# Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activase Protein Prevents the *in Vitro* Decline in Activity of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase[1]

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## ABSTRACT

The rate of $CO_2$ fixation by ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) following addition of ribulose 1,5-bisphosphate (RuBP) to fully activated enzyme, declined with first-order kinetics, resulting in 50% loss of rubisco activity after 10 to 12 minutes. This *in vitro* decline in rubisco activity, termed fallover, was prevented if purified rubisco activase protein and ATP were added, allowing linear rates of $CO_2$ fixation for up to 20 minutes. Rubisco activase could also stimulate rubisco activity if added after fallover had occurred. Gel filtration of the RuBP-rubisco complex to remove unbound RuBP allowed full activation of the enzyme, but the inhibition of activated rubisco during fallover was only partially reversed by gel filtration. Addition of alkaline phosphatase completely restored rubisco activity following fallover. The results suggest that fallover is not caused by binding of RuBP to decarbamylated enzyme, but results from binding of a phosphorylated inhibitor to the active site of rubisco. The inhibitor may be a contaminant in preparations of RuBP or may be formed on the active site but is apparently removed from the enzyme in the presence of the rubisco activase protein.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (rubisco[2]) catalyzes both the carboxylation of RuBP, which is the initial step in photosynthetic carbon reduction, and its oxygenation, which is the first reaction of the photorespiratory carbon oxidation cycle. Measurement of rubisco activity *in vitro* has been complicated by the fact that the enzyme can exist in more than one form. One inactive form (E) can be converted to an active form (ECM) by incubation with $CO_2$ and $Mg^{2+}$ at alkaline pH, resulting in carbamylation of a lysine residue near the active site (9). However, even with fully activated enzyme, the initial rate of carboxylation upon addition of RuBP is not maintained, and most assays are normally conducted for 1 min or less. This decline in activity of rubisco *in vitro* has been informally termed "fallover"[3] and is apparent in a number of published reports (1, 2, 5, 10, 11,

17). RuBP binds tightly to the inactive form of the enzyme (3) and fallover may represent the formation of this inactive rubisco-RuBP complex (ER), which would only very slowly undergo reactivation by $CO_2$ and $Mg^{2+}$ (7). Alternatively, fallover may result from binding of inhibitory degradation products present in the RuBP used for *in vitro* assays (8, 11).

Whatever the mechanism, it is obvious either that fallover does not occur *in vivo* or, if it does, that some repair mechanism exists to reverse the effect. Thus, Sicher *et al.* (17) found no evidence for a decline in rubisco activity in intact isolated chloroplasts but fallover was observed once the chloroplasts were lysed. Considerable evidence now exists that activation of rubisco *in vivo* is not a spontaneous process but is catalyzed by a specific protein, rubisco activase (12, 15, 18, 20). In the presence of rubisco activase and ATP, activation of rubisco occurs *in vitro* at physiological $CO_2$ concentrations and in the presence of RuBP (12, 14, 20). We have investigated the possible involvement of rubisco activase in maintaining rubisco activity. The results indicate that rubisco activase can both prevent and reverse the *in vitro* decline in rubisco activity and suggest that fallover results from binding of a phosphorylated inhibitor to the active site of rubisco.

## MATERIALS AND METHODS

### Chemicals and Enzymes

RuBP was synthesized enzymically from ribose 5-phosphate and purified as described previously (4). Rubisco activase protein was purified from spinach (*Spinacia oleracea* L.) leaves as described previously (14). Rubisco was purified as described previously (21), then inactivated by gel filtration at 25°C through Sephadex G-50 equilibrated with 20 mM Tricine, 0.2 mM EDTA, pH 8.0. The enzyme was activated by incubation at 25°C for 1 h in 100 mM Tricine, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM DTT, pH 8.0, at a protein concentration of 1 to 2 mg $mL^{-1}$. Alkaline phosphatase (bovine intestinal mucosa type VII-NL from Sigma[4]) was prepared as a stock solution in 100 mM Tricine, pH 9.0, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ to give 100 IU $mL^{-1}$.

[2] Abbreviations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate.

[3] This descriptive term was introduced by D. Edmonson and J. Andrews at the RUBISCO '87 Meeting in Tucson, AZ.

[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.

## Assay of Rubisco Activity

Rubisco activity was determined at 25°C by a one-step assay (Figs. 1 and 2) or a two step assay (Figs. 3 and 4). In the one-step assay, activated rubisco was added to assay medium containing 100 mM Tricine, pH 8.0, 10 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM RuBP, 2 mM ATP, 8 mM phosphoenolpyruvate, 2 mM DTT, 20 IU $mL^{-1}$ pyruvate kinase, and 0.3 Ci $mol^{-1}$ of $^{14}CO_2$. Samples were withdrawn at various times, acidified, and dried to determine incorporation of $^{14}CO_2$ into acid-stable products by liquid scintillation counting. For the two-step assay, activated rubisco was added to reaction medium (100 mM Tricine [pH 8.0], 10 mM $MgCl_2$, 10 mM $NaHCO_3$, and 4 mM RuBP). At various times, 50-$\mu$L samples were removed and added to 450 $\mu$L of assay medium (100 mM Tricine [pH 8.0], 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.5 mM RuBP, and 1 Ci $mol^{-1}$ of $^{14}CO_2$), and the assay was terminated with acid after 0.5 min to determine $^{14}CO_2$ fixation. For the experiment described in Figure 3, ER was prepared by adding 0.5 mM RuBP to inactivated rubisco and incubating for 30 min at 25°C.

## Other Procedures

Rubisco protein was determined spectrophotometrically using $A_{280} \times 0.61 = $ mg $mL^{-1}$ (21) and rubisco activase protein by the dye binding assay (19).

## RESULTS

Addition of fully activated rubisco to assay medium with RuBP and $^{14}CO_2$ resulted in initial rates of fixation of 1.0 to 1.5 $\mu$mol $min^{-1}$ $mg^{-1}$ but there was a continual decline in the activity (Fig. 1). After 10 to 12 min the rate of $CO_2$ fixation was only half the initial rate, and after 30 min it had decreased by 70 to 80%. The same decline in rubisco activity was observed in a two-step assay, where rubisco was incubated with RuBP but without labeled $CO_2$, and samples were diluted 10 fold into fresh medium with $^{14}CO_2$ and terminated 0.5 min later. The in vitro decline in rubisco activity appeared to be a first-order process from a kinetic analysis of the data.

In the presence of rubisco activase protein, ATP, and an ATP-regenerating system, the in vitro decline in rubisco activity was largely prevented, and rates of $^{14}CO_2$ fixation were linear for up to 20 min (Fig. 1). Controls in which ATP was omitted, or with rubisco activase, which had been boiled, were essentially the same as in the absence of rubisco activase. The extent to which fallover was prevented was dependent on the rubisco activase concentration (data not shown). Addition of rubisco activase also subsequently reversed the effects of fallover, as shown in Figure 2. After 40 min, rubisco activity had declined to 20% of the initial rate but addition of rubisco activase restored activity to 72%. There was only a short lag before recovery of rubisco activity and $CO_2$ fixation was then linear for 20 min.

If fallover represents formation of a tight complex between the inactive form of rubisco and RuBP (ER), this could explain the prevention and reversal of fallover shown in Figures 1 and 2, since rubisco activase is known to promote activation of rubisco in the presence of RuBP (12, 14, 20).
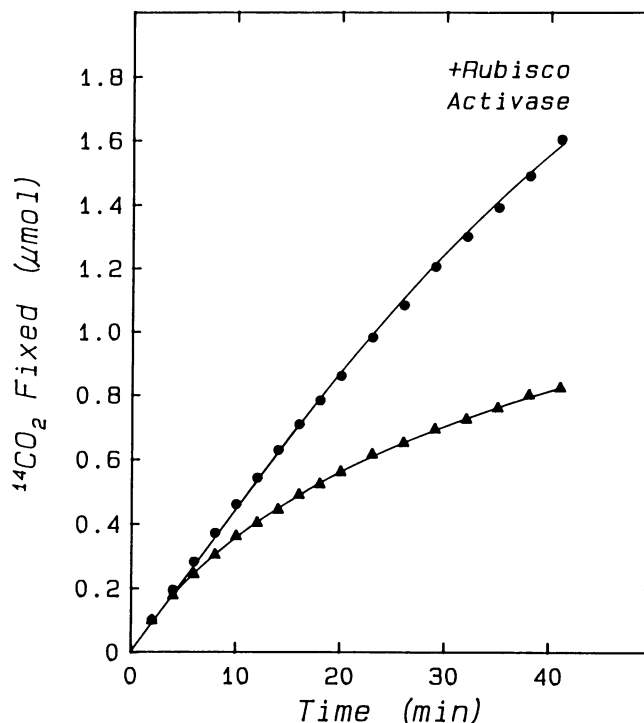


**Figure 1.** Time course of $CO_2$ fixation by rubisco in the presence and absence of rubisco activase. Rubisco concentration was 40 $\mu$g $mL^{-1}$ and the upper curve also contained 100 $\mu$g $mL^{-1}$ rubisco activase. The initial rate of $^{14}CO_2$ fixation was 1.25 $\mu$mol $min^{-1}$ $mg^{-1}$ rubisco.

However, the results shown in Figure 3 are not consistent with such a mechanism. When rubisco in the ER form was incubated with $CO_2$, $Mg^{2+}$, and RuBP, activation was very slow and, even after 30 min, the specific activity was only 0.12 $\mu$mol $min^{-1}$ $mg^{-1}$ (Fig. 3A). After gel filtration in the presence of $CO_2$ and $Mg^{2+}$ to remove any unbound RuBP, there was a rapid activation of rubisco and within 10 min the specific activity was equal to fully activated enzyme (Fig. 3A). This presumably reflects dissociation of RuBP from inactive rubisco and its removal by gel filtration or consumption by active enzyme, allowing spontaneous activation of all the rubisco present. The same experiment was performed starting with fully activated rubisco, and after 30 min in the presence of RuBP the activity had declined to 30% of the initial activity (Fig. 3B). Gel filtration of this enzyme only recovered 30% of the decline in activity resulting from fallover, and the maximum specific activity achieved was only 50% of the fully activated enzyme.

The results in Figure 3 suggest that an inhibitor is tightly bound to rubisco during fallover. If this inhibitor is phosphorylated, the inhibition should be reversible by phosphatase treatment. Addition of alkaline phosphatase to rubisco that had been allowed to fallover for 30 min resulted in an increase in rubisco activity after a lag of 6 to 8 min (Fig. 4). Fifty percent reversal of fallover inhibition occurred after an additional 5 min, and 90 min after adding phosphatase the rubisco activity was equal to the initial activity of fully activated enzyme.
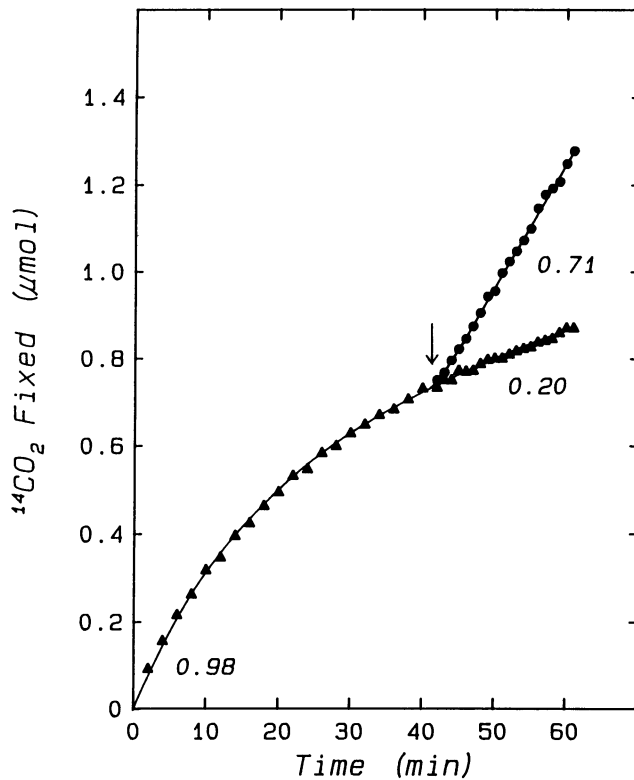
**Figure 2.** Time course of $CO_2$ fixation by rubisco and effect of addition of rubisco activase. Rubisco concentration was 40 $\mu$g mL$^{-1}$ mg$^{-1}$. After 41 min (arrow) rubisco activase (100 $\mu$g mL$^{-1}$) was added to one sample (circles). The numbers indicate rates of $^{14}CO_2$ fixation, in $\mu$mol min$^{-1}$ mg$^{-1}$ rubisco.

## DISCUSSION

The results in Figures 1 and 2 show that the in vitro decline in rubisco activity can be prevented and reversed by rubisco activase. The ATP requirement for this effect is consistent with the activation of rubisco in the presence of RuBP, which also requires rubisco activase and ATP, and is inhibited by ADP (14, 20). The stabilizing effect of rubisco activase may have contributed to the improvement of the spectrophotometric assay of rubisco in crude extracts by an ATP-regenerating system reported by Lilley and Walker (6).

The prevention of fallover by rubisco activase could be explained if fallover results from formation of ER, since this form of the enzyme would be reactivated by the rubisco activase protein under these conditions (14, 20). However, the results shown in Figure 3 are not consistent with such a mechanism. Spontaneous activation of the ER form of rubisco occurred readily once the unbound RuBP was removed by gel filtration, whereas the effects of fallover were only partially reversed under the same conditions. Furthermore, direct measurement has shown that the extent of carbamylation of rubisco does not decrease during fallover (J. Pierce, personal communication). This indicates that fallover does not result from decarbamylation of activated rubisco.

Alternatively, the results would be consistent with binding of an inhibitor to the activated form of the enzyme. The failure of gel filtration to reverse the effects of fallover (Fig. 3B) implies the presence of a relatively tight binding inhibitor and the reversal of fallover by alkaline phosphatase (Fig. 4) suggests that the inhibitor is phosphorylated. A similar pattern of inhibition of rubisco is observed with the endogenous inhibitor, carboxyarabinitol 1-phosphate, which binds to the activated form of rubisco. Inhibition by carboxyarabinitol 1-phosphate is only partially reversed by gel filtration but can be overcome by alkaline phosphatase treatment (16). Rubisco activase can also reverse carboxyarabinitol 1-phosphate inhibition of rubisco, apparently by increasing the dissociation rate of the bound compound from the enzyme, since carboxyarabinitol 1-phosphate is not metabolized by rubisco activase (13). By analogy, rubisco activase may prevent fallover by increasing the dissociation rate of an inhibitor from the active site of rubisco. The inhibitor responsible for fallover apparently does not bind as tightly to rubisco as carboxyarabinitol 1,5-bisphosphate, which also binds to the activated form of rubisco. Inhibition by carboxyarabinitol 1,5-bisphosphate cannot be reversed by alkaline phosphatase (16) nor by rubisco activase (13).

The inhibitor which binds to rubisco during fallover may be a contaminant present in RuBP preparations. Paech et al. (11) found two degradation products in preparations of RuBP, one that was identified as xylulose 1,5-bisphosphate and the other tentatively identified as deoxypentodiulose 5-phosphate.

Xylulose 1,5-bisphosphate is a slow, tight binding inhibitor of rubisco (8), which would be consistent with the observed
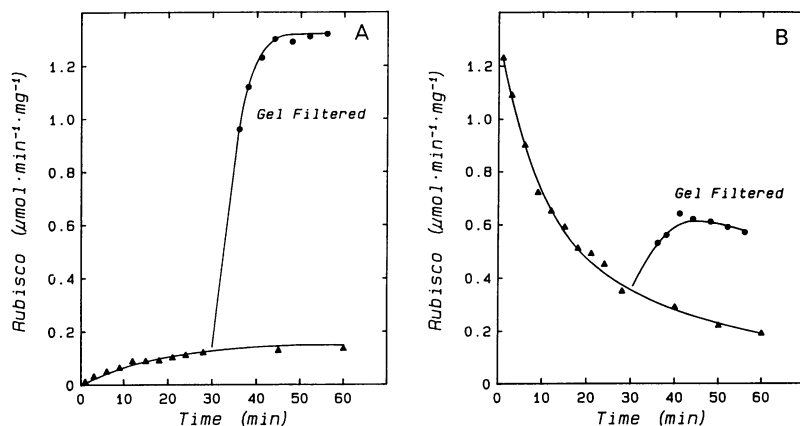


**Figure 3.** Effect of gel filtration on rubisco activity. Rubisco, in the ER form (A) or in the activated form (B) was added to a final concentration of 100 $\mu$g mL$^{-1}$ to medium containing 100 mM Tricine, 10 mM NaHCO$_3$, 10 mM MgCl$_2$, and 4 mM RuBP. Aliquots were removed for assay of rubisco activity at the times indicated with the two-step assay. At 30 min, a sample was removed for gel filtration (with the same medium), the protein peak was collected, and aliquots were also removed for assay of rubisco activity at the times indicated.
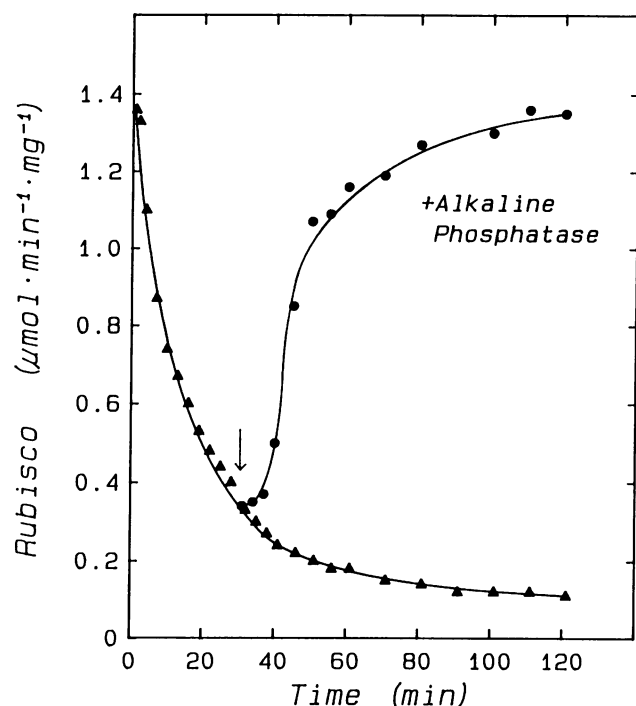
**Figure 4.** Reversal of *in vitro* decline in rubisco activity by alkaline phosphatase. The activated form of rubisco was added to a final concentration of 100 $\mu$g mL$^{-1}$ to medium described in Fig. 3. After 30 min (arrow), alkaline phosphatase was added to part of the mixture (circles) to give a final concentration of 2 IU mL$^{-1}$. Rubisco activity was determined in aliquots of the mixture with the two-step assay at the times indicated.

kinetics of fallover. If fallover *in vitro* is the result of an inhibitor in RuBP, its reversal by rubisco activase may be purely fortuitous in that rubisco activase may enhance dissociation of the inhibitor from the active site in the same way as it apparently enhances the dissociation of carboxyarabinitol 1-phosphate (13).

It is also possible that fallover results from a low frequency catalytic misfire of rubisco, resulting in formation of an inhibitor on the active site of the enzyme. From the kinetics of fallover (Figs. 1, 3, and 4), 50% loss of activity occurred after 700 to 800 enzyme turnovers, which would require a misfire every 1400 to 1600 turnovers. In this case, fallover may also occur *in vivo* and rubisco activase would be necessary to prevent a decline in rubisco activity. If rubisco activase only enhances the dissociation of the inhibitor from rubisco, some mechanism to metabolize it would probably be necessary.

The observation that a tight binding inhibitor is retained on rubisco after rapid gel filtration of the enzyme (Fig. 3B) has allowed the isolation of the inhibitor (AR Portis, SP Robinson, unpublished results). Further characterization of the inhibitor should facilitate the elucidation of its role in the *in vitro* decline in rubisco activity. This would also clarify the role of rubisco activase in preventing fallover and its relevance to rubisco activity *in vivo*.

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