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Alteration of Spinach Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase Activities by Site-Directed Mutagenesis

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ABSTRACT

Site-directed mutagenesis was performed on the 1.6 and 1.9 kilobase spinach (*Spinacea oleracea*) ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase cDNAs, encoding the 41 and 45 kilodalton (kD) isoforms of the enzyme, to create single amino acid changes in the putative ATP-binding site of Rubisco activase (Lys-107, Gln-109, and Ser-112) and in an unrelated cysteine residue (Cys-256). Replacement of Lys-107 with Met produced soluble protein with reduced Rubisco activase and ATPase activities in both isoforms. Substituting Ala or Arg for Lys-107 produced insoluble proteins. Rubisco activase activity increased in the 41-kD isoform when Gln-109 was changed to Glu, but activity in the 45-kD isoform was similar to the wild-type enzyme. ATPase activity in the Glu-109 mutations did not parallel the changes in Rubisco activase activity. Rather, a higher ratio of Rubisco activase to ATPase activity occurred in both isoforms. The mutation of Gln-109 to Lys inactivated Rubisco activase activity. Replacement of Ser-112 with Pro created an inactive protein, whereas attempts to replace Ser-112 with Thr were not successful. The mutation of Cys-256 to Ser in the 45-kD isoform reduced both Rubisco activase and ATPase activities. The results indicate that the two activities of Rubisco activase are not tightly coupled and that variations in photosynthetic efficiency may occur *in vivo* by replacing the wild-type enzyme with mutant enzymes.

Rubisco (EC 4.1.1.39) catalyzes the initial steps of photosynthesis and photorespiration (10). To become catalytically competent, Rubisco must first be activated by carbamylation of a lysine residue on the large subunit of the enzyme (9). Rubisco activase has been shown to be required for the activation of Rubisco *in vivo* and in the presence of physiological concentrations of RuBP[2] *in vitro* (12, 17), and its activity is ATP-dependent (15, 21). Sequence analysis of Rubisco activase cDNA identified a glycine-rich consensus segment (G–G-GK[111]S) (26), which is homologous to other ATP-binding proteins (3, 5, 24). Directed mutations of Rubisco activase cDNA that modified the Lys-111 site completely abolished Rubisco activase activity,[3] confirming the essentiality of this residue for activity (19).

Spinach (*Spinacea oleracea*) Rubisco activase has two isoforms with molecular masses of about 41 and 45-kD that are derived from alternate splicing of the same or very similar primary transcripts (25). The mechanism by which Rubisco activase activates Rubisco in the presence of RuBP, the nature of the association of Rubisco and Rubisco activase, the relationship of the Rubisco activase and intrinsic ATPase activities, and the role of the two isoforms are still unclear. To begin to address these questions, expression clones for the two isoforms were constructed (19) and mutant proteins with single amino acid substitutions were synthesized in *Escherichia coli*. A series of mutations for three amino acids (Lys-107, Gln-109, and Ser-112) in the consensus ATP-binding site were prepared to determine the contribution of each individual amino acid to ATP binding and enzyme activity. Another residue, Cys-256, which is not included in any known consensus nucleotide-binding sequence and located further downstream of this ATP-binding site, was also chosen for mutagenesis because of the possibility it might be involved in disulfide bond formation or subunit interaction.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Clone Construction

Mutant clones were prepared by the site-directed mutagenesis procedure described earlier (19) and modified from Kunkel *et al.* (7). The full-length cDNA pRCA1.9 (25) was subcloned into the *Eco*R1 site of pTZ18U, which was then used to prepare single-stranded templates for the mutagenesis reactions. The following synthesized oligonucleotides (base mismatches underlined) were used to construct mutant clones: (a) 5′-GGATTTACCTTGACCCGCGCCTCCCCA-AAC-3′, to change Lys-107 to Ala; (b) 5′-CCTTGACCC-NTGCCTCCCC-3′ (N = G or T), to change Lys-107 to Arg or Met; (c) 5′-GGATTTACCTTNACCCTTGCC-3′ (N = C or T), to change Gln-109 to Glu or Lys; (d) 5′-CATTGGAAGGNTTTACCTTGACC-3′ (N = G or T), to change Ser-112 to Thr or Pro; (e) 5′-CTTGAAAATACCGGT-AGTGACACCAATACGG-3′, to change Cys-256 to Ser. The

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[2] Abbreviation: RuBP, ribulose-1,5-bisphosphate.

[3] In Shen *et al.* (19), the numbering system for amino acid residues started with the first amino acid of the prepolypeptide, which included the 58 amino acid Rubisco activase transit peptide. In this paper the numbering system has been changed to begin with the first amino acid of the mature polypeptide. Thus, Lys-111 here corresponds to Lys-169 in Werneke *et al.* (25).

transformed cells were screened and identified by sequencing of single or double-stranded DNA from mini-scale preparations. The *Kpn*I fragment (position 550–869) (26) containing the mutant ATP-binding sequence was purified from low-melting agarose and subcloned into the *Kpn*I site of pPLEX1.6 or pPLEX1.9 from which the wild-type *Kpn*I fragment had already been removed. For the mutation at Cys-256, a 1249 base pair *Hin*dIII fragment from position 661 (26) to the *Hin*dIII site in the polylinker, was inserted into the *Hin*dIII site of pPLEX1.6 from which the wild-type *Hin*dIII fragment had been removed. Mutant clones were screened by immunoblot analysis of protein in extracts of transformed cells cultured overnight to select the clones with the correct orientation. To confirm that only the mutation of interest was present, double-stranded DNA sequencing was conducted again throughout the entire *Kpn*I fragment or the *Hin*dIII fragment in the mutant expression clones. Rubisco activase activity was first assayed in the crude lysates of the mutants in the 41-kD isoform (pPLEX1.6). Only mutations with activity were subcloned into the 45-kD isoform (pPLEX1.9). We intended to change the Ser-112 residue to Thr or Pro using the same mixed oligonucleotide. However, all the mutants obtained encoded a Pro residue at position 112, and no Thr mutant was recovered. Ser-256 was constructed only in the 45-kD isoform due to subcloning difficulties with pPLEX1.6 in this instance.

Protein Isolation and Assay

Rubisco activase proteins were produced in transformed *E. coli* strain UT421 containing the appropriate pPLEX expression vector, extracted and purified as described previously (19). Rubisco protein was determined spectrophotometrically from the formula mg Rubisco/mL = $A_{280} \times 0.61$ (27). All other protein determinations were made with the dye-binding assay according to the manufacturer (Bio-Rad[4]), using BSA as a standard. Average values were determined from duplicate measurements. SDS-PAGE was performed using the buffer system described by Laemmli (8), using a 5% polyacrylamide stacking gel and a 12% resolving gel.

Rubisco Activase Assay

The Rubisco activase assay was performed as described by Robinson and Portis (15). Purified spinach Rubisco was desalted by gel filtration and RuBP was added to form the inactive Rubisco-RuBP complex. The Rubisco activation assay mixtures (100 μL) contained 50 mM Tricine-KOH (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 4 mM RuBP, varied concentrations of ATP (0–1 mM), 1 mM phospho*enol*pyruvate, 20 units/mL pyruvate kinase, 1 mg/mL Rubisco (as Rubisco-RuBP), and 50 μg/mL Rubisco activase protein. Rubisco activase was added 1 min before initiating the reaction at 25°C with Rubisco-RuBP complex. Rubisco activity was determined at 25°C by adding a 50-μL aliquot of the assay mixture after 1 min to 450-μL of 50 mM Tricine-KOH (pH 8.0), 10 mM $NaH^{14}CO_3$ (0.3 Ci/mol), 10 mM $MgCl_2$, 0.5 mM RuBP. The reaction was terminated after 0.5 min by the addition of 100 μL of 4 N formic acid/1 N HCl. The samples were dried and incorporation of $^{14}CO_2$ into acid-stable products determined by liquid scintillation counting. One unit of Rubisco activation specific activity was defined as 1 μmol CO_2 fixed per min/mg Rubisco per mg Rubisco activase protein/min.

ATPase Assay

ATPase activity was measured in a coupled spectrophotometric assay utilizing pyruvate kinase and lactic dehydrogenase (15). Reaction mixtures contained 50 mM Tricine-KOH (pH 8.0), 20 mM KCl, 5 mM $MgCl_2$, varied concentrations of ATP (0–1 mM), 1 mM phospho*enol*pyruvate, 0.3 mM NADH, 12 units/mL pyruvate kinase and 12 units/mL lactic dehydrogenase in a total volume of 0.4 mL. The reaction was started by the addition of 30 μg Rubisco activase protein, and the rate of ATP hydrolysis determined from the rate of NADH oxidation measured at 25°C. One unit of ATPase activity was defined as 1 μmol ADP produced per min/mg Rubisco activase protein.

RESULTS

Predicted Secondary Structure of Spinach Rubisco Activase

Many nucleotide-binding domains of known structure exhibit similar tertiary folding topology and are classified as doubly wound, parallel α/β domains (13). The predicted secondary structure of spinach Rubisco activase is shown in Figure 1 (Garnier-Robson Structural Predictions, DNASTAR) (4, 16, 22). The extra C-domain in the 45-kD isoform contains a short α-helix followed by a long β-strand. One nucleotide-binding region, sequence A (24), is located in a turn connecting a β-strand and a short β-strand in front of an α-helix. This turn terminated with the absolutely conserved Lys residue (position 111 in spinach Rubisco activase). In adenylate kinase, this region has been designated a flexible loop, and was suggested to be involved in binding of one of the phosphate groups of ATP (3). A second conserved nucleotide-binding region, sequence B, was located in a hydrophobic β-strand ending with an Asp residue (position 169 in spinach Rubisco activase), and was proposed to interact with the Mg^{2+} of the MgATP complex (2, 3, 24).

Rationale for Mutations in the ATP-Binding Site and Cys-314

Figure 2 shows the amino acid sequence of spinach Rubisco activase and the residues that are most conserved among the ATP-binding proteins described thus far (3, 5, 6, 24). Lys-111 is an absolutely conserved residue and has been proposed to associate with one of the phosphate groups of ATP (3, 24). It was chosen for the first target of mutagenesis and was shown to be essential for Rubisco activase activity (19). Lys-107 and Gln-109 were not conserved in this Gly-rich consensus sequence, and were considered to be good candidates for site-directed mutagenesis for modifying the structure of the molecule without completely losing activity. Lys-107 was

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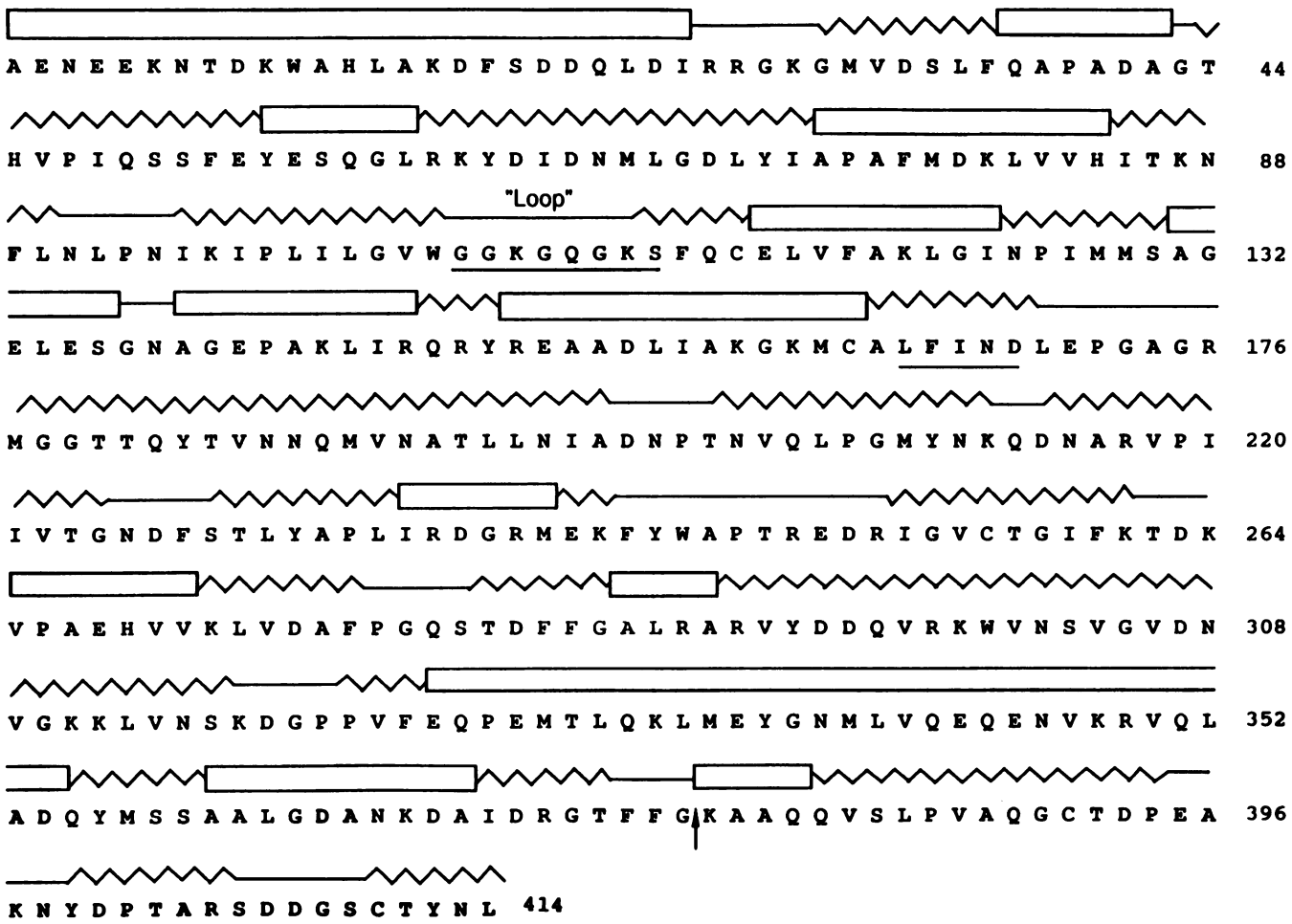

Figure 1. Predicted secondary structure of spinach Rubisco activase. The primary sequence is from Werneke *et al.* (26). The secondary structure is based on the Garnier-Robson rules (4, 16, 22) as embodied in the DNASTAR Protein Program. Areas of sequence homology with other nucleotide-binding proteins (3, 5, 24) are underlined (positions 105–112, 165–169). Ala-1 is the first amino acid of the mature 41-kD and 45-kD polypeptides, and Gly-377, indicated by the arrow, is the carboxyl terminus of the 41-kD isoform (25); (open box) α-helix, (wavy line) β-strand, and (straight line) turn.

mutated to Ala, Arg, or Met. Ala was chosen to mimic the sequences of the β-subunit of *E. coli* and bovine F_1-ATPase, which contain Ala in the corresponding position (24). Arg has the same positive charge, and Met has a similar side-chain bond length and bond angles as Lys, but is uncharged. Gln-109 was mutated to Glu or Lys, which changed the original uncharged residue to either a negatively (Glu) or positively charged (Lys) residue with a side chain of similar size. An unsuccessful attempt was made to replace Ser-112 with the more commonly conserved Thr residue. Pro was another product from this mutagenesis reaction. Cys-256 was changed to Ser in order to replace an —SH group with an —OH group, which would prevent disulfide bond formation if that were a functional role for this Cys residue.

Site-Directed Mutagenesis

The frequency of *in vitro* mutagenesis was routinely 25 to 50%. A *Nco*I site was created at the junction of the transit peptide and mature coding region in the expression clones, which eliminated all the extra amino acids from the *lacZ* gene

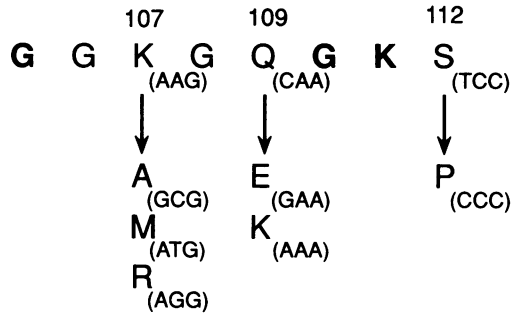

Figure 2. Mutations made in the ATP-binding domain of Rubisco activase. Lys-107 was changed to Ala, Met, and Arg; Gln-109 was changed to Glu and Lys; and Ser-112 was changed to Pro. Additionally, Cys-256 was changed to Ser. Gly-105, Gly-110, and Lys-111 are conserved in all nucleotide-binding proteins (3, 5, 24). Rubisco activase polypeptides in which Lys-111 was replaced with Arg, Ile, or Thr were completely inactive (19).

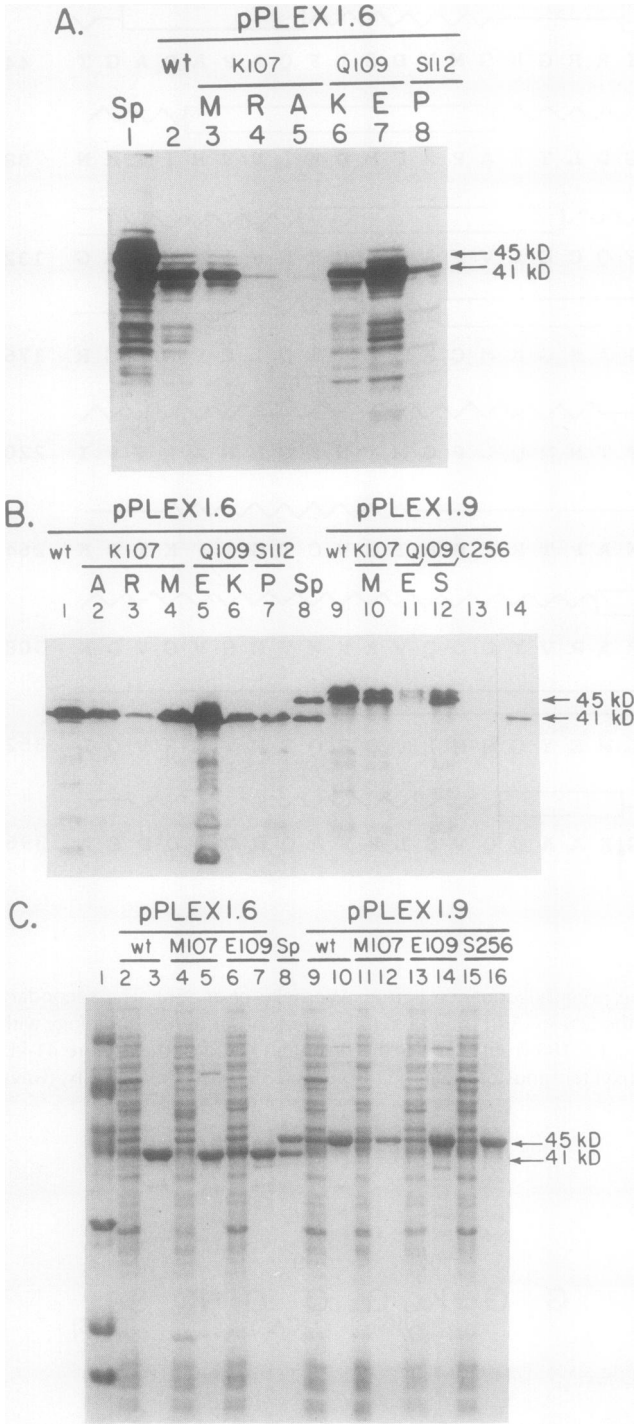

Figure 3. Gel analysis of native and cloned Rubisco activase polypeptides. A, Immunoblot of spinach Rubisco activase (Sp) and soluble protein from extracts of transformed *E. coli* containing wild-type (wt) or mutant pPLEX1.6 substituted at Lys (K)-107 with Met (M), Arg (R), and Ala (A); at Gln (Q)-109 by Lys (K) or Glu (E); and at Ser (S)-112 by Pro (P). Each lane contained 7.5 μg protein. B, Immunoblot of total protein from transformed *E. coli* containing either wild-type or mutant spinach Rubisco activase cDNA. Amino acid substitutions are as described in A. Only those mutations yielding active enzyme were introduced into the 45-kD polypeptide. Cys (C)-314 was replaced by Ser (S) only in the 45-kD isoform. Lanes 1 to 7 and 9 to 13 are crude extracts of *E. coli* cells equivalent to 100 μL of overnight culture. Lane 8 (Sp) is 1.2 μg purified spinach Rubisco activase. Lane 13 is a negative control containing extract from *E. coli* transformed with pPLEX vector without an insert. Lane 14 is 0.2 μg Rubisco activase purified from *E. coli* transformed with pPLEX1.6-wt. C, SDS-PAGE analysis of Rubisco activase polypeptides purified from transformed *E. coli* and spinach. Lanes 2, 4, 6, 9, 11, 13, and 15 contained crude extracts of the indicated wild-type or mutant clone and lanes 3, 5, 7, 10, 12, 14, and 16 the corresponding purified polypeptide. Purified spinach Rubisco activase is in lane 8. Lane 1 contains molecular mass markers (130-, 75-, 50-, 39-, 27-, and 17-kD). Each lane contained 7.5 μg protein.

product, linker, and transit peptide at the N terminus, and left only two residues in front of the mature protein (19). The $_f$Met was retained for the initiation of protein synthesis in *E. coli*, and Ala was the last amino acid of the transit peptide (26). The $_f$Met was assumed to remain in the cloned Rubisco activase because amino acid sequencing from the N terminus of the purified cloned protein was blocked, presumably because of the formyl group (data not shown). The cDNA sequences of mutant clones were confirmed by sequencing double-stranded DNA from mini-scale preparations after the mutant fragment (*Kpn*I or *Hin*dIII fragment) was subcloned into the expression plasmid.

Protein Accumulation in Transformed *E. coli*

The levels of cloned Rubisco activase that accumulated in the transformed *E. coli* cells were significantly changed by the single amino acid substitutions. Protein from transformed *E. coli* containing wild-type or mutant Rubisco activase cDNAs were analyzed by SDS-PAGE or immunoblots. Mutant Rubisco activase proteins encoded by pPLEX1.6-Met-107, pPLEX1.9-Met-107, and pPLEX1.6-Lys-109 accumulated at levels comparable to wild type, whereas the proteins from pPLEX1.6-Pro-112 and pPLEX1.9-Ser-256 accumulated at a level somewhat lower than wild type, and protein from pPLEX1.6-Glu-109 and pPLEX1.9-Glu-109 accumulated at a greater level than wild type (Fig. 3). The Rubisco activase polypeptide was not present in the pPLEX1.6-Ala-107 soluble fraction and was greatly diminished in pPLEX1.6-Arg-107 (Fig. 3A). The color reaction in the western immunoblot of Figure 3A was considerably overdeveloped to detect trace amounts of Rubisco activase protein encoded by pPLEX1.6-Ala-107 and pPLEX1.6-Arg-107. Analysis of uncentrifuged crude extracts of *E. coli* cells containing pPLEX1.6-Ala-107 and pPLEX1.6-Arg-107 indicated the presence of significant levels of Rubisco activase (Fig. 3B), and thus they were present largely as insoluble proteins. Further characterization of these two mutant proteins was not pursued. There was no Rubisco activase protein from *E. coli* transformed with the pPLEX vector without an insert (Fig. 3B, lane 13).

Protein Purification

Protein synthesis in the two Rubisco activase expression clones (pPLEX1.6 encoding the 41-kD isoform, and pPLEX1.9 encoding the 45-kD isoform) was driven by a strong λP_L promoter in a constitutive expression system (19), providing a high yield of Rubisco activase polypeptide (Fig. 3C) with

Table I. *Specific Activity and Ratio of Rubisco Activase to ATPase Activity in Cloned Wild-Type (wt) and Mutant Rubisco Activase Polypeptides*

Clone	Molecular Mass	Rubisco Activase	ATPase	Rubisco Activase/ATPase
	kD	*units/mg protein*		*ratio*
pPLEX1.6-wt	41	1.43 ± 0.36	0.72 ± 0.13	2.0
pPLEX1.6-Met-107	41	0.72 ± 0.12	0.45 ± 0.15	1.6
pPLEX1.6-Glu-109	41	3.33 ± 0.30	0.65 ± 0.13	5.1
pPLEX1.6-Ala-107	41	NA[a]	NA	NA
pPLEX1.6-Arg-107	41	NA	NA	NA
pPLEX1.6-Lys-109	41	nd[b]	nd	nd
pPLEX1.9-wt	45	1.05 ± 0.19	0.53 ± 0.11	2.0
pPLEX1.9-Met-107	45	0.36 ± 0.04	0.31 ± 0.20	1.2
pPLEX1.9-Glu-109	45	1.02 ± 0.08	0.29 ± 0.03	3.5
pPLEX1.9-Ser-256	45	0.37 ± 0.05	0.29 ± 0.06	1.3

[a] Data not available due to insolubility of mutant polypeptide. [b] Enzyme activity not detectable.

no apparent harmful effect on *E. coli* growth by the overproduced protein. Wild-type and mutant proteins were purified by the procedure described earlier (19). After $(NH_4)_2SO_4$ precipitation, the Rubisco activase polypeptides were highly purified by Q-Sepharose fast protein liquid chromatography (Fig. 3C). The high yield and purity made it possible to study enzyme kinetics in each wild-type or mutant isoform.

Rubisco Activase and ATPase Activities of the Two Spinach Isoforms

Activity of Rubisco activase from the cloned wild-type cDNA was compared with the Rubisco activase purified from spinach. The specific activities, apparent affinity for ATP, and Rubisco activase/ATPase activity ratio of the enzymes were found to be comparable to earlier work by Robinson and Portis (14, 15). Thus, the structure of the cloned polypeptides obtained from *E. coli* is probably very similar to the native form of spinach Rubisco activase, and the presence of the two additional N-terminal amino acids in the cloned enzyme expressed in *E. coli* is without major effect on activity.

Mutation of Lys-107, Gln-109, Ser-112, and Cys-256

Rubisco activase assays were first conducted with crude lysates of the mutant clones in the 41-kD isoform. From the

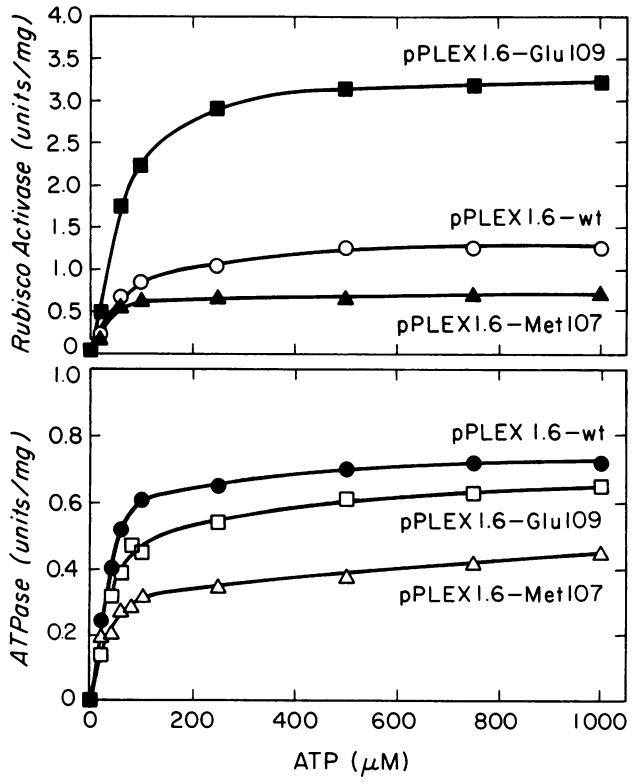

Figure 4. ATP concentration response of Rubisco activase and ATPase activities in the cloned 41-kD isoforms of wild-type, Met-107 mutant, and Glu-109 mutant polypeptides.

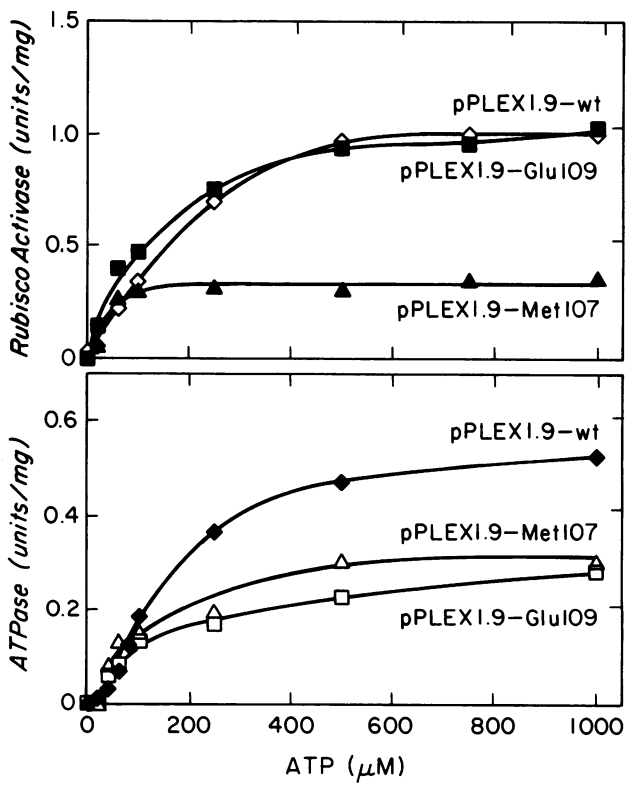

Figure 5. ATP concentration response of Rubisco activase and ATPase activities in the cloned 45-kD isoforms of wild-type, Met-107 mutant, and Glu-109 mutant polypeptides.

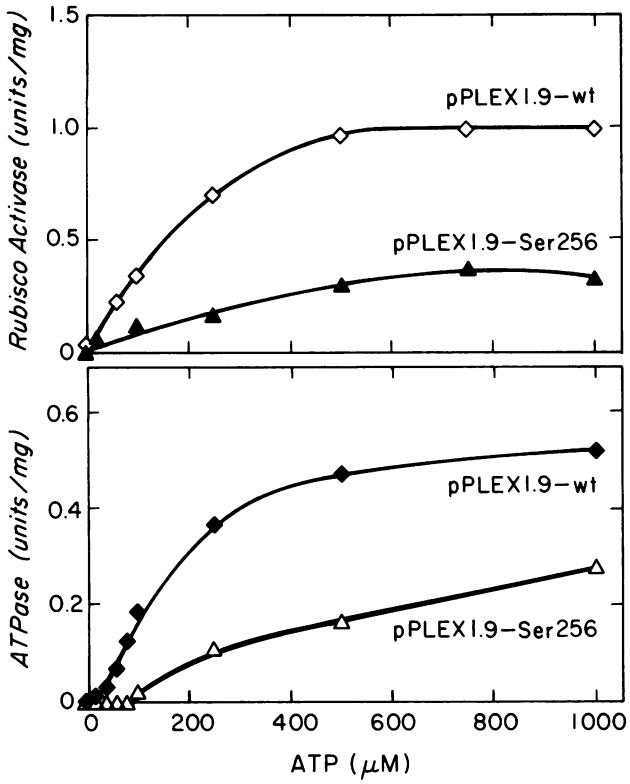

Figure 6. ATP concentration response of Rubisco activase and ATPase activities in the cloned 45-kD isoforms of wild-type and Ser-256 mutant polypeptides.

six altered cDNAs produced, activity was found only in the Met-107 and Gln-109 mutant proteins (encoded by pPLEX1.6-Met-107 and pPLEX1.6-Glu-109) (Table I, Fig. 4). These two mutants were then chosen to construct the 45-kD mutant isoforms (encoded by pPLEX1.9-Met-107 and pPLEX1.9-Glu-109). Additionally, pPLEX1.9-Ser-256 also encoded a soluble protein with activity.

The kinetics of both Rubisco activase and ATPase activities were examined for the purified wild-type and mutants in both isoforms. Replacement of Lys-107 with Met in both isoforms led to reduced Rubisco activase activity (Figs. 4 and 5). Unexpectedly, the substitution of Glu for Gln-109 in the 41-kD isoform increased Rubisco activase activity about threefold (Fig. 4), but the Glu-109 mutation in the 45-kD isoform had a Rubisco activase activity similar to wild type (Fig. 5). ATPase activities were reduced in the Met-107 mutant proteins, consistent with the reduced Rubisco activase activities. The Glu-109 mutation in both isoforms showed reduced ATPase activities, even though there was a higher Rubisco activase activity in the 41-kD isoform (Fig. 4) and a similar Rubisco activase activity in the 45-kD isoform (Fig. 5). Therefore, the ratio of Rubisco activase to ATPase activity was considerably increased by the Glu-109 mutation (Table I). The replacement of Cys-256 with Ser resulted in reduced Rubisco activase and ATPase activities (Fig. 6). The specific activities and ratio of Rubisco activase/ATPase activities of the two wild-type and five mutant enzymes are summarized in Table I.

DISCUSSION

In addition to the absolutely conserved Lys-111, which is essential for Rubisco activase and ATPase activities (19), there is another Lys residue at position 107 in spinach Rubisco activase (Figs. 1 and 2). Mutation of Lys-107 to Ala or Arg rendered the protein largely or completely insoluble (compare Fig. 3, A and B). Changing the Lys-107 to Met in both the 41- and 45-kD isoforms created polypeptides in which both the Rubisco activase and ATPase activities were decreased without a significant change in the apparent affinity for ATP. This suggests that Met-107 does not affect the binding of ATP, but rather alters catalytic efficiency. Although the existence of a second Lys in this Gly-rich region is not common, the transcription termination factor ρ protein of *E. coli* contains an additional Lys, Lys-181, which is near the highly conserved Lys-184 (1). From a prediction of the tertiary structure, it was proposed that Lys-184 might be able to interact with the α-phosphate of ATP, whereas Lys-181 was situated in a region near the β-and γ-phosphorous atoms (1). This hypothesis does not seem to apply to Rubisco activase, because the highly conserved Lys-111 could not be replaced by either Arg, Ile, or Thr in the presence of the Lys-107 residue (19). The positive charge of Lys-107 does not seem to be essential, because activity was retained in the presence of the nonpolar Met residue.

Although Gln-109 is not part of the consensus ATP-binding region as described by Walker *et al.* (24), it is located in the middle of the ATP-binding loop (Fig. 1). When Gln-109 was replaced by Lys, there were three Lys residues (Lys-107, Lys-109, and Lys-111) in this region and the enzyme possessed no activity. The positive charges could repulse each other to strain the loop, leading to the loss of the ability to bind ATP. However, replacing Gln-109 with Glu in the 41-kD isoform led to a threefold higher Rubisco activase activity (Table I, Fig. 4). The negative charge of Glu-109 may therefore help to balance the positive charges from the two Lys residues on either side and stabilize the loop structure. The change in ATPase activity in this mutant protein did not parallel the change in Rubisco activase activity but remained similar to wild type, thereby increasing the Rubisco activase/ATPase activity ratio (Table I).

The observation that most ATP-binding proteins contain either a Thr or Ser at the corresponding 112 position of spinach Rubisco activase led to the prediction that the hydroxyl group or the short side chain of the residue could be important in providing an environment to promote ATP-binding. The Thr-112 mutant could not be obtained to test this hypothesis. The Pro-112 mutant protein was synthesized and found to be inactive. This absence of activity may occur because the bulky ring structure of Pro perturbs the loop structure, or alternatively, blocks the accessibility of the ATP molecule to the ϵ-amino group of Lys.

The insolubility of the mutant 41-kD isoform containing either Ala-107 or Arg-107 precluded efforts at further characterization. The 107 position of spinach Rubisco activase is equivalent to Pro-17 in adenylate kinase, which may be important in stabilizing the Gly-rich flexible loop (3). A change in solubility has also been reported for mutant proteins of chicken adenylate kinase with the substitution of Gly

or Val for Pro-17. After solubilization by acid and reneutralization by NaOH, the mutant adenylate kinase proteins possessed comparable enzyme activities, but with lower affinities for ATP and AMP (23).

Cys-256, removed from the conserved ATP-binding regions in the primary sequence of spinach Rubisco activase, resides in a β-strand of the predicted secondary structure (Fig. 1). Although a potential for forming intra- or intermolecular disulfide bonds was not assessed, the sulfhydryl group was apparently not essential for activity (Table I). The lower apparent affinity for ATP and lower activities of Rubisco activase and ATPase with this polypeptide (Fig. 6, Table I) indicate that the Cys-256 residue may have some role in ATP binding and catalytic efficiency. The β-strand in which it resides could be part of the nucleotide-binding domain, which is composed of four to five parallel β-strands from x-ray crystallography predictions of the adenylate kinase structure (3).

The results reported here indicate that single amino acid substitutions in the ATP-binding domain are sufficient to eliminate or alter the Rubisco activase and ATPase specific activities. The changes in these two activities of the mutant proteins were not directly correlated, indicating that the Rubisco activase and ATPase activities are not tightly coupled. These observations could have significant implications regarding the overall efficiency of photosynthesis. If the stoichiometry of Rubisco activase to ATPase activities can be increased *in vivo*, a more energy-efficient regulation of Rubisco activity could occur. More importantly, perhaps, is the ability to alter the specific Rubisco activase activity. Changes in photosynthetic rate in response to environmental parameters such as light occur not by substrate limitation but by changes in Rubisco activation state (11, 18). If the photosynthetic mechanism has evolved to be overly conservative in this regulation, so that at less than saturating light intensities, for example, the decline in Rubisco deactivation is greater than the decline in the ability of the chloroplast to regenerate RuBP, then an increase in steady-state photosynthesis could be obtained by maintaining a higher Rubisco activation state. It may be possible to test this hypothesis by transforming the Rubisco activase-deficient *Arabidopsis thaliana* mutant (20) with mutant Rubisco activase enzymes possessing varied specific activities.

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