

Activation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (Rubisco) by Rubisco Activase¹

Effects of Some Sugar Phosphates

Ross McC. Lilley² and Archie R. Portis, Jr.*

U.S. Department of Agriculture, Agricultural Research Service, Photosynthesis Research Unit,
1102 S. Goodwin Avenue, Urbana, Illinois 61801

ABSTRACT

The activation of purified ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) has been studied in the presence of sugar phosphates, and the effect of rubisco activase on this process determined. During an 11-minute time course at pH 7.7 and 11 micromolar CO₂, the activation of rubisco was strongly inhibited by ribulose-1,5-bisphosphate (4 millimolar), fructose-1,6-bisphosphate (1 millimolar) and ribose 5-phosphate (5 millimolar), but this inhibition was overcome by the addition of rubisco activase and activation then proceeded to a greater extent than spontaneous activation of rubisco. Glycerate 3-phosphate (20 millimolar) slowed the initial rate but not the extent of activation and rubisco activase had no effect on this. The activation of rubisco was shown to be affected by phosphoenolpyruvate (3 millimolar) but not by creatine phosphate (3 millimolar) or ATP (3 millimolar), and the creatine-phosphate/creatine phosphokinase system was used to generate the high ATP/ADP quotients required for rubisco activase to function. ATP was shown to be required for the rubisco activase-dependent rubisco activation in the presence of fructose-1,6-bisphosphate (1 millimolar). It is concluded that rubisco activase has a mixed specificity for some sugar phosphate-bound forms of rubisco, but has low or no activity with others. Some possible bases for these differences among sugar phosphates are discussed but remain to be established.

Purified rubisco³ is inactive and the formation of an enzyme-carbamate metal complex is necessary for catalytic activity which requires the presence of CO₂, Mg²⁺, and alkaline

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² Permanent address: Department of Biology, University of Wollongong, P. O. Box 1144 Wollongong, N.S.W. 2500, Australia.

³ Abbreviations: rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; CA1P, 2-carboxy-D-arabinitol-1-phosphate; CABP, 2-carboxy-D-arabinitol-1,5-bisphosphate; creatine-P, creatine phosphate; E, inactive (CO₂ and Mg²⁺-free) form of ribulose-1,5-bisphosphate carboxylase/oxygenase; ECM, active (CO₂ and Mg²⁺-bound) form of ribulose-1,5-bisphosphate carboxylase/oxygenase; FBP, fructose-1,6-bisphosphate; glycerate 3-P, glycerate 3-phosphate; ribose 5-P, ribose 5-phosphate; RuBP, ribulose-1,5-bisphosphate; PEP, phosphoenolpyruvate.

pH (13). The binding site for the substrate (RuBP) is not very selective in that a wide variety of phosphate compounds at millimolar concentrations (3, 10, 12, 13) can also bind to this site and such binding can occur to both the active and inactive forms of rubisco. Recent structural studies of rubisco indicate that access to the carbamate and Mg²⁺ sites is severely restricted when the active site is occupied (1). Consequently, these phosphates can have either stimulatory or inhibitory effects on the activity observed under conditions that normally allow only partial activation, depending on their order of addition with respect to CO₂ and Mg²⁺ prior to the initiation of catalysis by RuBP (3, 10, 12, 13). For example, rubisco activity can be stimulated by 6-phosphogluconate or inhibited by ribose 5-P, and either inhibited or stimulated by FBP depending on the length of the preincubation period with the FBP (10). RuBP seems to have similar effects (9, 10) although they are more difficult to characterize because of its consumption by catalysis. Nevertheless, it is clear that changes in the activation state of rubisco in response to changes in one or more of pH, CO₂, and Mg²⁺ concentrations can be greatly altered when sugar phosphates, particularly RuBP, are present.

Rubisco activase, a soluble chloroplast protein composed of monomers of about 42 and 45 kD (21, 23), can promote the rapid conversion of the inactive E-RuBP complex to an active form (16) which is also carbamylated (30) in the presence of otherwise inhibitory concentrations of RuBP. Rubisco activase appears to be present in all higher plants (26), requires ATP and Mg²⁺ (28) for activity, has an intrinsic ATPase activity (19), and is affected by the ATP/ADP quotient (4, 17). The inhibitory effect of RuBP on changes in rubisco activation appears to be one reason why rubisco activase is required for the proper functioning of rubisco *in vivo* and is a major factor in the light-dependent activation of rubisco (16, 23, 24).

Rubisco activase is also capable of restoring activity (18) to rubisco which has been pretreated with the *in vivo* regulator (27) CA1P, but not with the reaction intermediate analog (15), CABP. Because the ECM form of the enzyme used in the experiments was already complexed with CO₂ and Mg²⁺, this result indicated that rubisco activase is capable of accelerating the release of the CA1P from the enzyme. Similarly, it reduces the decline in (or even restores) activity observed when the enzyme is assayed *in vitro* (20). The enzyme also

remains carbamylated during the loss in activity and the decline appears to be due to the formation of an inhibitory sugar phosphate from the catalytically bound RuBP before normal reaction with CO_2 or O_2 . An accelerated release of this inhibitor would again account for the observed effects of rubisco activase on activity.

The activation of rubisco can also be demonstrated in intact chloroplast systems, where the rubisco activase can be investigated under physiologically relevant conditions (17, 22), as well as in reconstituted chloroplast systems where the effect of added metabolites can be more easily characterized (16). Using a lysed chloroplast system, Parry *et al.* (14) reported that FBP and RuBP together were capable of supporting rubisco activase activity in the absence of added adenylate.

In an attempt to further clarify the effects of sugar phosphates on rubisco activation promoted by rubisco activase, in this report we have followed the activation of purified rubisco in the presence of several physiologically relevant sugar phosphates and determined the effect of rubisco activase on this process. From the standpoint of kinetic analysis, the procedure for such studies would be to preincubate free rubisco with the sugar phosphate, then transfer the sugar phosphate-bound enzyme to conditions having the appropriate concentrations of CO_2 and Mg^{2+} , but lacking exogenous sugar phosphate, and determine the time-course of activation. However, the chloroplast stroma contains substantial concentrations of some sugar phosphates and rubisco must normally function in the presence of these. For this reason, this work was undertaken in more physiologically relevant conditions by determining the time-course of activation of sugar phosphate-bound rubisco in the presence of the sugar phosphate. The sugar phosphate was maintained at the same concentration in the preincubation and activation stages. It is shown that rubisco activase promotes the activation of rubisco in the presence of concentrations of sugar phosphates that would otherwise be inhibitory.

MATERIALS AND METHODS

Enzyme Preparation

Rubisco was prepared from spinach (*Spinacea oleracea*) by the procedure of Salvucci *et al.* (25) and stored as 1 mL aliquots under liquid nitrogen. When required, a sample was thawed in water at 25°C and allowed to stand at that temperature for 1 h, then converted to the CO_2 and Mg^{2+} -free (E) form by the following procedure: The sample (0.8 mL, approximately $7 \text{ mg protein} \cdot \text{mL}^{-1}$) was applied to a Sephadex⁴ G50 column ($18 \times 1 \text{ cm}$ diameter), equilibrated and eluted with 20 mM Tricine, 0.2 mM EDTA (pH 8.0), flushed with N_2 . Care was taken to exclude bicarbonate during preparation of the buffer. The eluate contained a single peak of protein, and 2 mL of the most concentrated protein was collected. This was placed in a Centricon 30 tube (Bio-Rad) and centrifuged at $500g$ for 20 min, reducing the volume to 0.9 mL.

Rubisco activase was prepared by the procedure of Robinson *et al.* (21) and was stored under liquid nitrogen.

Enzyme Assays

The activation state of rubisco was determined in a three-stage procedure at 25°C . While rubisco was preincubated and assayed at pH 8.0, the intermediate activation stage was performed at pH 7.7 in order to slow the activation to rates more easily measurable with sampling at 60 s intervals. Rubisco (E form, $5 \text{ mg} \cdot \text{mL}^{-1}$) was preincubated with the appropriate sugar phosphate in Mg^{2+} -free and CO_2 -free conditions for 2 h in 20 mM Tricine, 0.2 mM EDTA (pH 8.0) under N_2 . Activation was initiated by injecting $100 \mu\text{L}$ of enzyme preincubation mixture into $400 \mu\text{L}$ of activation mixture containing an identical concentration of the appropriate sugar phos-

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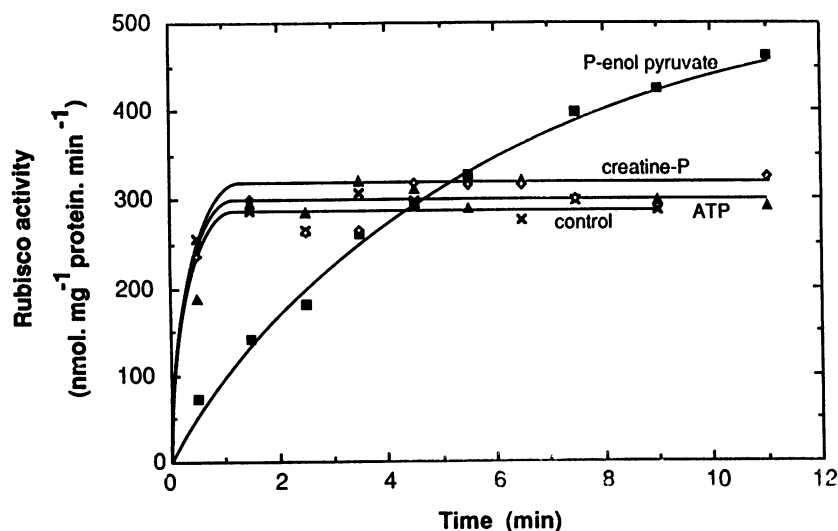


Figure 1. Effect of ATP, creatine-P, and PEP (3 mM) on the activation of rubisco. The enzyme was preincubated and activated in the presence of each sugar phosphate as described in "Materials and Methods." No effector (x), ATP (▲), creatine-P (◇), PEP, (■).

phate. The activation mixture was preflushed for 5 min with a gas mixture containing 315 ppm CO₂ and 2% O₂, and the flushing maintained during the time-course of enzyme activation. After addition of the enzyme, the concentrations of other components in the complete activation assay were: MgCl₂, 10 mM; dithiothreitol, 2 mM; carbonic anhydrase, 500 units mL⁻¹; K⁺-Hepes, 100 mM (pH 7.7). Additionally, ATP, an ATP-regenerating system and rubisco activase were present as specified. At 30 s and subsequent 60 s intervals, 10 μL of activation mix was withdrawn and mixed with 490 μL of assay mixture of the following composition: MgCl₂, 10 mM; NaH¹⁴CO₂, 10 mM (17 GBq·mol⁻¹ C or 450 mCi·mol⁻¹ C); RuBP, 0.5 mM; K⁺-Tricine, 100 mM (pH 8.0). After 30 s, 100 μL of 4 M formic acid, 1 M HCl was injected into the assay mixture, and the vial dried at 65°C, before determining the fixed carbon radioactivity by liquid scintillation counting.

RESULTS

Under the optimum conditions of saturating CO₂ (10 mM bicarbonate) and pH 8.0, the extent of spontaneous activation of the E form of the enzyme was more than 95% complete after 30 s (data not shown). In order to slow the activation of rubisco to rates measurable by the technique used here, the activation stage of the assay was performed under the suboptimal conditions of pH 7.7 and 315 ppm CO₂. Even under these conditions, the initial rate of activation was too rapid to be determined with any accuracy for the spontaneous activation of the E form of rubisco, but the rates of activation in the presence of sugar phosphates could be followed.

The requirement for a high and constant ATP/ADP quotient for maximum rubisco activase activity necessitates the inclusion in assays of an ATP-regenerating system. For the purposes of this work it was important to exclude any effects of the substrate for the ATP-regenerating system on rubisco activity and activation. When the enzyme was activated in the absence of rubisco activase and RuBP, the 11 min time course of activation (Fig. 1) was essentially unaffected when 3 mM ATP or creatine-P were included in the preincubation and activation stages. When 3 mM PEP was included, the initial rate of activation was much lower, but the extent of activation was higher after 10 min. PEP has previously been shown to enhance rubisco activation (12). On this basis, the creatine-P/creatine phosphokinase system was used in preference to PEP/pyruvate kinase in subsequent experiments.

The concentration of dissolved CO₂ in the activation assay (in equilibrium with a gas phase of 315 ppm CO₂ at 25°C) was 10.6 μM (11). The rubisco activity measured under these conditions after the E form of the enzyme had activated spontaneously to its maximum extent was about 300 nmol·mg⁻¹ protein·min⁻¹ (Figs. 1–3). When preincubation and activation of rubisco was performed in the presence of saturating CO₂ (10 mM NaHCO₃) and 10 mM Mg²⁺, the rate observed after full activation averaged 1936 nmol·mg⁻¹ protein·min⁻¹ for rubisco preparations used in the experiments of Figures 1 to 3. Thus, the extent of spontaneous activation under 315 ppm CO₂ (pH 7.7), represented about 15% of the available active sites.

The presence of 4 mM RuBP in the preincubation and activation stages inhibited completely the activation of rubisco during the 11 min time-course unless rubisco activase was also present (Fig. 2). Activation was restored in the presence of RuBP when rubisco activase was added, but the initial rate was less than the initial rate of spontaneous activation of the enzyme without RuBP added. After 11 min, however, the extent of activation with 100 μg activase·mL⁻¹ was 25% higher than the spontaneously activated enzyme. Rubisco activase did not increase the rate or extent of activation of the free enzyme (data not shown).

The effect of glycerate 3-P, a metabolite that is normally present at substantial concentrations in the chloroplast stroma (6) on the activation of rubisco was also investigated (Fig. 2). The higher concentrations required for this weak inhibitor of activation result in significant reduction of free Mg²⁺. The concentration of MgCl₂ was, accordingly, increased to 20 mM

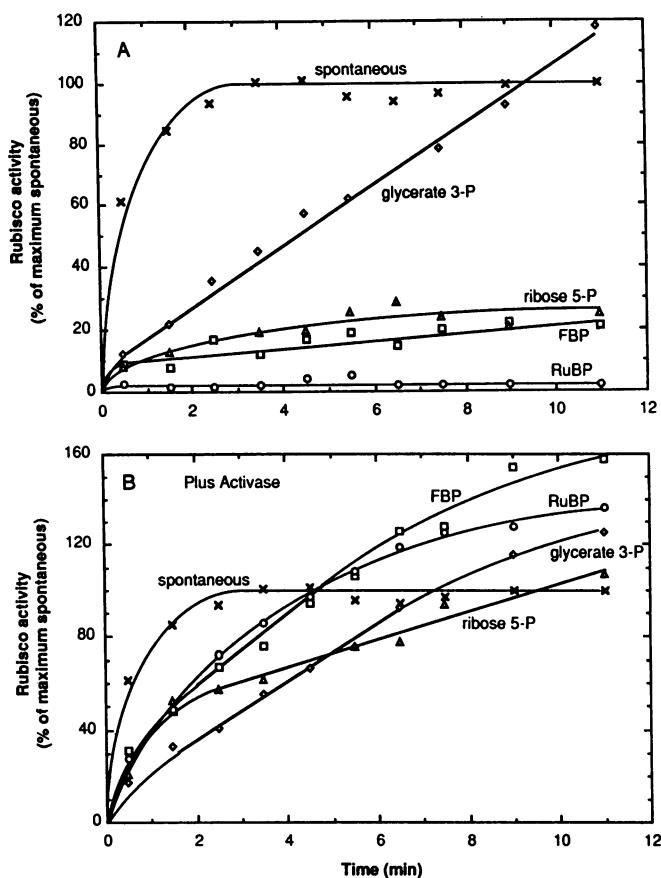


Figure 2. Effect of sugar phosphates on rubisco activation in the absence (A) and presence (B) of rubisco activase. Rates (averages of two separate experiments) are expressed as the percentage of the rubisco activity observed after 11 min spontaneous activation in the absence of effector (average 100% rate; 321 nmol·mg⁻¹ protein·min⁻¹). Each activation assay contained 1 mM ATP, 3 mM creatine-P, 20 units·mL⁻¹ creatine phosphokinase, and 100 μg·mL⁻¹ rubisco activase in (B). Procedure is described in "Materials and Methods." No effector (x), 4 mM RuBP (○), 20 mM glycerate 3-P (◇), 1 mM FBP (□), 5 mM ribose 5-P (△).

for 20 mM glycerate 3-P. This gave 10 mM concentrations of both free glycerate 3-P and Mg^{2+} , assuming a binding coefficient of $100 M^{-1}$ for the Mg-glycerate 3-P complex (10). Although the initial rate of activation of rubisco was substantially inhibited by glycerate 3-P, the activity increased almost linearly and after 11 min was slightly higher than for spontaneous activation of the free enzyme (Fig. 2A). With rubisco activase present, slightly higher activities were observed throughout the time-course, but the initial rate of activation of rubisco in the presence of glycerate 3-P was not increased substantially (Fig. 2B).

As expected from a previous report (10), FBP (1 mM) and ribose 5-P (5 mM) both inhibited the activation of rubisco over the 11 min time-course, although the inhibition was not as complete as with 4 mM RuBP (Fig. 2A). In contrast to the results with glycerate 3-P, the addition of rubisco activase ($100 \mu g \cdot mL^{-1}$) increased the activation rate substantially in both cases (Fig. 2B). Although the initial rates of activation were then slower than the spontaneous activation of the free enzyme, the activity increased continually and at 11 min exceeded that for spontaneous activation. The activity at 11 min with FBP was higher than that for RuBP, while that with ribose 5-P was lower.

In view of the results of Parry *et al.* (14), the effect of ATP on the activation of FBP-bound rubisco by rubisco activase was examined (Fig. 3). In this experiment, rubisco activase increased the activity of rubisco substantially. However, in the absence of ATP, no activation was seen and what low activity was observed was identical to that of FBP-bound rubisco in the absence of rubisco activase.

DISCUSSION

Creatine and creatine-P have no known role in plant metabolism. The lack of effect of creatine-P, but not PEP, on rubisco activation demonstrated here (Fig. 1) suggests that creatine-P is generally preferable to the pyruvate kinase system used previously (21) to maintain the high ATP/ADP ratios required for maximal rubisco activase activity.

Robinson *et al.* (21) proposed a working model for the activation of rubisco by the rubisco activase in which the protein promotes the release of RuBP from the inactive E-RuBP complex. The accelerated release of RuBP then allows CO_2 and Mg^{2+} to bind more rapidly to the enzyme, with consequent activation. The apparent ability of the rubisco activase to increase the extent of activation at suboptimal CO_2 concentrations (16, 30) may actually be the result of the binding of RuBP to the activated enzyme since RuBP has been shown to inhibit severely the rate of activity loss when CO_2 and Mg^{2+} are rapidly reduced (10). It is also possible that rubisco activase alters the intrinsic affinity of rubisco for CO_2 in some manner but evidence of this has not been demonstrated. Some support for the Robinson model was obtained when it was found that rubisco activase promoted the release of the *in vivo* inhibitor CA1P (18) and prevents the decline in rubisco activity during catalysis (20). However only limited comparisons can be made with the present results because in these cases the rubisco activase is interacting with a form of rubisco which is already carbamylated and complexed with Mg^{2+} .

The present results show that rubisco activase can overcome the inhibitory effects of two additional sugar phosphates, FBP and ribose 5-P, on the spontaneous activation of rubisco by CO_2 and Mg^{2+} (Fig. 2). They provide considerably more support for a model in which rubisco activase accelerates the activation of rubisco by promoting the release of the sugar phosphate from the uncarbamylated complex allowing a more rapid access of CO_2 and Mg^{2+} to the active site. Activation in the presence of FBP, RuBP, and, if a longer time course could have been followed, possibly ribose 5-P resulted in a higher activation state than in the absence of the sugar phosphate. This seems to be due in part to the ability of these sugar phosphates to inhibit the loss (or exchange under steady state conditions used here) of CO_2 and Mg^{2+} from the enzyme (10). However, because it is clear that rubisco activase can also interact with the carbamylated form of the enzyme (*e.g.* ECM-CA1P), one might expect the binding of these sugar phosphates to the carbamylated form also to be affected by the

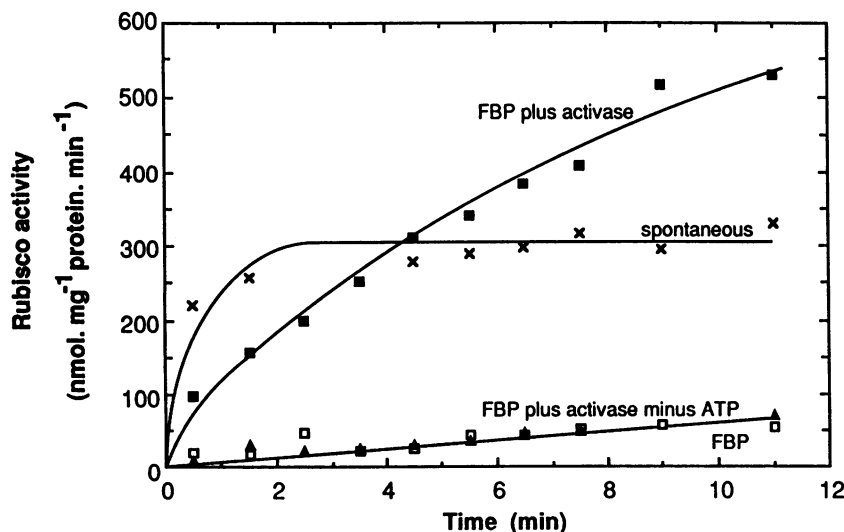


Figure 3. Requirement for ATP for rubisco activase activation of rubisco in the presence of FBP. Each activation assay contained 3 mM creatine-P and 20 units $\cdot mL^{-1}$ creatine phosphokinase. Procedure is described in "Materials and Methods." No effector (x), 1 mM FBP plus 1 mM ATP (□), 1 mM FBP plus rubisco activase ($100 \mu g \cdot mL^{-1}$) (▲), 1 mM FBP plus 1 mM ATP and activase ($100 \mu g \cdot mL^{-1}$) (■).

presence of rubisco activase with a possible reduction in steady state activation. Evidence for this was not observed here, but other types of experiments are more suitable to examine this question further.

The mechanism by which rubisco activase promotes the release of the bound sugar phosphate remains unknown. The most likely possibility is that an association of some kind between the rubisco activase and rubisco is required. An accelerated dissociation rate would seem to imply a decrease in affinity but without additional evidence it is possible both the association and dissociation rates are increased with little effect on the binding constant. An additional problem for the interpretation of the effect of rubisco activase on the relationships between the kinetics of CO₂ and Mg²⁺ binding (*i.e.* activation) and sugar phosphate binding to the various forms of rubisco is their complexity even in the absence of rubisco activase (10, 29). For example, equilibrium binding studies indicate that there seems to be no relationship between the relative affinities of the inactive and active forms of rubisco and the ability of a sugar phosphate to promote increased activation (10). A mechanism involving a kinetic stabilization of the ECM form was proposed to explain the otherwise anomalous results. At present, one can only speculate as to the possible involvement of the rubisco activase in such a mechanism.

The demonstration (Fig. 3) that the activation of the E-FBP complex by rubisco activase is dependent on the presence of ATP is at variance with the report of Parry *et al.* (14). However, their studies with a lysed chloroplast system were not well defined with respect to metabolite composition. The presence of sufficient endogenous adenylates for activation is a likely explanation pending further characterization of the system.

The ability of FBP and ribose-5P, but not glycerate 3-P, to substitute for RuBP in the activation of rubisco by rubisco activase is of some relevance to the possible role of other metabolites (2, 5) besides RuBP in the regulation of rubisco activation and activity *in vivo*. Of the photosynthetic metabolites in the chloroplast stroma, glycerate 3-P is present at the highest concentrations under most conditions. Gerhardt *et al.* (6) measured 100 nmol·mg⁻¹ Chl in darkness and 300 in the light which equates to about 4 and 12 mM, respectively, assuming no binding and a stromal volume of 25 μL mg⁻¹ Chl (8). Although glycerate 3-P at 20 mM inhibited the rate of rubisco activation and the presence of rubisco activase had no significant effect, the rate was still relatively rapid. It may be concluded that glycerate 3-P has no major role in the activation of rubisco under physiological conditions. Other possible effects of glycerate 3-P on the steady-state activity of rubisco have been considered previously (5).

Of the metabolites studied here, FBP was the most similar to RuBP in its effect on activation of rubisco in the absence and presence of rubisco activase. Under conditions where the stromal RuBP concentration is very low, FBP could potentially serve to increase or maintain the activation state of the enzyme. However, FBP would confer no apparent advantage under steady-state conditions, since the catalytic activity, already limited by the low RuBP concentration, might be further reduced by FBP which is a better competitive inhibitor

than most other metabolites (3). Normally the stromal level of FBP is about one-tenth that of RuBP (6), and given the much lower *K_d* of RuBP (9), it would not appear to have a significant role. Although ribose 5-P is also somewhat effective in replacing RuBP, this compound would not seem to have a significant role in rubisco activation *in vivo* as the measured concentration of all the pentose monophosphates in the stroma is only 0.04 mM (7).

In summary, the present results show that rubisco activase has a mixed specificity for sugar phosphate complexes with rubisco. Rubisco activase increases the activation rates with E-RuBP, E-FBP, and E-ribose 5-P (shown here) and restores the activity of ECM-CA1P (18). It has little effect on the activation of E-glycerate 3-P and cannot restore the activity of the ECM-CABP complex (18). The degree to which these properties result from differences in the steady-state affinity of the compounds for the various forms of rubisco, or in both the rates of dissociation and association, or possibly also reflect differences in the affinity of rubisco activase for rubisco, and the consequent effects on the kinetics of activator CO₂ and Mg²⁺ binding remain to be established. Additionally, the results further emphasize that the possible role of steric and conformational factors associated with access of the activator CO₂ and Mg²⁺ to and from the active site of rubisco in the presence of sugar phosphates and rubisco activase remain to be addressed.

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