Activation of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (Rubisco) Involves Rubisco Activase Trp16

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Received February 28, 1996; Revised Manuscript Received April 26, 1996[⊗]

ABSTRACT: The role of the N-terminal region of tobacco Rubisco activase in ATP hydrolysis and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activation was examined by construction of mutant proteins. Deletion of the first 50 amino acids of Rubisco activase almost completely eliminated the ability to activate Rubisco, without changing the ATP-hydrolyzing and self-associating properties of the enzyme. Thus, the N-terminus of Rubisco activase is distinct from the ATP-hydrolyzing domain and is required for Rubisco activation. Directed mutagenesis of the species-invariant tryptophan residue at position 16 inhibited Rubisco activation but not the binding or hydrolysis of ATP. The ability to activate Rubisco was less severely inhibited when Trp was replaced by a Tyr or Phe than by an Ala or Cys, indicating that an aromatic residue at position 16 and particularly a Trp is required for proper activation of Rubisco. Fluorescence quenching of the 7-nitrobenz-2-oxa-1,3-diazole-modified W16C mutant upon addition of nucleotide suggested that position 16 becomes more solvent accessible in response to nucleotide binding. However, changes in the intrinsic fluorescence of truncated and Trp16 mutants upon addition of ATP were similar to those of the wild type, evidence that Trp16 is not the residue reporting the conformational change that accompanies subunit association.

The primary enzyme of photosynthetic carbon fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco,[1] EC 4.1.1.39), is regulated by Rubisco activase, a chloroplastic enzyme that facilitates the release of tight-binding sugar phosphates from Rubisco. Without Rubisco activase, tight-binding sugar phosphates act as inhibitors of Rubisco by either hindering carbamylation of the activator lysine or blocking the substrate, ribulose 1,5-bisphosphate, from binding to the carbamylated enzyme [for reviews, see Portis (1990) and Hartman and Harpel (1994)]. The hydrolysis of ATP by Rubisco activase is required to convert Rubisco from the tight- to the loose-binding form. ATP hydrolysis presumably supplies a source of energy for the conformational changes that reduce the binding affinity of Rubisco for sugar phosphates. However, the nature of the interaction between Rubisco and Rubisco activase is unknown.

In previous studies, photoaffinity analogs of ATP were used to identify the subdomains of the ATP binding site of Rubisco activase (Salvucci et al., 1993, 1994). Photoaffinity labeling of the purified tobacco leaf enzyme with ATPγ−benzophenone identified peptides in the ATP γ-phosphate binding domain (Salvucci et al., 1993). Breakdown products of 38 and 39 kDa, present in the preparation, also photolabeled and had saturation and protection kinetics identical to those of the full-length 42 kDa polypeptide. The 38 and 39 kDa polypeptides commenced at Ser56 and Phe41, respectively, indicating that these breakdown products were truncated at the N-terminus. Thus, the N-terminus of Rubisco activase through Gln55 does not appear to be necessary for ATP binding.

Photoaffinity labeling of recombinant tobacco Rubisco activase was conducted with 2- and 8-N_3ATP to identify the subdomain involved in binding the adenine base. 2-N_3ATP labeled the peptide Asn68 to Asp74, and incorporation into this peptide was prevented by ADP and ATP (Salvucci et al., 1994). The azido-substituted ATP analogs also labeled a peptide near the N-terminus of Rubisco activase. Labeling of this peptide was not prevented by ADP and ATP but was blocked by tryptophan and other indoles. On the basis of these observations, the binding of azidoadenine nucleotides to this site was ascribed to base-stacking interactions with Trp16, a species-invariant tryptophan that is one of the most highly conserved residues in the labeled peptide (Salvucci et al., 1994). Interestingly, modification in the vicinity of Trp16 inhibited Rubisco activation without affecting ATP hydrolysis. A similar effect on the two activities of Rubisco activase was recently reported for a deletion mutant of the spinach enzyme that lacked the N-terminal 12 residues through the analogous Trp residue (Esau et al., 1996).

In this study, we examine the involvement of the N-terminus of Rubisco activase, and particularly Trp16, in the interaction between Rubisco activase and Rubisco. For these studies, we use *in vitro* mutagenesis to construct a truncation mutant (Trunc) that begins at His51 and mutants with various substitutions at Trp16. Study of the recombinant mutant proteins showed that both types of mutations impair Rubisco activation, without affecting the binding or hydrolysis of ATP. Thus, the N-terminus of Rubisco activase and specifically Trp16 is not necessary for ATPase activity but is required for Rubisco activation.

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⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; NBD, 7-nitrobenz-2-oxa-1,3-diazole; N_3ATP, azidoadenosine 5′-triphosphate; ATPγS, adenosine 5′-(γ-thio)triphosphate.

S0006-2960(96)00490-4
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MATERIALS AND METHODS

In Vitro Mutagenesis and Protein Purification. A cDNA clone was previously engineered for expression of a mature form (i.e. minus chloroplast transit peptide) of tobacco Rubisco activase (Salvucci & Klein, 1994). This "wild type" clone is similar to the partial clone pJQ11 (GenBank Z14979) sequenced by Qian and Rodermel (1993) and to the full-length clone NtRCA342 (GenBank U35111) sequenced by G. W. Snyder (unpublished). However, the first five residues of our mature protein sequence (EEKDA) following the initiating M were engineered to correspond to protein sequence data (Wang et al., 1992) and differ from the predicted translation product of NtRCA342 (EQIDV).

An N-terminally truncated protein (Trunc mutant) was engineered by PCR, using the forward primer 5′TACTCCCATGGCTGTGTTGCAATCCTACGAATAC3′ and reverse primer 5′CTACTCCCACAAAAGTGCCCTTAAAA3′. The PCR product was digested with *Nco*I and *Sal*I and cloned into the expression vector pET21d (Novagen[2]). For site-directed mutagenesis of the Trp16 codon, an *Nco*I-*Hin*dIII fragment coding for the N-terminus of Rubisco activase was cloned into the M13 phage vector M13BM21 (Boehringer-Mannheim) for oligonucleotide-directed mutagenesis (Amersham). Initially, the W16A mutant was made using the mutagenic oligonucleotide 5′CGACAGTGACAGAGCGAAGGGTCTTGT3′, and an *Xba*I-*Hin*dIII fragment encompassing the mutation was used to replace an *Xba*I-*Hin*dIII fragment of pET21d containing the wild type cDNA cloned at *Nco*I and *Sal*I sites. Subsequently, a primer was designed for introduction of the W16Y, W16S, and W16F mutations. This primer was 5′CGACAGTGACAGAT(A/C/T)TAAGGGTCTTGTCC3′. A separate primer was designed for isolation of the W16C mutant, 5′CGACAGTGACAGATGTAAGGGTCTTGTCC3′. The W16Y/C/F mutant *Nco*I-*Sma*I fragments were cloned by replacement of the wild type fragment in the pET21d vector.

The mutagenized DNA fragments for the Trunc, W16A, W16Y, W16C, and W16F mutants were sequenced to ascertain that no extraneous mutations were introduced during PCR or cloning in the M13 phage. Wild type and mutant Rubisco activase proteins were expressed in the *Escherichia coli* strain BL21(DE3)pLysS (Novagen) by induction of an ($OD_{600} = 0.6$) M9ZB culture (Studier et al., 1990) with 0.4 mM isopropyl β-thiogalactoside at 37 °C. Cells were harvested 3.5 h after induction. Resultant poor expression (<5% of total protein) was not improved by increasing the isopropyl β-thiogalactoside concentration (up to 5 mM) but was greatly improved (>80% of total protein, >50% of soluble protein) by using the host strain lacking the plasmid pLysS. Similar expression was achieved in the pLysS-containing strain by using the vector pET23d, which differs from pET21d by the absence of the *lacI* gene encoding the *lac* repressor, and absence of the *lac* operator from the promoter.

Rubisco activase was purified from the pLysS-containing cells essentially as described (Salvucci & Klein, 1994), except that cells were lysed by thawing and then sonicated briefly to reduce the viscosity.

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

Photoaffinity Labeling. Photoaffinity labeling of recombinant wild type and mutant Rubisco activase with [γ-^{32}P]-2-N_3ATP was performed in 25 μL reaction mixtures as described previously (Salvucci et al., 1994) using the concentrations of [γ-^{32}P]-2-N_3ATP and Rubisco activase indicated in the figure legends. Photolabeled proteins were analyzed by direct autoradiography following separation of the labeled polypeptides by SDS−PAGE (Salvucci et al., 1993, 1994).

Assay of Rubisco Activase Activities. ATPase and Rubisco activation was measured in the presence of 5% (w/v) PEG-3350 as described previously (Salvucci, 1992) except that the concentrations of $MgCl_2$, dithiothreitol, pyruvate kinase, and lactate dehydrogenase in the ATPase assay were changed to 5 mM, 5 mM, 4.2 u mL^{-1}, and 6.0 u mL^{-1}, respectively. The concentrations of proteins used in the assays are indicated in the figure legend. Assays were conducted in triplicate (activation) or duplicate (ATPase), and the results presented are the means of representative experiments.

Chemical Modification of W16C. Wild type Rubisco activase and the W16C mutant were desalted in 50 mM potassium phosphate (pH 7.5) after precipitation with ammonium sulfate (Salvucci, 1992). The desalted proteins were incubated in the dark at 2 mg mL^{-1} (47.6 μM protomer) with a 2-fold molar excess of *N*,*N*′-dimethyl-*N*-(iodoacetyl)-*N*′-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine [(iodoacetyl)-NBD]. After 5 h at 23 °C, Rubisco activase protein was precipitated by addition of ammonium sulfate to 50% (w/v), collected by centrifugation, and then resuspended and desalted in 50 mM Hepes-KOH (pH 7.2), 50 mM sucrose, 2.5 mM $MgCl_2$, and 10 mM EDTA. Approximately 0.8 mol of NBD was incorporated per mole of W16C subunit on the basis of the A_{500} of the NBD adduct, compared to 0.59 mol/mol for the wild type. Automated Edman degradation analysis of reverse-phase HPLC-purified, *Staphlococcus aureus* V8 protease-generated peptides from (iodoacetyl)-NBD-modified W16C (Salvucci et al., 1994) verified that approximately 78% of the incorporated NBD was associated with the N-terminal peptide, i.e. the V8 peptide containing Cys16. Presumably, the remaining 22% of the incorporated NBD (i.e. 0.17 mol/mol of subunit) was associated with the four naturally occurring Cys residues.

Miscellaneous Techniques. Intrinsic fluorescence was measured using the conditions described in Wang et al. (1993). Fluorescence of the W16C−NBD was measured at 23 °C as described in the figure legend. Rubisco activase protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

RESULTS

In Vitro Mutagenesis and Protein Purification. A cDNA clone engineered to resemble the mature tobacco Rubisco activase protein was expressed in *E. coli* cells as the wild type Rubisco activase (Salvucci & Klein, 1994). An N-terminally truncated protein (Trunc) was expressed from a derived clone, in which the codon for His51 was replaced with the initiator Met codon. Site-directed mutagenesis was used to replace Trp16 of the wild type protein with Ala (W16A), Tyr (W16Y), Phe (W16F), or Cys (W16C). Wild type and mutant proteins were purified from *E. coli* cells expressing the appropriate clone by ammonium sulfate precipitation, rate-zonal centrifugation, and anion exchange

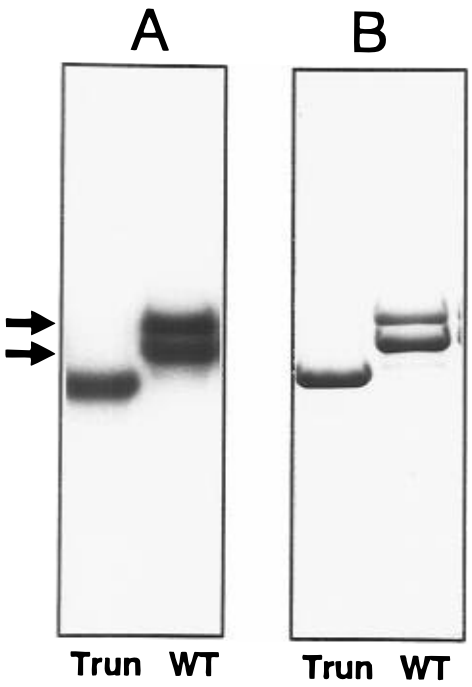

FIGURE 1: Photoaffinity labeling of Trunc and wild type (WT) Rubisco activase with 10 μM [γ-^{32}P]-2-N_3ATP. Wild type (10 μg, 9.3 μM protomer) and Trunc mutant (10 μg, 10.8 μM protomer) protein were photoaffinity labeled in a total volume of 25 μL: (A) autoradiogram and (B) Coomassie-stained gel. The positions of 42 and 44 kDa bands described in the text are indicated.

chromatography. Similar behavior through these various modes of separation indicated that none of the mutations caused major changes in the physical properties of the native enzymes.

ATP Binding. When wild type Rubisco activase is photoaffinity labeled with [γ-^{32}P]-2-N_3ATP, approximately 2 mol is incorporated per mole of subunit (Salvucci et al., 1994). 2-N_3ATP labels two distinct sites on the enzyme, the ATP-hydrolysis site and a separate site near the N-terminus. Modification of the ATP-hydrolysis site has no effect on the electrophoretic mobility of the polypeptide and can be prevented by inclusion of ATP or ADP. In the present study, ^{32}P was covalently incorporated into the 38 kDa polypeptide when the Trunc mutant was photoaffinity labeled with [γ-^{32}P]-2-N_3ATP (Figure 1). The modified polypeptide migrated as a single band on SDS−PAGE indistinguishable from the unmodified protein. The Trunc mutant was also modified when photoaffinity labeled with 10 μM [γ-^{32}P]-8-N_3ATP and was protected against photolabeling by ADP (data not shown). Previous results showed that, at this concentration, 8-N_3ATP preferentially labels the ATP-hydrolysis site (Salvucci et al., 1994). Thus, the results are consistent with the presence of a functional ATP-hydrolysis site in the Trunc mutant protein and the absence of the N-terminal site.

Judging from the intensity of the image on the autoradiogram, mutation of Trp16 to Ala, Tyr, Phe, or Cys had no effect on photoaffinity labeling of the ATP-hydrolysis site of Rubisco activase (42 kDa band) as compared to wild type (Figure 2). However, the W16A, W16Y, and W16F mutations abolished labeling of the N-terminal site (44 kDa band, Figure 2A−D). Slight labeling of this site occurred in the W16C mutant when a higher concentration of photoaffinity

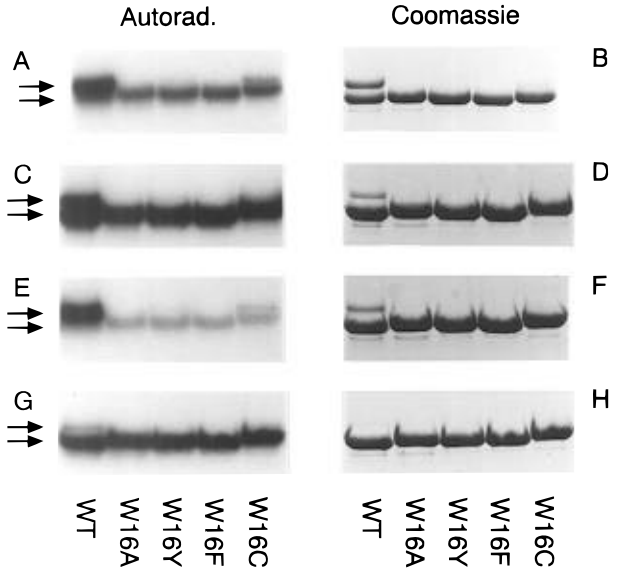

FIGURE 2: Photoaffinity labeling of wild type (WT) and Trp16 mutants of Rubisco activase with [γ-^{32}P]-2-N_3ATP. Wild type and mutant proteins (7 μg, 6.7 μM protomer) were photoaffinity labeled with 50 μM [γ-^{32}P]-2-N_3ATP (A and B) or 10 μM [γ-^{32}P]-2-N_3-ATP (C−H) with no addition (C and D) or with 200 μM ADP (E and F) or 100 μM tryptophan (G and H): (A, C, E, and G) autoradiographs and (B, D, F, and H) corresponding Coomassie-stained gels. The positions of 42 and 44 kDa bands described in the text are indicated.

Table 1: Kinetic Parameters for ATP Hydrolysis by Wild Type and Mutant Recombinant Rubisco Activase[a]

enzyme	V_{max}[b]	$[S]_{0.5}$[c]	Hill coefficient
wild type	1.07	0.124	1.52
Trunc	0.95	0.085	1.99
W16A	1.14	0.116	1.58
W16Y	0.95	0.116	1.53
W16F	1.05	0.111	1.63
W16C	0.95	0.111	1.82

[a] ATP hydrolysis was assayed spectrophotometrically by following coupled NADH oxidation. All reaction mixtures contained 0.466 μM activase. [b] (mol of ATP) (mol of activase)$^{-1}$ s^{-1}. [c] Half-maximal [ATP] in millimolar.

probe was used (Figure 2A,B). In the presence of 10 μM [γ-^{32}P]-2-N_3ATP, labeling of the ATP hydrolysis site (42 kDa band) was protected by 200 μM ADP, for both wild type and Trp16 mutant proteins (Figure 2E,F). In contrast, 100 μM tryptophan provided no protection against labeling of this site in wild type and the Trp16 mutants but protected against labeling of the N-terminal site in the wild type (44 kDa band, Figure 2G,H). These results demonstrate that mutation of Trp16 does not affect binding of an ATP analog to the ATP hydrolysis site but disrupts photoaffinity labeling of the N-terminal site.

ATP Hydrolysis. Purified Rubisco activase hydrolyzes ATP, even in the absence of Rubisco. Consistent with unimpaired ATP binding by the Trunc and Trp16 mutant proteins was the observation that these mutants also hydrolyze ATP. The V_{max} values, Hill coefficients, and half-maximal ATP concentrations for ATP hydrolysis by the mutants were similar to those of wild type enzyme (Table 1), indicating that removal of the N-terminus of Rubisco activase, or replacement of Trp16 with Ala, Tyr, Phe, or Cys, had no effect either on the affinity for ATP or on the maximal rate of ATP hydrolysis.

Table 2: Initial Rates of Rubisco Activation by Wild Type and Mutant Rubisco Activase[a]

	experiment 1		experiment 2[c]	
enzyme	rate[b]	% of WT	rate	% of WT
wild type	9.41		5.81	
Trunc	0.33	3.5		
W16A	0.83	8.8	0.58	10.0
W16Y			3.10	53.3
W16F			3.94	67.8
W16C			0.62	10.7

[a] Decarbamylated Rubisco complexed with RuBP was incubated at 4 (experiment 1) or 1.5 (experiment 2) mg mL^{-1} with 0.1 (experiment 1) or 0.2 (experiment 2) mg mL^{-1} Rubisco activase for 20 s. Rates are calculated by subtraction of the no-activase control. [b] (mol of Rubisco sites activated) (mol of activase)$^{-1}$ min^{-1}. [c] Experiment 2 corresponds to the time course in Figure 3.

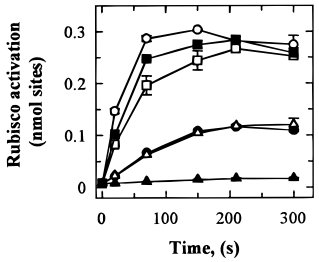

FIGURE 3: Rubisco activation by Trp16 mutants and wild type Rubisco activase. Decarbamylated Rubisco complexed with RuBP was incubated at 1.5 mg mL^{-1} in the absence (▲) or presence of 0.2 mg mL^{-1} wild type (○) or mutant W16F (■), W16Y (□), W16C (△), or W16A (●) Rubisco activase. At the indicated times, a 15 μL aliquot was removed for determination of Rubisco activity.

Rubisco Activation. Since selective modification of the N-terminal site of Rubisco activase with 2-N_3AMP inhibits Rubisco activation (Salvucci et al., 1994), we tested the Trp16 mutants for the ability to activate Rubisco. The initial rates of activation for the Trunc and W16A mutants were only 3 and 9% of that of the wild type, respectively (Table 2). In a separate set of experiments, the initial rates of activation for the W16A, W16C, W16Y, and W16F mutants were only 10, 11, 53, and 68% of that of wild type, respectively (Table 2). The decreased rates of Rubisco activation in the Trp16 mutants were not caused by a lower affinity for Rubisco since the K_m(Rubisco) values of the mutants were only slightly higher than those of the wild type (data not shown). The time course of Rubisco activation by the Trp16 mutants indicated that the W16C and W16A mutations had a marked effect on the final extent of Rubisco activation, whereas the W16Y and W16F mutations had a much smaller effect (Figure 3).

Activase−Activase Interactions. Rubisco activase is a self-associating protein, and its specific activity increases with the degree of aggregation (Salvucci, 1992). To investigate the ability of the W16A and Trunc mutants to undergo self-association, we measured ATPase activity at various concentrations of Rubisco activase. The specific ATPase activity of the mutants increased with the concentration of the enzyme in a relationship similar to that of wild type (data not shown). For example, specific activities increased approximately 50% with an increase in protein concentration from 0.116 to 0.466 μM.

ATPase activity of the Trunc and W16A mutant enzymes was additive with wild type enzyme in mixing experiments (data not shown), indicating that interactions between mutant and wild type enzymes were also normal. When mutant and wild type enzymes were mixed, the mutant enzymes had little effect on Rubisco activation by the wild type enzyme (data not shown). These results demonstrated that mixed complexes of wild type and W16A mutant maintained the ability to activate Rubisco, unlike complexes of wild type and the K247R mutant, a mutant with very low ATPase activity. The K247R mutant markedly inhibited activation of Rubisco by wild type Rubisco activase (Salvucci & Klein, 1994).

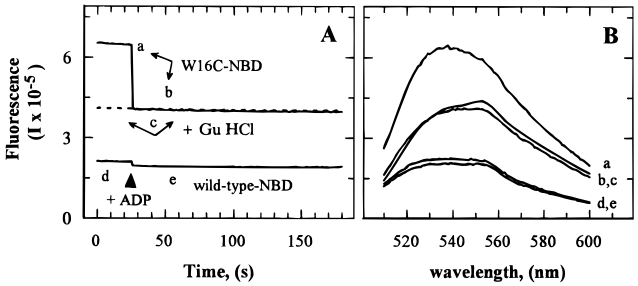

FIGURE 4: Effect of nucleotide and denaturants on the fluorescence emission of wild type and W16C Rubisco activases derivatized with (iodoacetyl)-NBD. Fluorescence of the derivatized proteins (40 μg) was measured in 100 mM Tricine-NaOH (pH 8.0), 10 mM $MgCl_2$, and 10 mM $NaHCO_3$ in the absence (a, b, d, and e) and presence (c) of 3 M guanidine hydrochloride (Gu-HCl) in a total volume of 1.5 mL. NBD fluorescence was excited at 467 nm. (A) Time course of the fluorescence emission at 537 nm for wild type (d and e) and mutant (a−c) Rubisco activase. ADP (0.2 mM) was added at the time indicated by the arrowhead. (B) The emission spectra of panel A before (a, c, and d) and after (b, c, and e) the addition of ADP.

Changes in Fluorescence of Chemically Modified W16C. To determine if changes in conformation occur in the vicinity of Trp16, the W16C mutant was chemically modified at Cys16 with (iodoacetyl)-NBD, an environmentally sensitive adduct whose fluorescence increases as the environment becomes more hydrophobic. Addition of decarbamylated Rubisco complexed with ribulose 1,5-bisphosphate to the chemically modified mutant either in the presence or in the absence of ATP, ADP, or ATPγ had no effect on NBD fluorescence (data not shown). However, addition of low concentrations of ADP caused a marked quenching of the NBD fluorescence and a shift in the emission spectrum to a higher wavelength (Figure 4, traces a and b). The effect was saturated at low concentrations of ADP (i.e. <50 μM) and also occurred with ATP and ATPγS (data not shown). In contrast, the fluorescence intensity of denatured W16C−NBD was unaffected by the presence of nucleotide (Figure 4, + Gu HCl, trace c) and was similar to the intensity of undenatured enzyme in the presence of nucleotide. As an additional control, wild type Rubisco activase, chemically modified with (iodoacetyl)-NBD, was also examined to exclude the possibility that one or more of the naturally occurring Cys residues were contributing to the quenching of NBD fluorescence. The results showed that the fluorescence intensity of wild type Rubisco activase was low compared with that of W16C−NBD and was relatively unaffected by addition of nucleotide (Figure 4, traces d and e). The contribution of one or more of the four naturally occurring Cys residues to NBD fluorescence would be even less in the W16C mutant since these residues were less heavily modified in the W16C mutant than in the wild type (see Materials and Methods).

Changes in Intrinsic Fluorescence. Intrinsic tryptophan fluorescence increases when Rubisco activase aggregates in

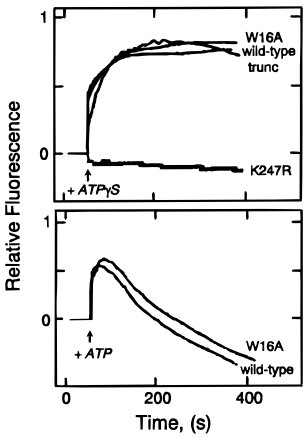

FIGURE 5: Intrinsic fluorescence of wild type and mutant Rubisco activases in response to addition of 0.2 mM ATPγS or ATP. ATP or ATPγS was added at the time indicated by the arrow. The wavelengths for excitation and emission were 296 and 345 nm, respectively.

response to Mg^{2+}−ATP or Mg^{2+}−ATPγS binding (Wang et al., 1993). In the presence of Mg^{2+} but in the absence of ATP, the intrinsic fluorescence of the W16A and Trunc mutants were approximately 75% of the wild type (data not shown), consistent with the removal of one of the four Trp residues. Addition of ATPγS caused a similar increase in the fluorescence of wild type, W16A, and Trunc but no increase in fluorescence of the K247R mutant (Salvucci & Klein, 1994), the negative control (Figure 5). As reported previously (Wang et al., 1993), ATP also induced an increase in fluorescence of wild type Rubisco activase, but the increase was only transient as accumulating ADP competed with ATP for binding. Similar effects of ATP on intrinsic fluorescence were observed with the W16A mutant. Thus, the conformational changes that are induced by ATPγS−Mg^{2+} or ATP−Mg^{2+} binding and subsequent aggregation of the protein appeared to be normal in the Trunc and W16A mutants.

DISCUSSION

Although the mechanism of interaction between Rubisco activase and Rubisco is not known, it is likely that energy derived from ATP hydrolysis is used by activase to change the conformation of Rubisco in a way that favors dissociation of the inhibitory sugar phosphate. In this study, directed mutants of Rubisco activase were generated to gain insights into the mechanism of interaction of these proteins. The choice of mutations was based on information from photoaffinity labeling studies which showed that (a) Rubisco activase missing as many as 55 amino acids from the N-terminus is still able to bind adenine nucleotide analogs and (b) modification of Rubisco activase in the vicinity of Trp16 markedly inhibits Rubisco activation without affecting ATP hydrolysis (Salvucci et al., 1993, 1994).

The Trunc mutant, in which the first 50 amino acids were deleted from the N-terminus of the protein, caused a specific disruption of the ability of Rubisco activase to activate Rubisco. To examine the role of the N-terminal domain in finer detail, the Trp residue at position 16 was changed to Ala, Cys, Phe, or Tyr. The W16A and W16C mutants were severely inhibited in their ability to activate Rubisco (Figure 3, Table 2) but were indistinguishable from wild type with respect to the ATP-hydrolyzing and self-associating properties of the enzyme. Possibly, Trp16 mediates a conformational change or other process within the activase protein that is indirectly required for interaction with Rubisco. Alternatively, Trp16 may directly mediate interaction between the two proteins. Analysis of surface residues in protein crystal structures has shown a predominance of hydrophobic residues such as Trp on protein surfaces mediating protein−protein interactions (Young et al., 1994). In either case, the less pronounced effect of the W16Y and particularly W16F mutants (Figure 3, Table 2) indicates the preference for an aromatic residue at this position. Hydrophobicity alone cannot explain this result, since Cys is more hydrophobic than Tyr (Young et al., 1994).

The W16A, W16Y, and W16F mutations also abolished labeling of the N-terminal site with [γ-^{32}P]-2-N_3ATP (Figure 2), indicating that Trp16 may be involved in the interaction with the azidoadenine nucleotide. Slight labeling of this site occurred in the W16C mutant when a higher concentration of photoaffinity probe was used (Figure 2). Therefore, the properties of this site that determine the ability to interact with Rubisco are not identical to those that determine the ability to bind the azidoadenine. Nevertheless, structural features necessary for Rubisco activation may provide an environment that is conducive to azidoadenine nucleotide binding.

The Trunc mutant provided an important insight into the domain structure of Rubisco activase. Proteins consist of one or more domains, which are defined as folding independently, and these domains may have separable functions. Frequently, the connecting loop between domains is relatively exposed and susceptible to proteolytic attack. The earlier identification of 39 and 38 kDa breakdown products of leaf Rubisco activase, lacking the first 40 and 55 amino acids, respectively, of the mature protein, and capable of binding ATPγ−benzophenone (Salvucci et al., 1993), indicated that this N-terminal region may be excluded from the ATP-binding domain. This conclusion was confirmed and extended in the present study (Figure 1) by showing that the Trunc mutant protein has unimpaired ATPase activity (Table 1). Similarly, concentration-dependent self-association of Rubisco activase was unperturbed by the Trunc mutation. Thus, the Trunc mutant is unimpaired in the ability to bind ATP, undergo self-association following ATP binding, and finally hydrolyze ATP. The N-terminal 50 amino acids deleted in the Trunc mutant are therefore not part of the domain(s) involved in ATP hydrolysis and self-association but define at least part of a domain specifically involved in activation of Rubisco. This result is consistent with the relatively distal position of the ATP-binding site in Rubisco activase, as compared with that of other ATPases (Traut, 1994).

The lack of effect of the Trunc and W16A mutant enzymes on Rubisco activation by wild type Rubisco activase was in sharp contrast to the pronounced inhibition caused by mixing wild type Rubisco activase with the K247R mutant, a mutant that is severely impaired in ATP hydrolysis (Salvucci & Klein, 1994). These results suggest that the mechanism of Rubisco activation involves cooperativity at the level of ATP hydrolysis, but not at the level of Rubisco activation. In this way, Rubisco activase may be similar to the GroEL chaperonins whose subunit toroids are allosterically connected via nucleotide hydrolysis (Hayer-Hartl et al., 1995).

Chemical modification of the W16C mutant with an environmentally sensitive fluorophor revealed that nucleotide binding causes a change in the conformation of Rubisco activase around position 16. Fluorescence quenching and the shifting of the emission spectrum to a longer wavelength upon addition of nucleotide (Figure 4) indicate that position 16 of Rubisco activase becomes more solvent accessible in response to nucleotide binding. Since this change occurred instantaneously and with very low concentrations of nucleotide, it is distinct from the changes in intrinsic fluorescence that accompany nucleotide-promoted aggregation of the enzyme [Wang et al. (1993) and see below]. Instead, the quenching of fluorescence that occurred upon addition of nucleotide to W16C−NBD more closely resembled the nucleotide-dependent changes in 1-anilinonaphthalene-8-sulfonic acid fluorescence reported by Wang and Portis (1992) and is consistent with the existence of an intermediate state of the enzyme−nucleotide complex (Wang et al., 1993). On the basis of the similarities between 1-anilinonaphthalene-8-sulfonic acid fluorescence and the fluorescence of the W16C−NBD, we believe that the region of Rubisco activase around Trp16 may be the major site of 1-anilinonaphthalene-8-sulfonic acid binding.

The increase in the intrinsic fluorescence of Rubisco activase upon ATP binding is thought to report a conformational change resulting in an increase in the hydrophobicity of the environment of a tryptophan residue (Wang et al., 1993). Which of the four Trp residues of Rubisco activase is involved in the aggregation-induced fluorescence increase is unknown. However, the Trunc and W16A mutants undergo this fluorescence increase upon ATP binding (Figure 5), demonstrating that Trp16 is not the residue involved. Of the remaining Trp residues, Trp109 and Trp250 are found in all plants sequenced to date, while Trp305 is replaced by Phe in *Arabidopsis thaliana* (Orozco et al., 1993). On the basis of differences in the kinetics of ATP binding and the ATP-induced fluorescence, Wang et al. (1993) argued that Trp109, which immediately precedes the nucleotide phosphate-binding domain (i.e. the P-loop), is not the residue involved in the fluorescence increase. We favor the possibility that Trp250 is the residue which reports a conformational change upon aggregation on the basis of its proximity to a highly reactive and essential lysine at position 247 (Salvucci, 1993; Salvucci & Klein, 1994). This conclusion is supported by the observation that there is no ATPγS-induced increase in intrinsic fluorescence of the K247R mutant (Figure 5).

ACKNOWLEDGMENT

Dr. R. R. Klein (USDA, Philadelphia, PA) contributed to construction of the Trunc mutant. We thank J. Anderson for skilled technical assistance, Dr. K. Hoober (Arizona State University) for use of the spectrofluorometer, and Dr. R. L. Houtz and the Department of Horticulture and Landscape Architecture at the University of Kentucky for providing laboratory space to F.J.L. during completion of this work. The [γ-^{32}P]-2-N_3ATP used in this study was a generous gift of Dr. B. E. Haley (University of Kentucky).

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BI9604901