K
Two residues of Rubisco activase involved in recognition of the Rubisco substrate
Cishan Li, Michael E. Salvucci and Archie R. Portis, Jr

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