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Photoaffinity Labeling of the ATP Binding Domain of Rubisco Activase and a Separate Domain Involved in the Activation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase

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ABSTRACT: Photoaffinity labeling of Rubisco activase with 2- and 8-N_3ATP was used to identify the adenine binding domain for ATP. Rubisco activase hydrolyzed both of these analogs of ATP and used their hydrolysis to support a low rate of Rubisco activation. When irradiated with ultraviolet light, these and other azido-substituted adenine nucleotides covalently modified Rubisco activase at two distinct binding sites. Competition binding experiments with ATP and ADP showed that one of the sites was the ATP binding domain. The other site was not a nucleotide binding domain *per se* but would bind adenine nucleotides if an azido moiety was present on the base. Tryptophan and other indoles prevented azidoadenine nucleotides from labeling this domain but afforded little protection to the ATP binding domain. The ability to selectively protect each of the two binding sites made it possible to localize the adenine binding domain for ATP to the region of Rubisco activase from N68-D74 and the other binding domain to a region near the N-terminus from Q10 to D14. Modification of the region from Q10 to D14 by photoaffinity labeling prevented Rubisco activase from promoting activation of Rubisco without affecting ATP hydrolysis. These data suggest that a specific region of Rubisco activase near the N-terminus may be a site of interaction with Rubisco. Binding of azidoadenine nucleotides in this region appears to be fortuitous and may involve base-stacking with the species-invariant Trp at position 16 and hydrogen bonding of the azido moiety.

The chloroplastic protein Rubisco activase promotes the controlled release of tight-binding sugar−phosphates from ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39)[1] (Robinson & Portis, 1988; Lilley & Portis, 1990; Wang & Portis, 1992). This action serves to "activate" Rubisco by freeing the Rubisco active-site of compounds that either hinder carbamylation of the activator lysine or block the substrate, RuBP, from binding to the carbamylated enzyme [reviewed in Portis (1992) and Hartman and Harpel (1994)]. Hydrolysis of ATP by Rubisco activase is required for activating Rubisco, presumably to supply energy for inducing specific conformational changes that alter the binding affinity of Rubisco for sugar−phosphates (Wang & Portis, 1992; Salvucci, 1992). The dependence of Rubisco activase activity on ATP serves an important regulatory function in photosynthesis by coupling the rate of CO_2 fixation via Rubisco to the rate of electron transport activity (Salvucci et al., 1985, 1986).

The primary structure of Rubisco activase includes a P-loop (Werneke et al., 1988; Shen et al., 1991), a glycine-rich region involved in nucleotide−phosphate binding (Pai et al., 1990; Saraste et al., 1990). In a previous study, photoaffinity labeling with ATPγBP, an analog of ATP with a photoactive *N*-(4-(benzoyl)phenylmethyl)phosphoramide in place of the γ-phosphate, was used to identify the regions of Rubisco activase that participate in binding of the ATP γ-phosphate (Salvucci et al., 1993). ATPγBP specifically modified the ATP binding domain of Rubisco activase, labeling the region adjacent to the P-loop and a second region from V223-T234. The latter segment is proximal to K247, a highly reactive lysyl residue that is required for maximal activity, but not for ATP binding (Salvucci, 1993; Salvucci & Klein, 1994). Chemical modification of K247 reduced the efficiency of photoaffinity labeling with ATPγBP, indicating that this residue may participate in interactions necessary for coordinating the γ-phosphate of ATP (Salvucci, 1993).

In the present study, photoaffinity labeling was conducted with the [γ-^{32}P]2- and 8-azido analogs of ATP. Originally our intent was to identify the adenine binding domain for ATP. However, the azido-substituted analogs of ATP unexpectedly labeled two separate sites on the protein. One of the sites was the ATP binding domain. The other site was separate from the ATP binding domain, but its modification interfered with the ability of Rubisco activase to

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[1] Abbreviations: ATPγBP, monoanhydride of ADP with *N*-(4-(benzoyl)phenylmethyl)phosphoramide; 2-N_3Ado, 2-azidoadenosine; 2-N_3AMP, 2-azidoadenosine 5′-monophosphate; 2-N_3ATP, 2-azidoadenosine 5′-triphosphate; 8-N_3Ap_4A, 8-azidoadenosine 5′,5″-tetraphosphate adenosine; 8-N_3ATP, 8-azidoadenosine 5′-triphosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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promote Rubisco activation. This second site was localized to a region of the protein near the N-terminus.

MATERIALS AND METHODS

Chemicals. Sodium [^{14}C]bicarbonate was purchased from Amersham Corp.[2] (Arlington Heights, IL). Ribulose 1,5-bisphosphate was synthesized from ribose-5-P as described by Horecker et al. (1956). 2-N_3Ado, [γ-^{32}P]2-N_3ATP, [α-^{32}P]2-N_3AMP, [γ-^{32}P]8-N_3ATP, [γ-^{32}P]8-N_3ADP, [β'-^{32}P]8-N_3Ap_4A, [γ-^{32}P]ATPγBP, and [γ-^{32}P]8-N_3ATPγBP were synthesized as described previously (Schaeffer & Thomas, 1958; Yoshikawa et al., 1967; Symons, 1968; Potter & Haley, 1983; Salvucci et al., 1992; Rajagopalan et al., 1993). [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc. (Irvine, CA). Poly(ethylene glycol)-3350, coupling enzymes, and other reagents, unless otherwise indicated, were from Sigma Chemical Co. (St. Louis, MO).

Protein Purification. Recombinant Rubisco activase was produced in and purified from *Escherichia coli* cells that harbor a cDNA clone engineered to resemble the mature tobacco enzyme (Salvucci & Klein, 1994). Rubisco was isolated from leaves of *Nicotiana tabacum* L. cv. KY-14 as described previously (Klein & Salvucci, 1992).

Enzyme Assays. Hydrolysis of [γ-^{32}P]ATP and [γ-^{32}P]2- and 8-N_3ATP was performed at 20 °C in 5 μL reactions containing 100 mM Tricine-NaOH, pH 8, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM nucleotide, and 0.5 μg of Rubisco activase (11.9 pmol of monomer). Reactions were initiated by addition of enzyme and terminated after 5 or 10 min by transfer of 0.5-μL aliquots to PEI-Cellulose TLC plates (Sigma Chemical Co.). [^{32}P]P_i was separated from the nucleotide substrate by ascending TLC using 0.25 M NH_4HCO_3 as the mobile phase, and the amount produced was determined by counting ^{32}P cpm directly on the plate using an AMBIS 4000 multiwire proportional counter (AMBIS, Inc., San Diego, CA). All assays were conducted in triplicate.

ATPase activity was also determined spectrophotometrically by monitoring ADP production via NADH oxidation (Salvucci, 1992). Assays were conducted at 25 °C and in duplicate. Activation of the decarbamylated Rubisco-RuBP complex was measured at 25 °C by the change in Rubisco activity that occurred after a 30-s incubation of 1.5 mg/mL Rubisco with 0.2 mg/mL Rubisco activase (Salvucci, 1992). The rate of activation is expressed as nmol Rubisco active sites carbamylated min^{-1} and is based on comparison with the activity of fully carbamylated Rubisco (Salvucci, 1992). The results presented represent the means of duplicate assays.

Photoaffinity Labeling. Photoaffinity labeling of Rubisco activase was performed as described previously with the additions noted in the text (Salvucci et al., 1993). Reaction mixtures were quenched by addition of SDS and dithiothreitol and electrophoresed in 7.5−15% SDS−PAGE gels (Klein & Salvucci, 1992). ^{32}P incorporation into the Rubisco activase subunit was either visualized by autoradiography or quantified directly in the dried gels as described above. For measurement of enzyme activity, photoaffinity labeling reactions were quenched by addition of dithiothreitol to 10 mM, and aliquots were removed for determination of ATPase or Rubisco activation.

To determine the stoichiometry of photoaffinity labeling, 50 μg of Rubisco activase (1.19 nmol) was photoaffinity labeled with 100 μM [γ-^{32}P]2- or 8-N_3ATP in 50 mM Hepes-KOH, pH 7.2, 50 mM sucrose, 2.5 mM $MgCl_2$, and 10 mM EDTA (buffer A) in a total volume of 100 μL. Protein in the reaction mixtures was immediately precipitated by addition of 1.5 volumes of saturated ammonium sulfate, pH 7.0, and was collected by centrifugation. The protein pellet was resuspended in 100 μL of buffer A and desalted by centrifugal gel filtration through a 1 × 2.5 cm column of Sephadex G-50-80 to remove noncovalently bound nucleotide (Salvucci, 1992). ^{32}P dpm and protein concentration in the desalted sample were determined in duplicate by liquid scintillation spectroscopy and a dye-binding assay (Bradford, 1976), respectively. Carry-over of unincorporated ^{32}P dpm was less than 0.1%. The results presented represent the means ± standard errors of triplicate samples. ^{32}P incorporation was also determined after SDS−PAGE of the desalted protein. Following electrophoresis in 11% minigels (Klein & Salvucci, 1992), polypeptides were visualized by staining (see below), and ^{32}P dpm in the stained bands were determined by liquid scintillation spectroscopy.

Isolation of Photolabeled Peptides. Photolabeled peptides were isolated from Rubisco activase by metal−ion chelate chromatography (Salvucci et al., 1992; Shoemaker & Haley, 1993). Rubisco activase was photoaffinity labeled in buffer A as described in the text, and, after two rounds of photolabeling, the modified protein was precipitated by the addition of two volumes of saturated ammonium sulfate, pH 7.0. After 30 min at 4 °C, precipitated protein was collected by centrifugation, resuspended in 50 mM ammonium acetate, pH 8.0, and desalted in this buffer by centrifugal gel filtration through a 1 × 2.5 cm column of Sephadex G-50-80. Urea was added to a final concentration of 2 M, and peptides were generated by incubation for 3 h at 25 °C with 10% (w/w) of the indicated proteases.

In preparation for chromatography, the pH of the peptide digest was adjusted to 6.0 by addition of an equal volume of 300 mM ammonium acetate, pH 6.0. The peptide mixture was applied to a 0.5-mL column of Al^{3+}−iminodiacetic acid−Sepharose at pH 6.0, and unmodified peptides were eluted with 20 mL of 0.1 M ammonium acetate, pH 6.0. Radioactivity in the buffer eluant was generally less than 10% of the amount applied to the column. The column was then treated with 5 mM KH_2PO_4, pH 7.5, to elute photolabeled peptides (Salvucci et al., 1992). The P_i eluant, which contained approximately 70−80% of the radioactivity applied to the column, was either further fractionated by reverse-phase HPLC (Salvucci et al., 1993) or concentrated by lyophilization and submitted directly for sequencing. Automated Edman degradation analysis was performed at the University of Kentucky Macromolecular Facility using an Applied Biosystems 477A pulse liquid protein sequencer with on-line 120A PTH identification.

Miscellaneous Techniques. SDS−PAGE was performed as described previously (Klein & Salvucci, 1992). Gels were stained with Coomassie brilliant blue R-250 and destained overnight to visualize the polypeptides (Klein & Salvucci, 1992). The concentration of Rubisco activase protein was determined by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard. Rubisco protein

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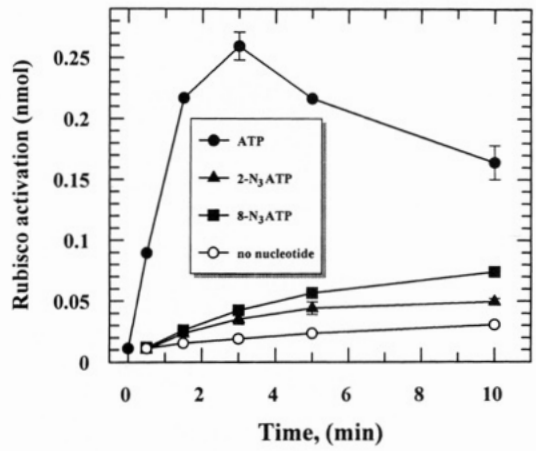

FIGURE 1: Effect of ATP and 2- and 8-N_3ATP on activation of the decarbamylated Rubisco−RuBP complex by Rubisco activase. Decarbamylated Rubisco complexed with RuBP (1.5 mg/mL) was incubated with recombinant tobacco Rubisco activase (0.2 mg/mL) in the presence of 2 mM ATP (●), 2-N_3ATP (▲), 8-N_3ATP (■), or in the absence of nucleotide (○). Aliquots containing 0.32 nmol of Rubisco protomer were removed at the indicated times for measurement of Rubisco activity. Rubisco activation, which is expressed as nmol Rubisco protomer carbamylated, was determined by the change in Rubisco activity.

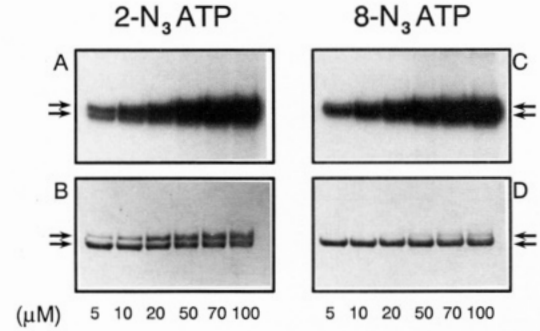

FIGURE 2: Photoaffinity labeling of recombinant tobacco Rubisco activase with [γ-^{32}P]2- and 8-N_3ATP. Autoradiogram (A and C) and Coomassie blue-stained gel (B and D) of Rubisco activase polypeptides, separated by SDS−PAGE, after incubation and photolysis with the indicated concentrations of [γ-^{32}P]2-N_3ATP (A and B) or 8-N_3ATP (C and D). The arrows indicate the positions of the two radiolabeled or stained bands at 42 and 44 kDa.

concentration was determined spectrophotometrically (Salvucci, 1992).

RESULTS

2-N_3ATP and 8-N_3ATP as Substrates for Rubisco Activase. Experiments were conducted to determine if Rubisco activase could hydrolyze 2- and 8-N_3ATP or use these analogs in place of ATP to promote activation of the decarbamylated Rubisco−RuBP complex. Product analysis showed that recombinant tobacco Rubisco activase produced [^{32}P]P_i from γ-^{32}P-labeled 8- and 2-N_3ATP. The rates of hydrolysis were 0.055 ± 0.004 and 0.019 ± 0.001 μmol min^{-1} mg of protein^{-1} for 2-N_3ATP and 8-N_3ATP, respectively, equivalent to about 15% and 5% of the rate of ATP hydrolysis.

Time course experiments showed that Rubisco activase used N_3ATP to promote activation of the decarbamylated Rubisco−RuBP complex (Figure 1). However, after correcting for the no nucleotide control, the initial rates of activation with saturating concentrations of 2- and 8-N_3ATP (see Figure 2) were only about 3% and 5%, respectively, of the rate with ATP. Control experiments showed that 2- and 8-N_3ATP alone did not increase the rate of Rubisco activation in the absence of Rubisco activase (data not shown).

Photoaffinity Labeling of Rubisco Activase with 2-N_3ATP and 8-N_3ATP. Recombinant Rubisco activase migrates as a single 42-kDa band on SDS−PAGE (Salvucci & Klein, 1994). When the enzyme was photoaffinity labeled with [γ-^{32}P]2- and 8-N_3ATP and subjected to SDS−PAGE, two distinct bands at 44 and 42 kDa were visible on the autoradiograms (Figure 2A,C) and Coomassie blue-stained gels (Figure 2B,D). Similar results were obtained when Rubisco activase was photoaffinity labeled with ^{32}P-labeled 2-N_3AMP, 8-N_3ADP, 8-N_3Ap_4A, and 8-N_3ATPγBP (data not shown). ^{32}P incorporation and the production of two labeled bands occurred only when Rubisco activase was photolyzed with ultraviolet light in the presence of azidoadenine nucleotides (data not shown). In contrast, photoaffinity labeling of recombinant (data not shown) or leaf Rubisco activase (Salvucci et al., 1993) with saturating concentrations of [γ-^{32}P]ATPγBP produced only a single labeled band at 42 kDa (see below). This result demonstrated that production of the 44-kDa polypeptide was associated with modification of Rubisco activase by azidoadenine nucleotides. Of the compounds examined, 2-N_3AMP exhibited the lowest apparent K_d and the highest efficiency for labeling of the 44-kDa polypeptide (data not shown).

The amount of [^{32}P]N_3ATP incorporated into Rubisco activase increased with increasing concentration of photoaffinity reagent, approaching saturation at concentrations of 100 μM N_3ATP (Figure 2A,C). For [γ-^{32}P]2-N_3ATP, the amount of ^{32}P associated with the 44-kDa band was greater at all concentrations than the amount associated with the 42-kDa band. The opposite was true for [γ-^{32}P]8-N_3ATP, in fact, at the lowest concentration of 8-N_3ATP only the 42-kDa band was visible on the autoradiogram.

Stoichiometry of Photoaffinity Labeling. The stoichiometry of photoaffinity labeling was determined from the amount of ^{32}P incorporated into Rubisco activase. Following removal of noncovalently bound nucleotide, there were 1.87 ± 0.2 mol 2-N_3ATP bound per mol Rubisco activase monomer after photoaffinity labeling with 100 μM 2-N_3ATP and 0.97 ± 0.1 mol of 8-N_3ATP bound per mol of monomer after photoaffinity labeling with 100 μM 8-N_3ATP. SDS−PAGE followed by a 1 h staining and overnight destaining in 40% methanol/7.5% acetic acid reduced the total amount of [γ-^{32}P]2- and 8-N_3ATP bound to 0.3 ± 0.02 and 0.4 ± 0.04 mol per mol of Rubisco activase monomer, respectively.

Effect of ATP and ADP on Photoaffinity Labeling. Thus far, the results show that covalent modification of Rubisco activase by azidoadenine nucleotides produced two types of modified polypeptides distinguishable by their electrophoretic mobility on SDS−PAGE gels. If we conclude that there are two potential binding sites for azidoadenine nucleotides on Rubisco activase based on the stoichiometry of photoaffinity labeling, then the two electrophoretic forms could be produced if the two binding domains are distinct and only one affects the electrophoretic mobility of the polypeptide on SDS−PAGE. Alternatively, the electrophoretic mobility may not change unless both sites are modified.

To distinguish between these possibilities, we examined the effect of natural ligands of the ATP binding domain on photoaffinity labeling of Rubisco activase with 10 μM [γ-^{32}P]2- and 8-N_3ATP (Figure 3). At this concentration of reagent, only photoaffinity labeling with 2-N_3ATP produced

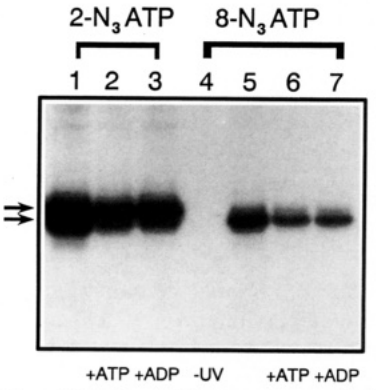

FIGURE 3: Effect of ATP and ADP on photoaffinity labeling of recombinant tobacco Rubisco activase with [γ-^{32}P]2- and 8-N_3ATP. Autoradiogram of Rubisco activase polypeptides, separated by SDS−PAGE, after incubation and photolysis with 10 μM [γ-^{32}P]2-N_3ATP (lanes 1−3) or 8-N_3ATP (5−7) in the absence (lanes 1 and 5) and presence of 300 μM ATP (lanes 2 and 6) or ADP (lanes 3 and 7). In lane 4, Rubisco activase was incubated with [γ-^{32}P]8-N_3ATP but not photolyzed. The arrows indicate the positions of the two radiolabeled bands at 42 and 44 kDa.

the modified form of Rubisco activase that migrated at 44 kDa (see also Figure 2). ATP and ADP afforded little protection against incorporation of [γ-^{32}P]2-N_3ATP into the 44-kDa polypeptide but afforded considerable protection against labeling of the 42-kDa polypeptide. This selective protection by ATP and ADP supports the existence of two distinct binding domains on Rubisco activase, both of which can bind azidoadenine nucleotides, but only one affects the electrophoretic mobility of the polypeptide. Photoaffinity labeling of the 42-kDa polypeptide probably represents modification of the adenine binding domain for ATP since natural nucleotide ligands effectively protected against labeling.

Effect of Tryptophan and Tryptophan Analogs on Photoaffinity Labeling. In competition binding experiments, ATP, ADP, AMP, GTP, NAD^+, NADH, $NADP^+$, and UTP all failed to block incorporation of 2-N_3ATP into the 44-kDa form of Rubisco activase (Figure 3 and data not shown). The inability of naturally occurring nucleotides to protect against photoaffinity labeling suggested that the domain associated with the 44-kDa polypeptide is not a nucleotide binding domain *per se*. For this reason, competition binding experiments were conducted with another group of naturally occurring aromatic compounds, the aromatic amino acids Phe, Tyr, and Trp. Of the three, only Trp reduced incorporation of 2-N_3ATP into the 44-kDa form of modified Rubisco activase (data not shown).

Figure 4 shows that micromolar concentrations of Trp afforded nearly complete protection against incorporation of [γ-^{32}P]2-N_3ATP into the 44-kDa form of modified Rubisco activase but were much less effective in blocking incorporation into the 42 kDa form. Included for comparison is ADP which had the opposite effect. The protection afforded by Trp was not due to shielding of the activating ultraviolet light since protection occurred with low concentrations of Trp and was selective for the 44-kDa polypeptide. Trp and the indole-based plant growth regulator, indole acetic acid, had no effect on ATP hydrolysis or Rubisco activation when included in the Rubisco activase assays at 1 mM (data not shown).

Tryptophan analogs also blocked incorporation of [γ-^{32}P]2-N_3ATP into the 44-kDa form of Rubisco activase (Table 1).

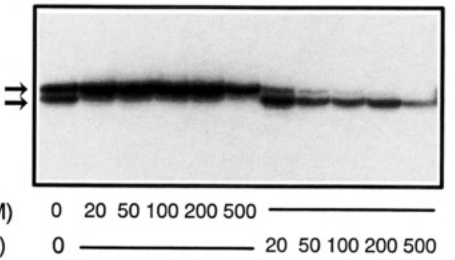

FIGURE 4: Effect of ADP and Trp concentration on photoaffinity labeling of recombinant tobacco Rubisco activase with [γ-^{32}P]2-N_3ATP. Autoradiogram of Rubisco activase polypeptides, separated by SDS−PAGE, after incubation and photolysis with 10 μM [γ-^{32}P]2-N_3ATP in the absence (lane 1) or presence of the indicated concentrations of Trp (lanes 2−6) or ADP (lanes 7−11). The arrows indicate the positions of the two radiolabeled bands at 42 and 44 kDa.

Table 1: Effect of Various Indole-Containing Compounds and Adenine Nucleotide Derivatives on Incorporation of [^{32}P]2-N_3ATP into the 44-kDa Form of Rubisco Activase[a]

compound	2-N_3ATP incorporated (relative units)
none	1.00
tryptophan	0.19
tryptamine	0.14
5-hydroxytryptamine	0.09
tryptophanamide	0.19
indole acetic acid	0.09
adenosine	0.97
2-chloroadenosine	1.04
6-benzyladenosine	0.84
2-azidoadenosine	0.29
ADP	0.94

[a] Photoaffinity labeling was conducted with 10 μM [γ-^{32}P]2-N_3ATP in the presence of 50 μM of the indicated indole or 20 μM [γ-^{32}P]2-N_3ATP in the presence of 100 μM of the indicated adenine nucleotides.

Both deaminated and decarboxylated analogs were effective in reducing incorporation of [γ-^{32}P]2-N_3ATP, some by as much as 91%. 2-N_3Ado also afforded considerable protection against incorporation of [γ-^{32}P]2-N_3ATP into the 44-kDa polypeptide. In contast, adenosine and the 2-chloro- and 6-benzyl- derivatives afforded little or no protection against incorporation.

Inhibition of Rubisco Activase Activity by Photoaffinity Labeling. The ability to selectively protect each of the two domains of Rubisco activase against modification with azidonucleotides enabled us to assess the relative involvement of the putative domain associated with the 44-kDa polypeptide in ATP hydrolysis and Rubisco activation. 2-N_3AMP was used for these experiments to obtain a higher efficiency of incorporation into this domain (see above). Photoaffinity labeling with 2-N_3AMP caused significant inhibition of both ATP hydrolysis and the ability of Rubisco activase to promote activation of Rubisco (Figure 5). However, the inhibitory effect was much greater toward Rubisco activation, which decreased almost 90% in response to photoaffinity labeling compared to a 60% decrease in ATPase activity. Trp protected against inactivation of ATPase and Rubisco activation, but as indicated by the ratio of activation/ATPase, the protective effect was much greater toward Rubisco activation. SDS−PAGE showed that restoration of Rubisco activation correlated with a decrease in the amount of the 44-kDa polypeptide formed in response to photoaffinity labeling with 2-N_3AMP (Figure 5). In contrast, ADP had little effect on the amount of the 44-kDa

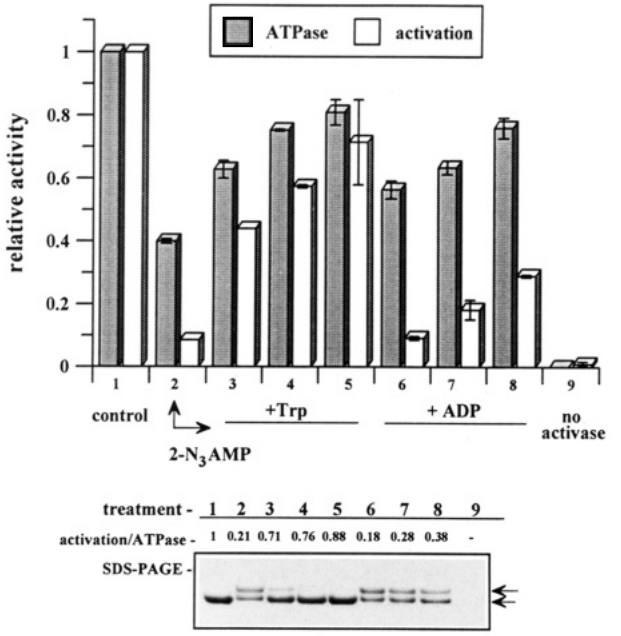

FIGURE 5: Effect of ADP and Trp concentration on the inhibition of Rubisco activase activity by photoaffinity labeling with 2-N_3-AMP. Recombinant tobacco Rubisco activase was photoaffinity labeled with 50 μM 2-N_3AMP in the absence (2) or presence of 50 (3, 6), 200 (4, 7), and 500 μM (5, 8) of Trp (3−5) or ADP (6−8). Aliquots were assayed for ATPase activity and the ability to promote activation of the decarbamylated Rubisco−RuBP complex or electrophoresed in an SDS−PAGE gel that was then stained with Coomassie brilliant blue (SDS-PAGE). Relative activity is expressed as v_i/v_c, where v_i refers to the activity of Rubisco activase after photoaffinity labeling in the presence of 2-N_3AMP and v_c refers to the activity of Rubisco activase that was photolyzed in the absence of 2-N_3AMP (1). The arrows indicate the positions of the two Rubisco activase polypeptides at 42 and 44 kDa.

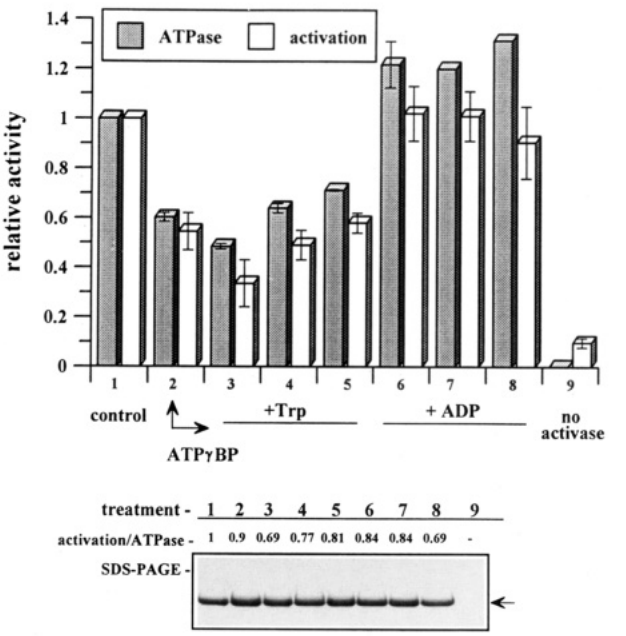

FIGURE 6: Effect of ADP and Trp concentration on the inhibition of Rubisco activase activity by photoaffinity labeling with ATPγBP. Recombinant tobacco Rubisco activase was photoaffinity labeled with 25 μM ATPγBP in the absence (2) or presence of Trp (3−5) or ADP (6−8) at 50 (3, 6), 200 (4, 7), and 500 μM (5, 8). Aliquots were assayed for ATPase activity and the ability to promote activation of the decarbamylated Rubisco−RuBP complex or electrophoresed in an SDS−PAGE gel that was then stained with Coomassie brilliant blue (SDS-PAGE). Relative activity is expressed as v_i/v_c, where v_i refers to the activity of Rubisco activase after photoaffinity labeling in the presence of ATPγBP and v_c refers to the activity of Rubisco activase that was photolyzed in the absence of ATPγBP (1). The arrow indicates the position of the Rubisco activase polypeptide at 42 kDa.

polypeptide produced. ADP was relatively ineffective in protecting against loss of the ability to activate Rubisco but was considerably more effective in protecting against loss of ATPase activity (Figure 5).

Previous results showed that ATPγBP selectively modifies tobacco leaf Rubisco activase at the ATP binding domain (Salvucci et al., 1993). In the present study, recombinant Rubisco activase was photoaffinity labeled with ATPγBP to compare the effects of this reagent with those of 2-N_3-AMP (Figure 5). Photoaffinity labeling of Rubisco activase with ATPγBP reduced ATPase activity and Rubisco activation, both by about 40% (Figure 6). Trp was relatively ineffective in protecting either activity against inactivation by ATPγBP; in fact, low concentrations of Trp enhanced the level of inhibition. In contrast, ADP afforded complete protection to both activities of Rubisco activase. SDS−PAGE showed that photoaffinity labeling with ATPγBP produced only the 42-kDa form of modified Rubisco activase (Figure 6).

The concentration dependence for inactivation of Rubisco activase activities by photoaffinity labeling with 2-N_3AMP is shown in Figure 7. The ability of Rubisco activase to promote activation of the decarbamylated Rubisco−RuBP complex was nearly abolished by 100 μM 2-N_3AMP. At this concentration, residual ATPase activity was equivalent to about 30% of the unmodified control. The apparent K_i for inactivation of Rubisco activation by photoaffinity labeling with 2-N_3AMP was 18 μM, approximately 3.4-fold lower than the apparent K_i for inactivation of ATPase

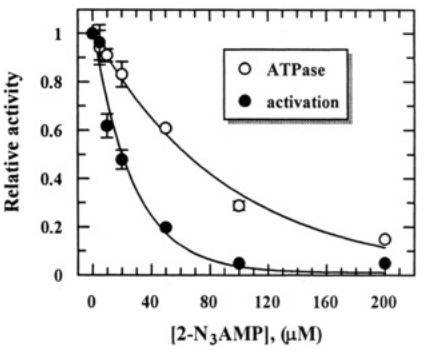

FIGURE 7: Effect of 2-N_3AMP concentration on the inhibition of Rubisco activase activity by photoaffinity labeling. Recombinant tobacco Rubisco activase was photoaffinity labeled with 2-N_3AMP at the indicated concentrations and then assayed for ATPase activity (○) and the ability to promote activation of the decarbamylated Rubisco−RuBP complex (●). Relative activity is expressed as v_i/v_c, where v_i refers to the activity of Rubisco activase after photoaffinity labeling with 2-N_3AMP, and v_c refers to the activity of Rubisco activase that was photolyzed in the absence of 2-N_3-AMP.

activity. In comparison, the K_i values for inactivation of these activities by photoaffinity labeling with ATPγBP were the same, both about 10 μM (Salvucci et al. 1993). 2-N_3-AMP had no effect on Rubisco activation when not covalently bound to Rubisco activase (i.e., incubated but not photolyzed). For example, the rates of Rubisco activation at 1 mM ATP were the same in the presence and absence of 0.5 mM 2-N_3AMP (data not shown).

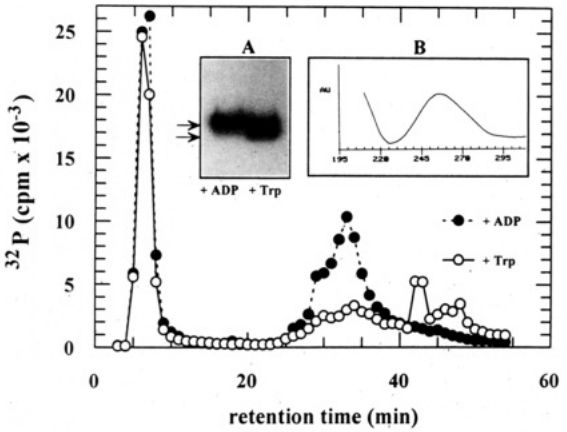

FIGURE 8: Separation of photolabeled peptides from Rubisco activase by reverse-phase HPLC. Rubisco activase (0.5 mg) was photoaffinity labeled with 50 μM [γ-^{32}P]2-N$_3$ATP in the presence of 300 μM ADP or 150 μM Trp in a total volume of 0.5 mL. After precipitation with saturated ammonium sulfate and desalting, aliquots of the desalted protein were subjected to SDS−PAGE. The remainder was digested with chymotrypsin, and photolabeled peptides were affinity purified on an Al^{3+}-chelate column and then separated by reverse-phase HPLC. (Inset A) Autoradiogram of Rubisco activase polypeptides, separated by SDS−PAGE, after photoaffinity labeling with [γ-^{32}P]2-N$_3$ATP in the presence of ADP or Trp. The main figure shows the ^{32}P profile of the reverse-phase HPLC separation of Al^{3+}-chelate chromatography-purified photolabeled peptides from Rubisco activase photoaffinity labeled with [γ-^{32}P]2-N$_3$ATP in the presence of ADP (●) or Trp (○). (Inset B) The ultraviolet spectra of the peptide peak corresponding to the radioactive peak at 33 min in the chromatogram (●).

Identification of Photoaffinity Labeled Peptides from the Two Binding Domains. The ability to selectively protect each of the two binding domains of Rubisco activase against modification also made it possible to identify modified peptides from each of the sites. SDS−PAGE and autoradiography confirmed that photoaffinity labeling with 50 μM [γ-^{32}P]2-N$_3$ATP in the presence of 400 μM ADP selectively modified the domain associated with the 44-kDa form of Rubisco activase (Figure 8, inset A), while photoaffinity labeling in the presence of 150 μM Trp primarily modified the ATP binding domain (i.e., the 42-kDa polypeptide).

To identify the two domains, photolabeled Rubisco activase was digested with chymotrypsin and the modified peptides were isolated by metal-ion chelate chromatography on an Al^{3+}-charged iminodiacetic acid-Sepharose column before separation by reverse-phase HPLC. Figure 8 shows that the reverse-phase HPLC profiles of ^{32}P-labeled peptides from Rubisco activase photoaffinity labeled in the presence of Trp and ADP differed considerably. Specifically, the major ^{32}P-labeled peak at 33 min that was present when ADP was included during photoaffinity labeling was reduced markedly when Trp was present. Also, two ^{32}P-labeled peptide peaks at 42 and 48 min were present when photoaffinity labeling was conducted in the presence of Trp but were absent when photoaffinity labeling was conducted in the presence of ADP. The A_{215} profiles corresponding to the chromatograms in Figure 8 showed that there was a small peak associated with the 33 min radioactive peak (data not shown). The ultraviolet spectrum of this peak exhibited a maximum absorbance at 256 nm, characteristic of a peptide containing a photoinserted adenine nucleotide (Figure 8, inset B). Very little A_{215} was associated with the 42 and 48 min radioactive peaks or the shoulder preceding the major portion of the radioactive peak at 33 min.

Table 2: Sequence Analysis of Labeled Chymotryptic Peptides from Rubisco Activase Photolabeled with [γ-^{32}P]2-N$_3$ATP in the Presence of 400 μM ADP or 150 μM Tryptophan

	PTH amino acid (pmol)[a]		
cycle	+ ADP	+ Trp	
1	M (120)	N (28)	M (21)
2	E (49)	L (60)	E (17)
3	E (45)	D (24)	E (13)
4	K (58)	N (31)	K (9)
5	D (14)	K (34)	D (21)
6	A (80)	L (43)	A (19)
7	D (19)	D (45)	D (45)
8	P (32)	X[b]	P (4)
9	K (29)	F (1)	X
10	K (35)		K (2)
11	Q (37)		Q (10)
12	T (31)		T (6)
13	D (8)		D (1)
14	S (8)		
15	D (9)		

[a] Picomole yield of PTH-amino acid residues. [b] X indicates predicted amino acid not identified in the cycle.

A considerable portion of the radioactivity recovered from the Al^{3+}-chelate column migrated as unretained material on the reverse-phase HPLC column. Previous studies have shown that photoinserted bonds are often labile to the conditions used for reverse-phase HPLC (Kim & Haley, 1992; Salvucci et al., 1992; Shoemaker & Haley, 1993). Also, photoinsertion can occur at multiple sites on a given peptide causing considerable peak broadening on reverse-phase HPLC (Salvucci et al., 1993). Consequently, we conducted automated Edman degradation analysis on the peptide material directly from the Al^{3+}-chelate column without further purification. When Rubisco activase was photoaffinity labeled with [γ-^{32}P]2-N$_3$ATP in the presence ADP (Figure 8, inset A), there was a single photolabeled peptide present in the material from the column (Table 2). The sequence of this peptide matched the N-terminus of recombinant Rubisco activase from M0[3] -D14. Two peptides were recovered from Rubisco activase when the enzyme was photoaffinity labeled in the presence of Trp. One of these photolabeled peptides matched a region of the protein from N68-F76. The other peptide matched the N-terminus of Rubisco activase, although the amount of this peptide was about 4−5-fold less than the amount obtained when photoaffinity labeling was conducted in the absence of Trp. The presence of a small amount of the N-terminal peptide in samples photoaffinity labeled in the presence of Trp was consistent with the trace of ^{32}P associated with the 44-kDa polypeptide (Figure 8, inset A) and the 33 min peak in the reverse-phase HPLC chromatogram (Figure 8).

In separate photoaffinity labeling experiments, Rubisco activase was modified with 2-N$_3$ATP in the absence of effectors and digested with different proteases to confirm the identity of the photolabeled peptides. In samples digested with chymotrypsin, sequence analysis after purification of the photolabeled peptides by metal-ion chelate chromatography detected amino acids corresponding to Rubisco acti-

[3] The N-terminal Met of the recombinant Rubisco activase is designated M0 to maintain a consistent numbering scheme between leaf and recombinant Rubisco activases.

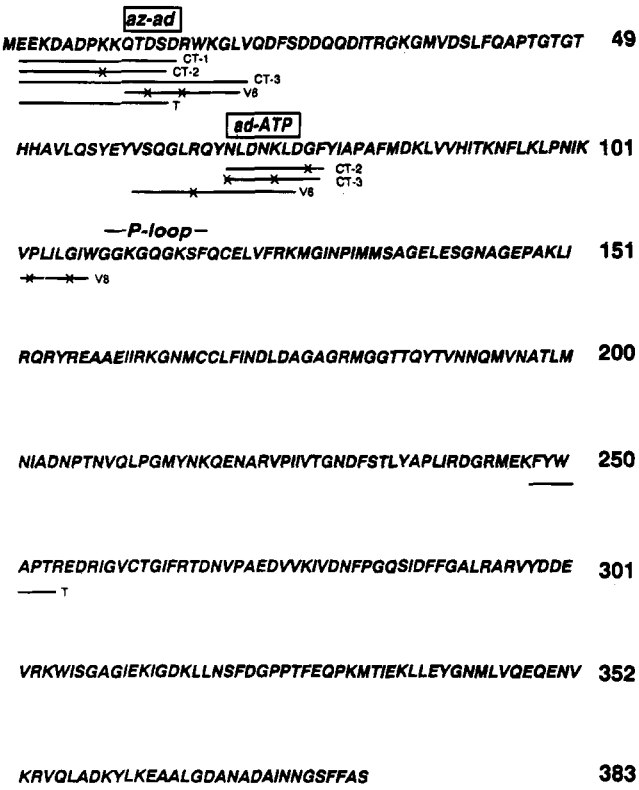

FIGURE 9: Positions of photolabeled peptides in the primary sequence of tobacco Rubisco activase. The figure includes the data for peptides recovered after digestion of photolabeled Rubisco activase with chymotrypsin (CT), trypsin (T), and V8 protease (V8). CT-1, CT-2, and CT-3 refer to chymotryptic peptides recovered after photoaffinity labeling with 2-N_3ATP in the presence of ADP and Trp (Table 2) or in the absence of effector, respectively. Assignment of the adenine binding domain for ATP (ad-ATP) and the azidoadenine nucleotide domain (az-ad) was based on selective protection of these peptides by ADP and Trp.

vase peptides M0-D14, N68-F76, and K10-L20 (Figure 9). Peptides present after digestion with V8 protease and purification by metal-ion chelate chromatography included M0-S13, Y59-L73, and F248-T253 (Figure 9). Peptides present after digest with trypsin included Q10-K18, K9-R15, and V102-W109 (Figure 9).

The positions of the photolabeled peptides in the primary structure of Rubisco activase are shown in Figure 9. The figure, which includes data from four separate experiments using three different proteases, shows that the peptides overlapped in two specific regions of the protein. We have designated these two regions as the adenine binding domain for ATP (ad-ATP) and a separate "azidoadenine-binding" domain (az-ad) based on selective protection with ADP and Trp, respectively (Table 2).

DISCUSSION

Photoaffinity labeling with azido-substituted nucleotides generally provides a straightforward approach for identifying nucleotide binding domains on proteins (Potter & Haley, 1983). These reagents bind specifically to the protein and, if activated with ultraviolet light, can usually form a stable bond with the region of the protein proximal to the azido group. For some proteins, covalent modification by the nucleotide decreases the electrophoretic mobility of the polypeptide on SDS−PAGE gels (c.f., Mimura et al., 1990). This change probably reflects a regional disruption in the binding of SDS at the site of the photoinserted nucleotide.

An unusual feature of Rubisco activase was the production of two labeled bands from a single polypeptide type upon photoinsertion of [γ-^{32}P]N_3ATP. Competition binding experiments established that the two bands, one at 42- and the other at 44-kDa, were produced by modification of two distinct binding sites on the protein. The presence of two binding sites for N_3ATP on each Rubisco activase monomer is consistent with the measured stoichiometry of photoaffinity labeling with 2-N_3ATP.

That one of the sites of Rubisco activase was the adenine binding domain for ATP was expected considering that the enzyme hydrolyzed both 2- and 8-N_3ATP and used these analogs to promote activation of Rubisco. Unexpectedly, azidoadenine nucleotides also labeled an apparently unrelated site on Rubisco activase. Modification of this "second" domain by photoaffinity labeling with azidoadenine nucleotides reduced the electrophoretic mobility of the polypeptide and prevented Rubisco activase from promoting activation of the decarbamylated Rubisco−RuBP complex without a corresponding effect on ATP hydrolysis (Figures 5 and 7). Photoaffinity labeling and competition binding (Figures 3 and 4 and Table 1) showed that this "second" site was not a nucleotide binding domain *per se* but could bind adenine nucleotides with relatively high affinity if they contain an azido substitution on the base.

An azido group on the base of a bound nucleotide can form hydrogen bonds with amino acid side chains thereby stabilizing otherwise weak binding. The added interaction explains why azido-substituted nucleotides can sometimes bind much more tightly within a binding domain than naturally occurring nucleotides. The propensity for forming hydrogen bonds would depend on the position of the azido group on the base and the relative distribution of nucleotide in the *syn-* and *anti-* conformations. For Rubisco activase, these factors alone or in combination could explain the preference of the second binding domain of Rubisco activase for 2- versus 8-N_3ATP (Figures 2 and 3). Steric interference by the nucleotide phosphates may also be a factor influencing this preference, as well as the preference for 2-N_3AMP versus 2-N_3ATP.

Identification of the photoaffinity labeled peptides provided definitive evidence for a relationship between the different electrophoretic forms of modified Rubisco activase and the labeling of two separate regions of the protein. The adenine binding domain for ATP was assigned to the region from N68 to D74 (Figure 9) on the basis of photoaffinity labeling results with 2-N_3ATP in the presence Trp and ADP (Figure 8 and Table 2). Of the residues in this region, N71 is conserved among the one green algal and seven higher plant sequences that are currently available, and an Asp is present at position 74 in all but one of the sequences (Wang et al., 1992, and references therein; Preisig-Muller & Kindl, 1992; Watillon et al., 1993). In the tobacco enzyme, the sequence containing N71 and D74, ^{71}NKLD, matches a highly conserved sequence motif (N/T)(K/Q)XD found in many guanine nucleotide binding proteins (Nuoffer & Balch, 1994, and references therein). In H-*ras* p21, the Asn and Asp of this sequence interact directly with the guanine base (Pai et al., 1990). Similarly, our photoaffinity labeling results indicate that the analogous residues in Rubisco activase may interact with the adenine base of ATP.

A second azidoadenine binding domain was assigned to a region near the N-terminus of Rubisco activase from around

Q10 to about D14. This region of the protein is not highly conserved, particularly between the algal (Roesler & Ogren, 1990) and higher plant (Wang et al., 1992) enzymes. However, all Rubisco activases from higher plant and green algal species contain a Trp at position 16 and a basic amino acid adjacent to W16 at position 15. Thus, binding of azidoadenine nucleotides to this region of the protein may involve purine−indole base-stacking between the species-invariant Trp and the azido-substituted adenine base. Indole−indole base-stacking may also occur at W16, which could account for the protective effect of Trp and other indole-based compounds on photoaffinity labeling with azidoadenine nucleotides. The physiological significance of these interactions is unknown, since reversible binding of indoles or unphotolyzed azidoadenine nucleotides to the N-terminal region of Rubisco activase had no effect on enzyme activity aside from protecting against photoaffinity labeling. Perhaps the changes in Rubisco activase conformational that occur upon ATP binding and hydrolysis (Wang et al., 1993) displace noncovalently bound compounds from this site.

Young et al. (1994) have shown that Trp residues are frequently part of hydrophobic clusters that participate in protein−protein interactions. Similarly, the region of Rubisco activase that includes W16 may be a site of interaction with Rubisco since modification of this domain by photoaffinity labeling prevented Rubisco activase from activating Rubisco. Protein−protein contact between Rubisco activase and Rubisco may involve base-stacking, hydrogen bonding, and other types of interactions that could also bind azidoadenine nucleotides. Alternatively, these interactions may have an indirect effect on Rubisco activation, perhaps through conformational changes in Rubisco activase. In either case, the binding of azidonucleotides to the N-terminal region of Rubisco activase appears to be fortuitous, occurring through interactions that are normally required for Rubisco activation.

Thus far, we have been unable to demonstrate an effect of Rubisco or Rubisco−RuBP on photoaffinity labeling of Rubisco activase by azidoadenine nucleotides (M. E. Salvucci, unpublished results). However, it was necessary to conduct these experiments at low molar ratios of Rubisco to 2-N_3ATP to avoid complications associated with the binding of azidonucleotide to Rubisco (Klein & Salvucci, 1991) and shielding of the activating ultraviolet light by high protein concentrations. Presently, we are using directed mutagenesis to investigate the involvement of W16 and the surrounding region of Rubisco activase in the interaction with Rubisco. Preliminary evidence from these studies supports the conclusion that W16 of Rubisco activase is involved in Rubisco activation, but not ATP hydrolysis (F. J. van de Loo and M. E. Salvucci, unpublished experiments).

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