Plant Physiol. (1988) 88, 1008–1014
0032-0889/88/88/1008/07/$01.00/0

# Purification and Assay of Rubisco Activase from Leaves[1]

Received for publication April 29, 1988 and in revised form July 11, 1988

SIMON P. ROBINSON, VIRGINIA J. STREUSAND,[2] J. MARK CHATFIELD, AND ARCHIE R. PORTIS, JR.*
*U.S. Department of Agriculture, Agricultural Research Service, Department of Agronomy, University of Illinois, Urbana, Illinois 61801* (S.P.R., V.J.S., J.M.C., A.R.P.), *and CSIRO, Division of Horticulture, Adelaide, 5000 South Australia* (S.P.R.)

## ABSTRACT

**Ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) activase protein was purified from spinach leaves by ammonium sulfate precipitation and ion exchange fast protein liquid chromatography. This resulted in 48-fold purification with 70% recovery of activity and yielded up to 18 milligrams of rubisco activase protein from 100 grams of leaves. Based on these figures, the protein comprised approximately 2% by weight of soluble protein in spinach (*Spinacia oleracea* L.) leaves. The preparations were at least 95% pure and were stable when frozen in liquid nitrogen. Addition of ATP during purification and storage was necessary to maintain activity. Assay of rubisco activase was based on its ability to promote activation of rubisco in the presence of ribulose-1,5-bisphosphate. There was an absolute requirement for ATP which could not be replaced by other nucleoside phosphates. The initial rate of increase of rubisco activity and the final rubisco specific activity achieved were both dependent on the concentration of rubisco activase. The initial rate was directly proportional to the rubisco activase concentration and was used as the basis of activity. The rate of activation of rubisco was also dependent on the rubisco concentration, suggesting that the activation process is a second order reaction dependent on the concentrations of both rubisco and rubisco activase. It is suggested that deactivation of rubisco occurs simultaneously with rubisco activase-mediated activation, and that rubisco activation state represents a dynamic equilibrium between these two processes.**

Ribulose 1,5-bisphosphate carboxylase/oxygenase is only catalytically competent when converted to an activated state. This is readily achieved *in vitro* by preincubation with $CO_2$ and $Mg^{2+}$ at alkaline pH (7–9) but activation of the enzyme *in vivo* apparently involves more than this spontaneous addition of $CO_2$ and $Mg^{2+}$. The activation of purified rubisco[3] *in vitro* does not occur in the presence of physiological levels of its substrate, RuBP, and requires $CO_2$ concentrations which are higher than those that exist *in vivo* (3–5). Somerville *et al.* (18) isolated a mutant of *Arabidopsis (rca)* in which there was no light activation of rubisco in the leaves, even though the enzyme could be spontaneously activated by $CO_2$ and $Mg^{2+}$ in the normal fashion once isolated from the plant. Salvucci *et al.* (13) subsequently identified two polypeptides which were absent from the *rca* mutant.

These experiments suggested that a specific protein, which has been named rubisco activase, is necessary to catalyze the activation of rubisco *in vivo*. The rubisco activase protein has been detected immunologically in a range of plants and has been purified from spinach chloroplasts (16). The gene for rubisco activase has been identified, cloned in *Escherichia coli* and sequenced (22). More recently, a simpler assay system consisting of RuBP, rubisco, and rubisco activase has been utilized and an ATP requirement for rubisco activation demonstrated (20). A protein isolated from *E. coli* transformed with a spinach rubisco activase cDNA clone has also been shown to mediate activation of rubisco in this assay system (21). In this paper we report an improved purification of rubisco activase and some properties of the purified protein in activating rubisco.

## MATERIALS AND METHODS

**Plant Material.** Spinach (*Spinacia oleracea* L. cv American Hybrid 424) plants were grown in hydroponic culture in a growth chamber as described (11).

**Chemicals.** RuBP was synthesized from ribose-5-phosphate and purified as described (5). All other biochemicals were obtained from Sigma.[4]

**Purification of Rubisco Activase.** Spinach leaves (100 g) were deribbed, frozen in liquid nitrogen, and ground to a fine powder using a mortar and pestle. The frozen leaf powder was slowly added to 200 mL of extraction buffer (10 mM BTP, 5 mM $MgCl_2$, 1 mM EDTA, 0.4 mM ATP, 15 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.01 mM leupeptin, pH 7.0) with continuous stirring over a period of 15 to 20 min during which time the temperature was kept below 0°C. All subsequent steps were carried out at 4°C. The extract was filtered through two layers of Miracloth and the filtrate centrifuged for 15 min at 48,000*g*. Saturated ammonium sulfate, adjusted to pH 7.0 with $NH_4OH$ was added to the supernatant dropwise with continuous stirring until 35% saturation was achieved. After stirring for a further 30 min the precipitate was collected by centrifugation for 5 min at 10,000*g*. The pellets were resuspended in 5 mL of buffer A (10 mM BTP, 100 mM KCl, 10 mM DTT, 0.2 mM ATP, pH 7.0) containing 35% $(NH_4)_2SO_4$ and washed by centrifugation for 5 min at 10,000*g*. The pellet was resuspended in 3 mL of buffer A, centrifuged, and the supernatant desalted on a column of Sephadex G25 equilibrated with buffer A. The protein peak was collected and frozen in liquid nitrogen. The extract was thawed, diluted with an equal volume of buffer containing 10 mM BTP, 10 mM DTT, 0.2 mM ATP (pH 7.0) and then centrifuged for 1 h at 30,000 RPM in a Beckman 70.1 Ti rotor. The supernatant was applied to a 0.5 × 5 cm Pharmacia FPLC

[1] This work was funded in part by the United States Department of Agriculture OGPS grant No. 86-CRCR-1-2017.

[2] Present address: Lilly Research Laboratories, P. O. Box 708, Greenfield, IN 46140.

[3] Abbreviations: rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; BTP, bis-tris-propane; FPLC, fast protein liquid chromatography; RuBP, ribulose 1,5-bisphosphate; AMP-PCP, adenylyl-β,γ-methylenediphosphate; AMP-PNP, adenylyl-imidodiphosphate

[4] 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.

column (Mono-Q, anion exchange), using 10 to 15 mg protein per injection. The column was washed with 20 mL of 20 mM BTP, 4 mM DTT (pH 7.0) before starting elution with a linear gradient of 0 to 500 mM KCl in the same buffer.

The gradient was run at a flow rate of 0.5 mL per min. Fractions (0.5 mL) were collected and assayed for rubisco activase activity or for ATP hydrolysis, as described below. ATP was added to the active fractions to give a final concentration of 0.2 mM, and they were stored in liquid nitrogen.

**Rubisco Activase Assay.** Rubisco was purified as described (14), then inactivated by gel filtration through a 0.7 × 27 cm column of Sephadex G50 equilibrated with 20 mM Tricine, 0.2 mM EDTA (pH 8.0). RuBP was added to a final concentration of 0.5 mM to form a rubisco-RuBP complex. The standard activation assay mixture contained 100 mM Tricine-KOH (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 4 mM RuBP, 1 mM ATP, 1 mM phosphoenolpyruvate, 20 units $mL^{-1}$ pyruvate kinase, 1 mg $mL^{-1}$ rubisco (as rubisco-RuBP), and 40 $\mu g$ $mL^{-1}$ rubisco activase protein. Rubisco activase was added 0.5 min before initiating the reaction with rubisco-RuBP complex. Rubisco activity was determined at intervals by adding a 50 $\mu L$ aliquot of the assay mixture to 450 $\mu L$ of 100 mM Tricine-KOH (pH 8.0), 10 mM $NaH^{14}CO_3$ (0.3 Ci $mol^{-1}$), 10 mM $MgCl_2$, 0.5 mM RuBP, and terminating this reaction 0.5 min later by addition of 100 $\mu L$ 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. Rubisco activase activity was based on the initial rate of increase in rubisco activity with time. One unit of rubisco activase was defined as an increase in the specific activity of rubisco of 1 $\mu mol$ $min^{-1}$ $mg^{-1}$ rubisco/min. Assays were conducted at 25°C. Addition of carbonic anhydrase (300 units $mL^{-1}$) did not increase the rate of rubisco activation in the standard assay (not shown) but was added when rubisco concentrations in excess of 1 mg $mL^{-1}$ were used to ensure rapid equilibration of $CO_2$ and $NaHCO_3$.

**ATP Hydrolysis.** Production of ADP was measured in a coupled spectrophotometric assay utilizing pyruvate kinase and lactic dehydrogenase. The reaction mixture contained 50 mM Tricine-KOH (pH 8.0), 20 mM KCl, 5 mM $MgCl_2$, 2.5 mM ATP, 1 mM phosphoenolpyruvate, 0.3 mM NADH, 12 units $mL^{-1}$ pyruvate kinase (Sigma P9136), 12 units $mL^{-1}$ lactic dehydrogenase (Sigma L1254), in a total volume of 0.4 mL. The reaction was started by the addition of rubisco activase (10–20 $\mu g$ protein) and the rate of ATP hydrolysis determined from the rate of NADH oxidation measured at 25°C. One unit was defined as 1 $\mu mol$ ATP hydrolyzed per min.

**Other Procedures.** Rubisco protein was determined spectrophotometrically using $A_{280} \times 0.61$ = mg $mL^{-1}$ (23). All other protein determinations were made with the dye binding assay (19) using BSA as a standard. SDS-PAGE was performed with 12% gels using the buffers of Laemmli (6) and stained with Coomassie blue.

## RESULTS

**Purification of Rubisco Activase.** In previous studies, intact chloroplasts were isolated from spinach leaves and used as a source of stromal proteins for purification of rubisco activase (13, 16, 20). We sought to increase the yield by purification directly from leaf homogenates and to find suitable conditions for storage of the purified protein without loss of activity. The purification table for the procedure adopted is given in Table I. Rubisco activase was precipitated in a 0 to 35% ammonium sulfate fraction with little loss in activity, provided the solution was at pH 7.0 and contained ATP and DTT. Rubisco is effectively removed from this fraction since it precipitates at higher ammonium sulfate (23). After desalting, the 0 to 35% fraction was frozen in liquid nitrogen and then thawed and ultracentrifuged to remove lipid vesicles and Chl. The supernatant was fractionated by ion exchange FPLC and the elution profiles for protein and rubisco activase activity are shown in Figure 1. There was a single peak of activity, eluting at 200 to 250 mM KCl. It is clear from Figure 1 that rubisco activase was the major protein present in the ammonium sulfate precipitate. The fractions from ion exchange FPLC had a specific activity of 3.5 to 4.5 units activation $mg^{-1}$ protein, and the overall purification in this instance was 48-fold with a 70% recovery of activity. In some instances, yields were as high as 18 mg protein from 100 g leaves. The purified rubisco activase also exhibited ATPase activity, and the measurement of ATP hydrolysis was useful for monitoring activity in fractions during the purification process. The purified enzyme hydrolyzed 1.0 to 1.5 $\mu mol$ ATP $Mg^{2+}$ $min^{-1}$ $mg^{-1}$ protein, and the ratio of activation activity to ATPase activity was virtually constant after the ammonium sulfate step (Table I). In the crude homogenate this ratio was lower, presumably due to the presence of other ATP-hydrolyzing enzymes.

Figure 2 shows SDS-PAGE analysis of the various fractions described in Table I. The peak fractions from ion exchange FPLC (lane 5) showed two predominant bands at 45 and 41 kD, which were both subunits of rubisco activase (16, 22). These bands stained very weakly with silver stain (data not shown). Analysis of the gels showed that the rubisco activase subunits comprised greater than 95% of the protein in this fraction. The two major bands in the leaf homogenate (lane 2) are the large and small subunits of rubisco. The ammonium sulfate step (lane 3) resulted in an 18-fold purification of rubisco activase and removed most of the rubisco. Ion exchange FPLC resulted in a further 2-fold purification and removed most other contaminating proteins. The 41 kD polypeptide of rubisco activase was clearly visible in the leaf homogenate (lane 2). Calculations based on the data in Table I suggested that the protein accounts for 1.5 to 2.0% (by weight) of the soluble proteins in a spinach leaf.

**Stability of Rubisco Activase.** The purified enzyme was relatively stable. When frozen and stored in liquid nitrogen in the presence of ATP and DTT, less than 10% of the activation activity was lost. The ATP requirement in stabilizing rubisco activase during purification and storage is illustrated in Figure 3. Purified protein was gel-filtered (at 2°C) to remove ATP with only minor loss of activity. If the desalted enzyme was incubated at 25°C in the absence of ATP, half the activity was lost after 30 min. In the presence of ATP very little activity was lost even after 4 h (Fig. 3). There was little effect of DTT on the stability at 25°C in the absence or presence of ATP (data not shown). The loss of activity shown in Figure 3 was greatly reduced if the protein was stored on ice in the absence of ATP. ADP also prevented the loss of activity at 25°C but was not as effective as ATP. AMP had no protective effect. The loss of activity was reversible. If ATP was added after incubation for 4 h at 25°C in the absence of ATP, activity recovered 25 to 35% after a further 2 h.

**Rubisco Activase Assay.** The assay is based on the ability of rubisco activase to catalyze the activation of rubisco in the presence of RuBP, as shown in Figure 4. When inactivated rubisco was added to a medium containing $CO_2$ and $Mg^{2+}$ there was a spontaneous increase in activity (circles), but if RuBP was added to the inactive rubisco first, the activation was prevented (squares). Even after 10 min in 10 mM $NaHCO_3$ and 10 mM $MgCl_2$, rubisco activity was normally less than 0.05 $\mu mol$ $min^{-1}$ $mg^{-1}$ protein in the presence of RuBP. In the presence of rubisco activase, activation occurred even in the presence of RuBP (triangles). With the concentrations of rubisco and rubisco activase used in Figure 3 (each 0.1 mg protein $mL^{-1}$) rubisco activity reached the same level as the spontaneous activation in the absence of RuBP.

**Nucleotide Specificity.** Rubisco activase activity required ATP

Table I. *Purification of Rubisco Activase from Spinach Leaves*

| Purification Step | Total Protein | Total Activity | Specific Activity | Recovery | Purification | Rubisco Activase / ATPase |
|---|---|---|---|---|---|---|
| | *mg* | *units* | *units mg*$^{-1}$ | *%* | *-fold* | *ratio*[a] |
| Homogenate | 900 | 78 | 0.087 | | | 1.0 |
| Ammonium sulfate | 51 | 78 | 1.53 | 100 | 18 | 3.4 |
| Freeze-thaw | 33 | 65 | 1.97 | 83 | 23 | 3.2 |
| FPLC-ion exchange | 13 | 55 | 4.13 | 70 | 48 | 3.4 |

[a] Ratio of rubisco activase activity (units) to ATP hydrolysis activity (units), both as defined in "Materials and Methods."

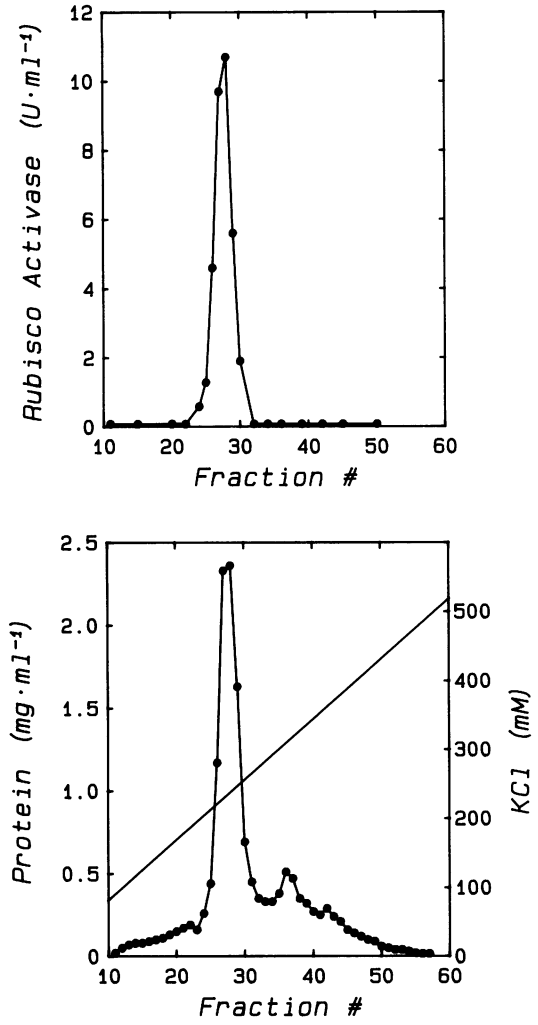


FIG. 1. Elution profiles for protein and rubisco activase activity from ion exchange FPLC using a Pharmacia Mono Q column. The straight line in the lower panel indicates the KCl gradient.

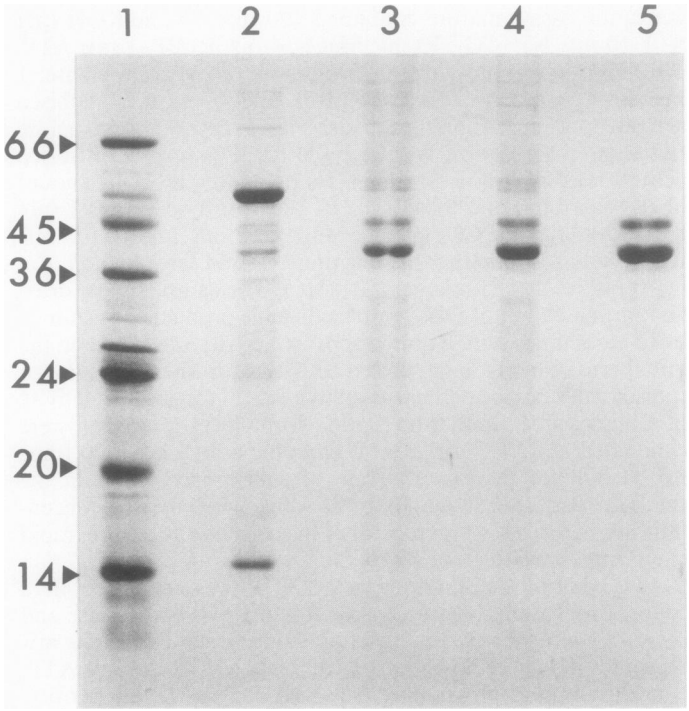


FIG. 2. SDS-PAGE of fractions described in Table I. Lane 1 contained mol wt standards and lanes 2 to 5 each contained 10 μg protein: lane 2, spinach leaf homogenate; lane 3, ammonium sulfate precipitate; lane 4, supernatant after freeze-thaw and ultracentrifugation; lane 5, peak fractions from ion exchange FPLC.

and was inhibited by ADP (Table II) as found previously (20). ATP could not be replaced by other nucleoside triphosphates (CTP, GTP, UTP) or the methylene and imido analogs of ATP. The low activity observed in the absence of ATP was due to the small increase in rubisco activity normally observed in the absence of rubisco activase. The effect of ADP in inhibiting rubisco activase activity also could not be mimicked by other nucleoside phosphates. In order to obtain maximal estimates of rubisco activase activity, an ATP regenerating system was required to maintain minimal levels of ADP.

**Effect of Metabolites.** The effect of a number of chloroplast metabolites was tested in the standard assay (with the ATP regenerating system) and in the absence of the regenerating system but with sufficient ADP to inhibit activity by 70% (Table III). In the standard assay, none of the metabolites tested altered activity by more than 10%, whereas in the presence of ADP, Pi increased activity and 3-phosphoglycerate, dihydroxyacetone-phosphate, ribose-5-phosphate, and fructose-1,6-bisphosphate all decreased activity.

**Interaction between Rubisco and Rubisco Activase.** At constant rubisco concentration, both the initial rate of increase of rubisco activity and the final specific activity of rubisco achieved increased in proportion to the concentration of rubisco activase (Fig. 5, left panel). Activity, calculated from the initial rate of increase of rubisco activity, was linearly dependent on the rubisco activase concentration (Fig. 5, right panel). Thus, the specific activity of rubisco activase as defined here was independent of concentration, at least up to 0.1 mg protein per mL. At constant rubisco activase concentration, the initial rate of increase of rubisco activity, was dependent on the rubisco concentration (Fig. 6, left panel). This relationship was not strictly linear, so the apparent specific activity of rubisco activase declined to a small extent with increasing rubisco concentration (Fig. 6, right

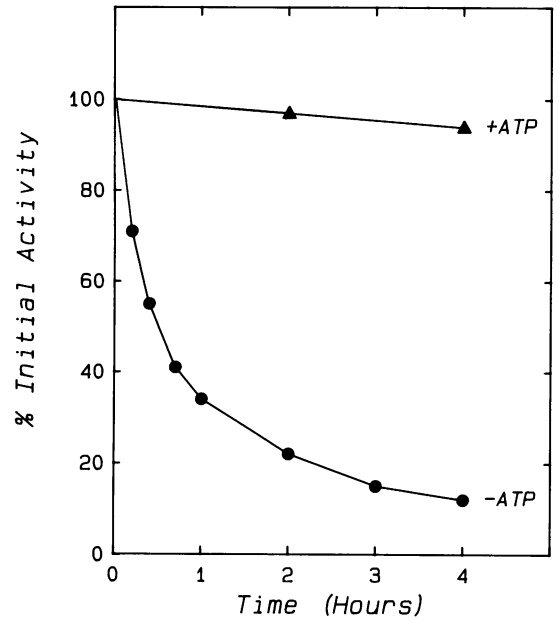


FIG. 3. Stability of rubisco activase following gel filtration in the absence of ATP. The protein was gel-filtered on a column of Sephadex G50 equilibrated with 20 mM BTP, 100 mM KCl, 5 mM DTT (pH 7.0) then ATP was added to one-half (final concentration, 1 mM) and the two samples incubated at 25°C. Initial activity was 3.22 units $mg^{-1}$ protein.

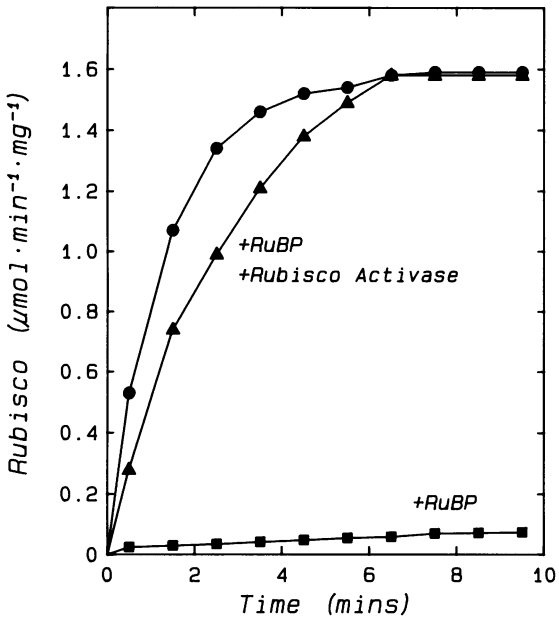


FIG. 4. Activation of rubisco in the presence and absence of RuBP and in the presence of RuBP and rubisco activase. The assays contained 100 mM Tricine-KOH (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 3 mM phosphoenolpyruvate, 20 units $mL^{-1}$ pyruvate kinase, 0.1 mg $mL^{-1}$ rubisco and, as indicated, 6 mM RuBP and 0.1 mg $mL^{-1}$ rubisco activase.

panel). The final activity of rubisco also increased with increasing rubisco concentration, but the relationship was not linear. Therefore the maximal rubisco specific activity also declined slightly with increasing rubisco concentration (Fig. 6).

## DISCUSSION

The purification procedure described gave relatively high yields of rubisco activase protein. Since this procedure does not require isolation of intact chloroplasts as reported earlier (16), it should be possible to purify rubisco activase from leaves of other plant species. The preparations contained some minor contaminants (Fig. 2) but these constituted less than 5% of the protein and the preparations were considered sufficiently pure for biochemical characterization. Rubisco activase was relatively stable when ATP was present during purification and storage. The ATP requirement for stability is consistent with the requirement for ATP in the rubisco activase assay (20) and the identification of a consensus nucleotide binding site in the protein (22). It will be of interest to determine whether the loss of activity upon incubation in the absence of ATP (Fig. 3) reflects release of bound nucleotide from the protein.

The assay for rubisco activase is based on its ability to promote activation of rubisco in the presence of physiological concentrations of RuBP. Assay blanks (minus rubisco activase or ATP) were typically only 7 to 8% of the activity in the standard assay, indicating that spontaneous activation is very slow under these conditions. Rubisco activase activity was determined from the initial rate of increase in rubisco activity rather than the extent to which rubisco was finally activated. Although both were dependent on the concentration of protein (Fig. 5), the rate of increase was directly proportional to the rubisco activase concen-

Table II. *Nucleotide Specificity of Rubisco Activase*

Specific activity was determined as described in "Materials and Methods" except that rubisco activase concentration was 0.048 mg $mL^{-1}$. The nucleotide concentrations were 2 mM (−ATP) and 1 mM (+2 mM ATP).

| Additions | Specific Activity | |
|---|---|---|
| | −ATP | +2 mM ATP |
| None | 0.33[a] | 3.23 |
| ADP | 0.32 | 0.78 |
| AMP | 0.36 | 3.58 |
| AMP-PCP | 0.36 | 3.31 |
| AMP-PNP | 0.10 | 2.96 |
| CTP | 0.33 | |
| GTP | 0.33 | |
| UTP | 0.30 | |
| CDP | | 3.77 |
| GDP | | 3.21 |
| UDP | | 3.58 |
| Regenerating system | | 3.79 |

[a] If this value is corrected for the spontaneous rate of rubisco activation in the presence of RuBP, the calculated specific activity is 0.

Table III. *Effect of Metabolites on Rubisco Activase Activity*

| Addition | Rubisco Activase[a] | |
|---|---|---|
| | Standard | + ADP |
| | *units* $mg^{-1}$ | |
| None | 3.58 | 1.09 |
| 1 mM Pi | 3.50 | 1.13 |
| 4 mM Pi | 3.65 | 1.30 |
| 1 mM 3-Phosphoglycerate | 3.58 | 1.08 |
| 4 mM 3-Phosphoglycerate | 3.28 | 0.83 |
| 1 mM Dihydroxyacetone phosphate | 3.28 | 0.85 |
| 1 mM Ribose-5-phosphate | 3.77 | 0.93 |
| 1 mM Ribulose-5-phosphate | 3.45 | 1.05 |
| 1 mM Fructose-1,6-bisphosphate | 3.43 | 0.95 |
| 1 mM Fructose-6-phosphate | 3.48 | 1.08 |
| 1 mM NADP | 3.68 | 1.08 |
| 1 mM NADPH | 3.40 | 1.08 |

[a] The standard assay contained 1 mM ATP and an ATP-regenerating system, as described in "Materials and Methods." The +ADP samples contained 0.5 mM ADP and the phosphoenolpyruvate and pyruvate kinase were omitted.

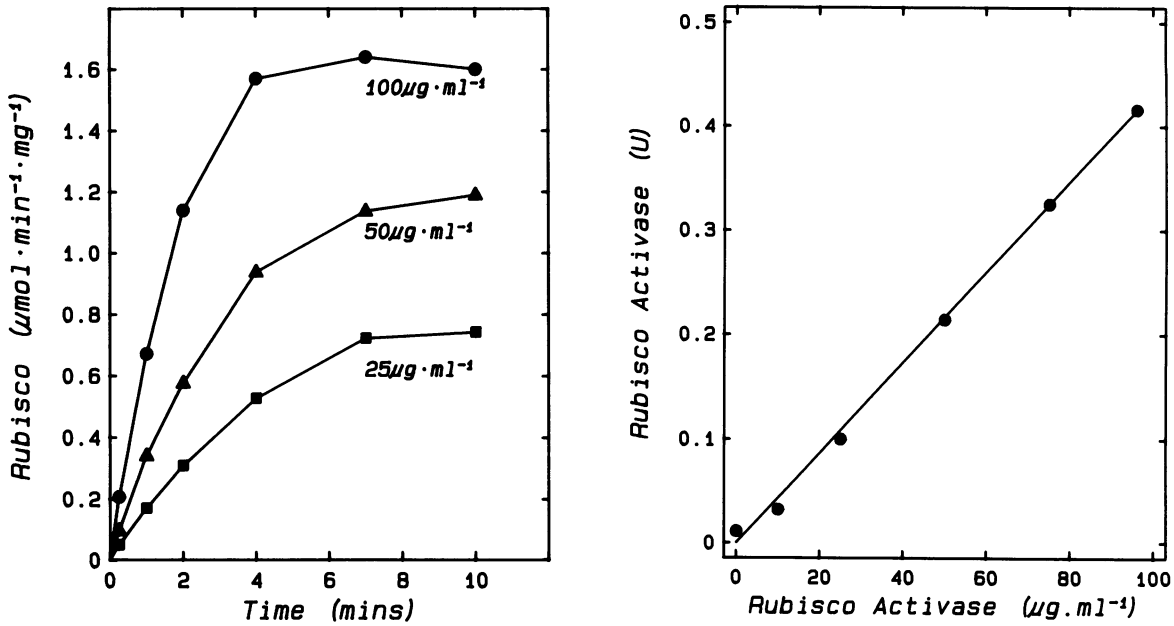


FIG. 5. Activation of rubisco with varying concentrations of rubisco activase. The left panel shows the increase in rubisco activity with time at the varying rubisco activase concentrations indicated. In this experiment the rubisco concentration was 0.6 mg $mL^{-1}$. The right panel shows activity determined from the initial rate of increase of rubisco activity as a function of rubisco activase concentration in the standard assay.

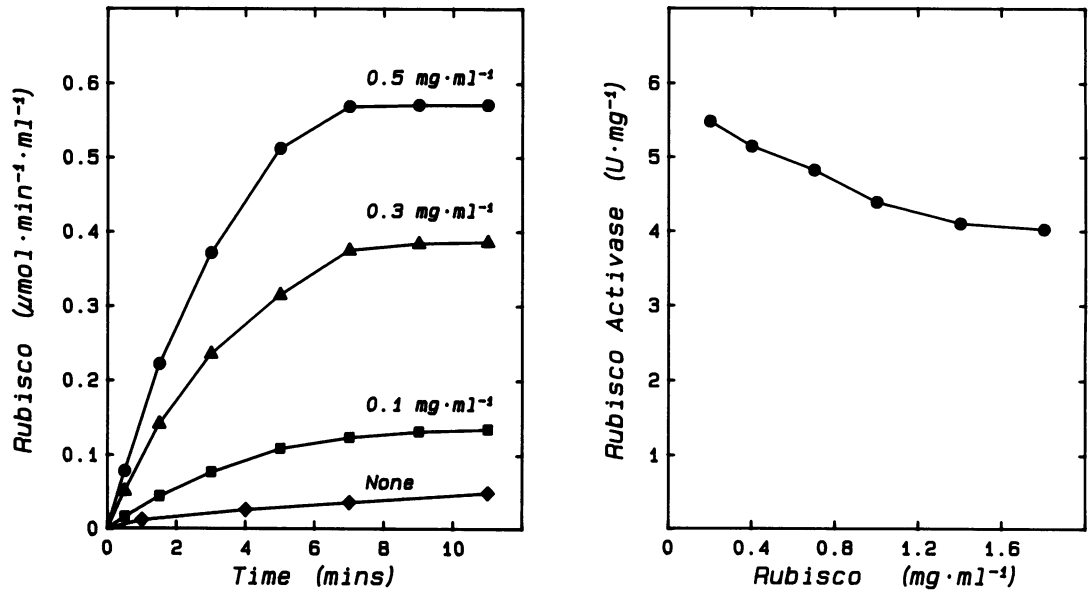


FIG. 6. Activation of rubisco by rubisco activase at varying rubisco concentration. The left panel shows the increase in rubisco activity with time at the varying rubisco concentrations indicated. Rubisco was added as the rubisco-RuBP complex. The assay contained 0.054 mg $mL^{-1}$ rubisco activase. After 11 min the rubisco specific activities (μmol $min^{-1}$ $mg^{-1}$ rubisco) were: 0.10 (minus activase), 1.35 (0.1 mg $mL^{-1}$), 1.29 (0.3 mg $mL^{-1}$), and 1.14 (0.5 mg $mL^{-1}$). The right panel shows rubisco activase specific activity, determined from the initial rate of increase of rubisco activity, as a function of rubisco concentration. The experiment in the right panel was with 0.054 mg $mL^{-1}$ rubisco activase and also contained 300 units $mL^{-1}$ carbonic anhydrase.

tration and is a more appropriate measure of activity. An alternative to the units used in this paper would be to calculate the number of mol of rubisco activated per min. This would have conventional enzyme unit dimensions (mol product $min^{-1}$) but would require measurement of the maximum specific activity of rubisco and would make assumptions about the rate of deactivation of rubisco and the interaction between rubisco activase and rubisco. The present unit also has the advantage that it can be more readily related to the experimental results in which the activity of rubisco is the measured parameter.

Assuming that rubisco is 50% of leaf soluble protein and rubisco activase comprises 2% (Table I), the rubisco:rubisco activase ratio *in vivo* would be 25:1, on a weight basis. The same ratio of proteins was chosen for the standard assay. If the mol wt of the holoenzyme is around 200 kD (16) this would indicate a molar ratio of 9:1 or 72 rubisco active sites per 1 activase. On this basis, it seems likely that each rubisco activase reacts with several rubisco holoenzymes, activating each one in turn.

The rate of activation was also proportional to the rubisco concentration (Fig. 6). This indicates that the activation process involves a second order reaction dependent on the concentrations of rubisco and rubisco activase:

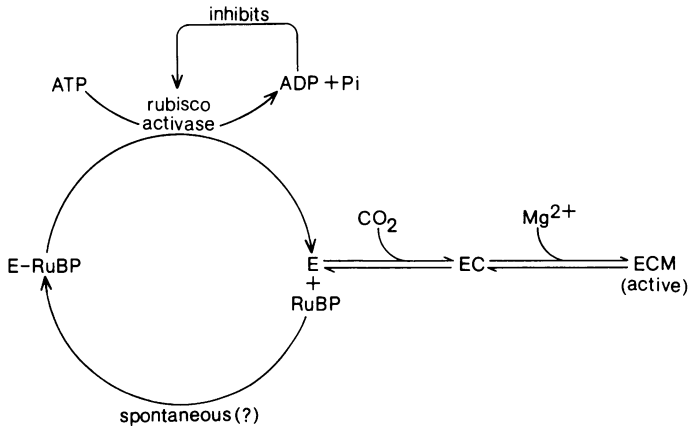


FIG. 7. Possible scheme for activation of rubisco by rubisco activase. Rubisco enzyme is represented as E.

$$\text{Activation rate} = k \cdot [\text{rubisco}] \cdot [\text{rubisco activase}].$$

However, the small but significant deviation from strict proportionality when the rubisco concentration was varied suggests that the actual kinetics are more complex. One obvious alternative is that formation of a putative rubisco activase-rubisco complex is kinetically important such that:

$$\text{Rubisco activase} + \text{rubisco} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \xrightarrow{k_2} \text{activated rubisco}.$$

Since [Rubisco Activase] ≃ [Rubisco], a modified form of the Michaelis-Menten equation would best describe the kinetics (2);

$$\text{Activation rate} = \frac{k_2\,[\text{rubisco activase}]\,[\text{rubisco}]}{\frac{k_{-1} + k_2}{k_1} + [\text{rubisco}] + [\text{rubisco activase}]}.$$

If we used this model to analyze the activation data, the $(k_{-1} + k_2)/k_1$ term was about 3.0 mg $mL^{-1}$. In order to rigorously examine the suitability of this model, a broader range of rubisco and especially rubisco activase concentrations would be required in order to detect the predicted deviation from linearity at higher rubisco activase concentration. Since the addition of $CO_2$ and Mg to form activated enzyme is not explicitly described, other possibilities also exist. More information about intermediate steps in the overall reaction will be required to formulate better kinetic descriptions of the process.

In the chloroplast, the concentration of soluble protein in the stroma may be as high as 400 mg $mL^{-1}$ (12) suggesting a rubisco concentration of 200 mg $mL^{-1}$. It is not practical to approach these concentrations *in vitro*; hence, the rubisco activase activity *in vivo* may be much greater than that measured with the purified protein. However, the *in vivo* activity would be decreased by the presence of ADP (11, 20).

The extent of rubisco activation was dependent on the concentration of rubisco activase (Fig. 5). This observation is consistent with an uncatalyzed deactivation of rubisco occurring simultaneously with the rubisco activase-catalyzed activation. Rubisco activity declines in the presence of RuBP *in vitro*, but this loss of activity is not correlated with a decarbamylation of the enzyme (J Pierce, personal communication; D Edmonson, J Andrews, personal communication). On the other hand, changes in rubisco activation *in vivo* were correlated with formation of tightly bound RuBP (1), suggesting that rubisco-RuBP is formed *in vivo*. The early experiments with rubisco activase suggested that it did not affect the rate of deactivation *in vitro*, although the experiments were conducted in a more complex reconstituted system without added ATP or ADP (10). Later experiments indicated that the activation of rubisco-RuBP by rubisco activase is associated with carbamylation of the enzyme (21). Whether conditions allowing a subsequent deactivation of rubisco in the presence of rubisco activase result in the decarbamylation of the enzyme is an important question for future work.

The scheme shown in Figure 7 would account for the kinetics of rubisco activation observed in Figures 5 and 6. In the presence of RuBP and an ATP regenerating system, the rate of activation would be proportional to the concentrations of both rubisco activase and rubisco, while the extent of activation would depend on the level of rubisco activase. Complete activation of rubisco would be achieved only when sufficient rubisco activase was present to catalyze activation at a rate in excess of the rate of deactivation of rubisco. In leaves, the levels of rubisco and rubisco activase would be constant in the short term, and the activation state of rubisco would be determined by the concentrations of RuBP, ATP, and ADP, as well as the pH and levels of $CO_2$ and $Mg^{2+}$. In the dark, the ATP/ADP ratio is low enough to largely inhibit rubisco activase, but RuBP levels are also low, so the activation state of rubisco would depend on $CO_2$ concentration, as has been observed (15, 17). In the light, RuBP levels are sufficiently high to prevent spontaneous activation of rubisco and to promote deactivation of activated rubisco, hence the level of activation would depend on rubisco activase activity. This, in turn, would depend on the levels of ATP and ADP, since rubisco activase requires ATP and is inhibited by ADP (20).

We have shown previously that in intact isolated chloroplasts the steady state rubisco activity was dependent on the concentration of ATP in the stroma (11), which is consistent with the scheme in Figure 7. In the presence of ADP, rubisco activase may also be modulated by other chloroplast metabolites (Table III) which could explain the decreased rubisco activity in isolated chloroplasts in the presence of dihydroxyacetone phosphate or 3-phosphoglycerate (11). In the intact chloroplast, changes in rubisco activity only followed changes in ATP after a short delay (11), and this may reflect the kinetics of deactivation of rubisco to achieve a new steady state following decreases in rubisco activase-mediated activation (Fig. 7).

The exact nature of the interaction between rubisco and rubisco activase is not yet established. The scheme in Figure 7 represents rubisco activase involvement in the conversion of rubisco-RuBP to rubisco, followed by spontaneous activation by $CO_2$ and $Mg^{2+}$, although it is also possible that rubisco activase is involved in the carbamylation process (21). The role of ATP in the activation process is also not known, although the observation that rubisco activase hydrolyzes ATP suggests there may be an energy requirement for the activation of rubisco in the presence of RuBP. The purification of rubisco activase described in this paper provides a procedure for preparation of sufficient quantities of protein, in a stable form, for elucidation of these mechanisms.

*Acknowledgment*—We thank W. L. Ogren for helpful comments.

## LITERATURE CITED

1. BROOKS A, AR PORTIS JR 1988 Protein-bound ribulose bisphosphate correlates with deactivation of ribulose bisphosphate carboxylase in leaves. Plant Physiol 87: 244–249
2. CHAPLIN MF 1981 The effect of enzyme concentration on the Michaelis-Menten equation. Trends Biochem Sci 6: vi
3. CHU DK, JA BASSHAM 1975 Regulation of ribulose, 1,5-bisphosphate carboxylase by substrates and other metabolites. Further evidence for several types of binding sites. Plant Physiol 55: 720–726
4. JORDAN DB, R CHOLLET 1983 Inhibition of ribulose bisphosphate carboxylase by substrate ribulose 1,5-bisphosphate. J Biol Chem 258: 13752–13758
5. JORDAN DB, WL OGREN 1981 A sensitive assay procedure for simultaneous determination of ribulose, 1,5-bisphosphate carboxylase and oxygenase activities. Plant Physiol 67: 237–245
6. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277: 680–685

7. LORMIER GH, MR BADGER, TJ ANDREWS 1979 The activation of ribulose 1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. Biochemistry 15: 529–635
8. LORIMER GH, HM MIZIORKO 1980 Carbamate formation on the e-amino group of a lysyl residue as the basis for the activation of ribulosebisphosphate carboxylase by $CO_2$ and $Mg^{2+}$. Biochemistry 19: 5321–5328
9. MIZIORKO HM, GH LORIMER 1983 Ribulose 1,5-bisphosphate carboxylase/oxygenase. Annu Rev Biochem 52: 507–535
10. PORTIS AR JR, ME SALVUCCI, WL OGREN 1987 Activation of ribulosebisphosphate carboxylase/oxygenase at physiological $CO_2$ and ribulosebisphospate concentrations by rubisco activase. Plant Physiol 82: 967–971
11. ROBINSON SP, AR PORTIS JR 1988 Involvement of stromal ATP in the light activation of ribulose 1,5-bisphosphate carboxylase/oxygenase in intact isolated chloroplasts. Plant Physiol 86: 293–298
12. ROBINSON SP, DA WALKER 1981 Photosynthetic carbon reduction cycle. *In* MD Hatch, NK Boardman, eds, The Biochemistry of Plants: A comprehensive Treatise, Vol 8. Academic Press, New York, pp 193–236
13. SALVUCCI ME, AR PORTIS JR, WL OGREN 1985 A soluble chloroplast protein catalyzes ribulosebisphophate carboxylase/oxygenase activation *in vivo*. Photosynth Res 7: 191–203
14. SALVUCCI ME, AR PORTIS JR, WL OGREN 1986 Purification of ribulose 1,5-bisphosphate carboxylase/oxygenase with high specific activity by fast protein liquid chromatography. Anal Biochem 153: 97–101
15. SALVUCCI ME, AR PORTIS JR, WL OGREN 1986 Light and $CO_2$ response of ribulose 1,5-bisphosphate carboxylase/oxygenase activation in *Arabidopsis* leaves. Plant Physiol 80: 655–659
16. SALVUCCI ME, JM WERNEKE, WL OGREN, AR PORTIS JR 1987 Purification of species distribution of rubisco activase. Plant Physiol 84: 930–936
17. SCHNYDER H, F MACHLER, J NOSBERGER 1984 Influence of temperature and $O_2$ concentration on photosynthesis and light activation of ribulosebisphosphate carboxylase/oxygenase in intact leaves of white clover (*Trifolium repens* L.) J Exp Bot 35: 147–156
18. SOMERVILLE CR, AR PORTIS JR, WL OGREN 1982 A mutant of *Arabidopsis thaliana* which leaks activation of RuBP carboxylase *in vivo*. Plant Physiol 70: 381–387
19. SPECTOR T 1987 Refinement of the coomassie blue method of protein quantitation. A simple and linear spectrophotometric assay for >0.5 to 50 μg of protein. Anal Biochem 86: 142–146
20. STREUSAND VJ, AR PORTIS JR 1987 Rubisco activase mediates ATP-dependent activation of ribulosebisphosphate carboxylase. Plant Physiol 85: 152–154
21. WERNEKE JM, JM CHATFIELD, WL OGREN 1988 Catalysis in ribulosebisphosphate carboxylase/oxygenase activation by the product of a rubisco activase cDNA clone expressed in *Escherichia coli*. Plant Physiol 87: 917–920
22. WERNEKE JM, RE ZIELINSKI, WL OGREN 1988 Structure and expression of spinach leaf cDNA encoding rubisco activase. Proc Natl Acad Sci USA 85: 787–791
23. WISHNICK M, MD LANE 1971 Ribulose diphosphate carboxylase from leaves. Methods Enzymol 23: 570–577