Subsaturating Ribulose-1,5-Bisphosphate Concentration Promotes Inactivation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (Rubisco)

We developed a continuous-addition method for maintaining subsaturating concentrations of ribulose-1,5-bisphosphate (RuBP) for several minutes, while simultaneously monitoring its consumption by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This method enabled us to observe the effects of subsaturating RuBP and CO₂ concentrations on the activity of Rubisco during much longer periods than previously studied. At saturating CO₂, the activity of the enzyme declined faster when RuBP was maintained at concentrations near its Kᵣ value than when RuBP was saturating. At saturating RuBP, activity declined faster at limiting than at saturating CO₂, in accordance with previous observations. The most rapid decline in activity occurred when both CO₂ and RuBP concentrations were subsaturating. The activity loss was accompanied by decarbamylation of the enzyme, even though the enzyme was maintained at the same CO₂ concentration before and after exposure to RuBP. Rubisco activase ameliorated the decline in activity at subsaturating CO₂ and RuBP concentrations. The results are consistent with a proposed mechanism for regulating the carbamylation of Rubisco, which postulates that Rubisco activase counteracts Rubisco’s unfavorable carbamylation equilibrium in the presence of RuBP by accelerating, in an ATP-dependent manner, the release of RuBP from its complex with uncarbamylated sites.

During photosynthesis, the activity of Rubisco (EC 4.1.1.39) must be modulated to balance the rate of RuBP consumption by carboxylation and oxygenation with the rate of RuBP regeneration. Metabolic conditions in the chloroplast are subject to variations induced by environmental fluctuations (such as varying irradiance) or by developmental changes (such as varying sink strength). Extensive study of the regulation of Rubisco has shown that, under steady-state conditions, the activity is regulated by variations in the activation state of the enzyme rather than by fluctuations in RuBP concentration in the subsaturating range (Portis, 1992; Geiger and Servaites, 1994). The activation state is varied by changing the carbamylation status of the enzyme (the fraction of active sites having carbamylated Lys at residue 201, which permits binding of the catalytically essential divalent metal). In a minority of plant species, the activity is also varied by the inhibitor, CAIP, which reduces activity by binding to ECM, particularly in darkness. As a consequence of this mode of Rubisco regulation, RuBP generally remains at saturating levels in the stroma during illumination. RuBP also binds to E with a Kᵣ of approximately 20 nM, forming a stable, inactive complex (Jordan and Chollet, 1983). The activation state of Rubisco in non-CAIP plants, therefore, represents a balance between the ECM and E forms of the enzyme, both of which are virtually completely saturated with RuBP as the forms ECMR and ER.

In vitro, RuBP is released from the isolated ER complex very slowly compared to the rate at which it is released from the ECMR complex by catalytic conversion to PGA. In vivo, the increase in activity and carbamylation that is required when irradiance increases is mediated by the stromal protein Rubisco activase (Portis, 1992). Activase facilitates the conversion of ER to ECMR, apparently by accelerating the dissociation of RuBP from the former (Wang and Portis, 1992), thus permitting carbamylation and divalent-metal binding, which can occur only with free E. Activase has not been reported to have any direct effect

Abbreviations: CABP, 2'-carboxy-o-arabinitol 1,5-bisphosphate; ECM, metal-bound carbamylated Rubisco; ECMR, metal- and RuBP-bound carbamylated Rubisco; ER, RuBP-bound uncarbamylated Rubisco; Kᵣ, Kᵣ, and Kᵣ, dissociation constants; Kᵣ', apparent Michaelis constant for RuBP at a particular CO₂ and O₂ concentration; PGA, 3-phospho-D-glyceric acid; RuBP, o-ribulose-1,5-bisP; t½, half-time of a first-order decay process; Vₘ, maximum rate.
on the rate of decarbamylation of ECM or ECMR, and the manner in which this process occurs in vivo or in vitro has not been established.

Since RuBP binds to E with a $K_m$ 3 orders of magnitude lower than its $K_A$ for ECM (Jordan and Chollet, 1983), we would expect that Rubisco must be almost entirely in the ER form at equilibrium when activase is inactive or absent. However, experiments show that ECMR decarbamylates extremely slowly, if at all, when saturating concentrations of RuBP are present (Edmondson et al., 1990b). A further complication was revealed by observations that the activity of ECMR slowly declines during catalysis under optimal conditions, without decarbamylation. This slow decline in activity occurs in concert with the appearance of at least two RuBP-derived inhibitors within the active site (Edmondson et al., 1990a, 1990b, 1990c, 1990d; Zhu and Jensen, 1991a, 1991b). This type of inactivation might also be exploited for the regulation of Rubisco in vivo, but that possibility remains to be clarified.

It may be significant that the decarbamylation induced following a reduction in irradiance in vivo occurs during a period when the RuBP concentration is low and limiting (Mott et al., 1984; Prinsley et al., 1986; Brooks and Portis, 1988), perhaps the only circumstance under which this condition applies in illuminated leaves. The sudden reduction in the rate of RuBP supply induces an imbalance between supply and demand by carboxylation, which causes the RuBP concentration to decrease to limiting levels for several minutes until Rubisco’s activity declines by decarbamylation to a point at which the carboxylation rate again matches the rate of RuBP supply; thereafter, the RuBP concentration resumes its usual saturating level (Woodrow and Berry, 1988). It may be that a decrease in stromal RuBP concentration to subsaturating levels is a prerequisite for decarbamylation. The present study was designed to examine this possibility in vitro.

The CO$_2$ concentration in assays can be easily buffered at subsaturating levels resembling those pertaining in vivo by equilibration with bicarbonate in the presence of carbonic anhydrase. However, maintenance of subsaturating RuBP concentrations in the presence of appreciable amounts of active Rubisco in vitro presents some difficulties. In the experiments reported here, the continuous measurement of Rubisco activity for several minutes at limiting substrate concentrations has been achieved for the first time by continuous addition of RuBP to reaction mixtures at known rates. Activity is monitored spectrophotometrically by coupling PGA formation to NADH oxidation (Lilley and Walker, 1974), allowing calculation of the RuBP concentration present at any time as the difference between the total RuBP added to that time and that consumed.

After validation of this technique, we used it to examine the effect of low substrate concentrations on Rubisco’s activity in the presence and absence of activase. We confirmed earlier observations (Edmondson et al., 1990a) that exposure to RuBP promotes a decline in the activity of Rubisco, even though the CO$_2$ and Mg$^{2+}$ concentrations were the same before and after such exposure. In this study, we showed that this decline is more extensive at subsaturating RuBP concentrations (compared to saturating RuBP concentrations) and that the decline is further exacerbated if the CO$_2$ concentration is also maintained at subsaturating levels before and after RuBP addition. The loss in activity induced by subsaturating RuBP concentrations was accompanied by decarbamylation. We examined the effects of activase on the kinetics of Rubisco under subsaturating RuBP conditions and compared them with earlier observations.

MATERIALS AND METHODS

Chemicals and Enzymes

Rubisco, isolated from tobacco (Nicotiana tabacum, Wisconsin 38) leaves using the method described by Servaites (1985) with only minor variations, was a gift of Dr. B. Entsch (University of New England, Armidale, Australia). Spinach and tobacco Rubisco activase and spinach Rubisco were isolated using the methods described by Wang et al. (1992). All other enzymes were obtained from Boehringer or Sigma, and preparations obtained as ammonium sulfate suspensions were desalted before use. RuBP was prepared as described by Edmondson et al. (1990a).

Assay Method, Calculations, and Equipment

RuBP consumption was assayed by enzymatically coupling the production of PGA to NADH oxidation (Lilley and Walker, 1974) yielding a stoichiometry of 4 NADH oxidized per RuBP consumed if the oxygenase activity is suppressed by a high CO$_2$ to O$_2$ ratio. Under low CO$_2$ conditions (13.5 $\mu$M, equivalent to 1.1 mM bicarbonate at pH 8), 254 $\mu$M O$_2$ (equivalent to 21% O$_2$) and using a CO$_2$/O$_2$ specificity factor of 84, the predicted stoichiometry of 3.64 was used to calculate the amount of RuBP consumed. The coupling enzyme system consisted of 60 units of phosphoglycerate kinase, 25 units of glyceraldehyde 3-phosphate dehydrogenase, 60 units of triose phosphate isomerase, 20 units of glyceraldehyde 3-phosphate dehydrogenase, and 40 units (or 60 when Rubisco activase was active) of creatine phosphokinase per mL of reaction. Also present in the reaction mixture (2 mL) were 100 mM Tricine-NaOH (pH 8), 1 mM ATP, 5 mM phosphocreatine, 12 mM MgCl$_2$, either 1 or 0.34 mM NADH, either 10 or 1.1 mM NaHCO$_3$, 35 Wilbur-Anderson units mL$^{-1}$ carbonic anhydrase, either 40 or 10 $\mu$g mL$^{-1}$ Rubisco, and Rubisco activase as indicated. In all experiments, the reaction mixture was preincubated for 5 min after the addition of Rubisco to allow temperature and the carbamylation status of the enzyme to reach equilibrium before RuBP addition was commenced.

A Hewlett-Packard diode array spectrophotometer (HP 8452A) was used in the kinetics mode, which permitted simultaneous data collection (1 s average) at 10-s intervals. Data from the 330- to 350-nm and/or 370- to 380-nm wavelength ranges were summed and averaged to improve the

---

2Product names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the product, and the use of the name by U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.
signal to noise ratio. Extinction coefficients for NADH (0–1 mM) in these wavelength ranges, relative to that at 340 nm, were determined on this instrument and used in the calculations. The coupling system was validated by additions of known amounts of PGA. The A in these wavelength ranges was a linear function of the added PGA as long as the A remained between 2.3 and 0.2 (0.5 for the 370- to 380-nm range).

Injection of NADH and PGA solutions were also used to estimate the pre-steady-state delay in the system due to both mixing and the build-up of intermediates of the coupling system. Solutions (NADH and PGA in the tests or RuBP in the experiments) were injected into the cuvette by using a 500-µL syringe coupled via a wormdrive to a stepper motor. Calibration of the device was performed to obtain an injection rate of 20 µL min⁻¹ into the 2-mL reaction volume. The syringe was connected to polyethylene tubing (Intramedic PE 10 [Becton Dickinson], 0.28 mm i.d., 0.61 mm o.d.), whose outlet was positioned 1 to 2 mm above the magnetic cuvette stirrer (Cell Spinbar; Bel-Art Products, Pequannock, NJ) immediately before each injection commenced. The solution was stirred as rapidly as possible without cavitation, and control experiments showed that the decline in Rubisco activity was less than 5% if the solution was stirred in this manner for up to 10 min prior to assay. Calculations of rates and RuBP concentrations were corrected for the increase of the reaction volume (always less than 10%) during the injection period.

Rubisco Carbamylation Level

Rubisco carbamylation was measured using the method described by Mate et al. (1993) with the following variations. [14C]NaHCO₃ was included in the reaction mixtures to obtain a specific activity of 9 or 0.9 Ci mol⁻¹ with 1.1 and 10 mM NaHCO₃, respectively. At the indicated times, aliquots of 1 mL were mixed with 0.1 mL of 1 mM CABP in 200 mM NaHCO₃ and left for 4 h. Then 1 mg of unlabeled Rubisco-CABP complex was added as a carrier, and the complexes were precipitated by adding PEG-3350 and MgCl₂ to 20% (w/v) and 20 mM, respectively. The mixtures were placed on ice for 30 min and centrifuged at 4°C for 5 min. The pellets were resuspended and washed four times using 1 mL of 50 mM Tricine (pH 8), 1 mM EDTA, 20 mM MgCl₂, 20 mM NaHCO₃, and 20% (w/v) PEG. Aliquots of the supernatants were counted to ensure that uncomplexed [14C]CO₂ and fixed carbon had been reduced to insignificant levels. The pellets were then dissolved in 500 µL of 0.1 N NaOH and added to 5 mL of Bio-Safe II fluid (Research Products International Corp., Mount Prospect, IL). Retained [14C] was determined by liquid scintillation counting and corrected for background using a reaction mixture that did not contain Rubisco initially.

\[ [\text{RuBP}] = \frac{[\text{Addition rate} - \text{Consumption rate}] \times t}{V_{\text{ol}}} \]

Consumption rate = \[\text{[RuBP]} \times V_{m} \times E_{r} / (K_{m} + [\text{RuBP}]) \]

where \( V_{\text{ol}} \), \( E_{r} \), and \( V_{m} \) are the volume of the reaction, the enzyme concentration, and the RuBP-saturated activity, respectively, at time \( t \). To approximate the decline in activity due to inhibitor formation, the effect of a first-order decline in the \( V_{m} \) can also be simulated by the following equation (Edmondson et al., 1990a):

\[ V_{m} = (V_{m_{0}} - V_{m_{inf}}) \times e^{-kt} + V_{m_{inf}} \]

where \( V_{m_{0}}, V_{m_{inf}}, \) and \( k \) are the initial and final RuBP-saturated activities and the rate constant of the decay, respectively. Theoretical time courses for RuBP concentration and consumption rate were generated iteratively using small time increments (Fig. 1). In the present experiments, RuBP concentrations near the \( K_{m} \) of 20 to 25 µM were desired. A maximal enzyme concentration of 40 µg mL⁻¹ was selected for the experiments because the maximum rate of RuBP consumption at RuBP and CO₂ saturation would be 1.33 µM s⁻¹, assuming a specific activity of 2 µmol mg⁻¹ min⁻¹. Although mixing in the cuvette and lags in the coupling system take several seconds (see below), the difference between the calculated and actual RuBP concentrations due to these factors is still quite small relative to the 20 to 25 µM concentration desired. Also, with this enzyme concentration, a maximal rate change in \( A_{14CO₂} \) of 0.033 s⁻¹ is observed. By measuring the \( A \) changes

**RESULTS**

Theoretical Time Courses for Continuous RuBP Addition

The expected time course for the change in RuBP concentration and Rubisco activity when RuBP is added to a reaction mixture is described by the following equations:

\[ V_{m} = \frac{V_{m_{0}} - V_{m_{inf}}}{1 + e^{-kt}} \]

**Figure 1.** Predicted time course of RuBP concentration (A) and consumption rate (B) when RuBP is injected at 40 nmol min⁻¹ into a 2-mL reaction containing 80 µg of Rubisco with (solid) and without (dashed) an exponential decline in \( V_{m} \). Other parameters used for the simulation were: Rubisco \( V_{m} \) of 2 µmol min⁻¹ mg⁻¹; \( K_{m}(\text{RuBP}) \) of 25 µM, and \( t_{0} \) of 5 min for the decay of \( V_{m} \) to a final value of 0.
averaged over the 370- to 380-nm spectral range (where the NADH A is only one-third of that at 340 nm) and using an initial concentration of 1 mM NADH, we could follow Rubisco activity spectrophotometrically at this enzyme concentration for several minutes.

When RuBP is added at a rate of 40 nmol min⁻¹ (approximately 20 μM min⁻¹) to a reaction containing 40 μg mL⁻¹ Rubisco, the calculated RuBP concentration reaches a maximum value of 8.3 μM (assuming a Kₚ(RuBP) of 25 μM) (Fig. 1A) and the calculated carboxylation rate increases rapidly to a steady-state value of 500 nmol min⁻¹ mg⁻¹ by 120 s (Fig. 1B). Different RuBP concentrations can be achieved by varying the addition rate, and they can be calculated from Equations 1 and 2.

Figure 2. Results of a typical RuBP-injection experiment with tobacco Rubisco showing A₃₄₀ and calculated consumption rate (A) and total RuBP (injected and consumed) and RuBP concentration (B). Start and end of continuous addition of RuBP (20 nmol min⁻¹ obtained by injection of 1 mM RuBP at 20 μL min⁻¹ into an initial volume of 2 mL) are indicated by the arrows. The consumption rate (A, right axis, ○) was calculated from the difference between successive A(Abs) measurements at 10-s intervals. RuBP concentration (B, right axis, ▲) was calculated from the difference between cumulative RuBP added (dotted line) and RuBP consumed (solid line).

Observed Time Courses

The results of a typical RuBP-injection experiment and the method of calculation are shown in Figure 2. A minor problem is caused by accumulation of errors. Theoretically, the calculated RuBP concentration should eventually return to zero after addition of RuBP is stopped. However, even very small uncertainties in the RuBP concentration of the solution in the syringe, or in the volume delivered by the syringe, or in the initial volume of the reaction solution, will accumulate to produce an apparent nonzero RuBP concentration after exhaustion of RuBP. In this experiment, the discrepancy was only 2 nmol out of a cumulative total of 137 nmol injected, but this was sufficient to cause an error in the calculated RuBP concentration of approximately 1 μM in the later stages of the experiment. Since the uncertainties in concentrations and volumes would be no less than approximately 2% in each case, the discrepancy is of the expected magnitude. Some variation in the calculated final RuBP concentration following cessation of RuBP addition was noticed even if the same stock RuBP solution and the same syringe starting and ending positions were used, indicating that small variations in the initial volume of the solution in the cuvette are, indeed, a contributing factor. The reasonable assumption that the RuBP concentration must return to zero eventually after injection ceases provides a simple way to correct this problem and provides assurance that cumulative errors do not affect the calculated RuBP concentration. Any one of the above parameters—we chose the RuBP concentration of the injected solution—can be adjusted empirically so that the calculated RuBP concentration returns to zero after injection ceases.
The enhanced inactivation that occurs during exposure to subsaturating RuBP concentrations has another feature that distinguishes it from inactivation occurring at saturating RuBP concentrations. The former recovers completely after exhaustion of RuBP (Fig. 5), whereas the latter does not. After exhaustion of a subsaturating amount of RuBP (80 nmol or 40 \( \mu \)M initially), addition of saturating RuBP returned the activity to a level (80% of initial) exceeding that seen when saturating RuBP had been present from the outset (65% of initial). Further incubation of the enzyme for several minutes after RuBP exhaustion before addition of saturating RuBP caused a slight further recovery to 87% of initial activity. When the same total amount of RuBP (80 nmol) was added continuously over 240 s, maintaining the concentration below 20 \( \mu \)M, a saturating addition of RuBP restored only 54% of the initial activity. However, when the saturating addition was delayed

consistent with the slow inactivation during catalysis due to inhibitor formation (see the introduction). However, further experiments showed that the loss of activity occurring during catalysis at subsaturating RuBP concentration was more severe than that seen at RuBP saturation. This was demonstrated in two-stage experiments: a period of continuous addition of RuBP, maintaining subsaturating concentrations, was followed by a single, large addition of RuBP, giving a step change to a saturating concentration. The activity increased after the second addition but did not attain the activity of the control, which had experienced saturating RuBP from the outset of the experiment (Fig. 4). In the control at 200 s, 15% of the initial activity was lost while, at the same time in the two-stage experiments (160 s of subsaturating RuBP followed by a saturating addition), a decline of 29% occurred. Thus, the inactivation must have occurred faster at subsaturating RuBP (\( t_{1/2} \) approximately 3.0 min) than at RuBP saturation (\( t_{1/2} = 6.3 \) min). This observation eliminates one potential cause for the enhanced loss of activity. It cannot be due to the accumulation of a competitive (with respect to RuBP) inhibitor in the solution because saturation with RuBP does not overcome it.

![Figure 3](image-url)  
**Figure 3.** Time course of RuBP concentration (A) and consumption rate (B) during addition of RuBP at different rates at a saturating concentration of CO\(_2\) (using 10 mM NaHCO\(_3\)). Tobacco Rubisco was added to a concentration of 40 \( \mu \)g mL\(^{-1}\) and allowed to preactivate for 5 min before RuBP was added at the indicated rates starting at 32 s and ending at 302 s. RuBP concentrations were calculated using the small correction described in the text.

![Figure 4](image-url)  
**Figure 4.** Time courses of RuBP concentration (A) and consumption rate (B) at saturating CO\(_2\) (using 10 mM NaHCO\(_3\)) after a single addition of 1000 nmol of RuBP (\( \bullet \)) or during continuous addition of RuBP at 40 (\( \square \)) or 80 (\( \triangle \)) nmol min\(^{-1}\), followed by a subsequent single addition of 1000 nmol of RuBP to estimate \( V_m \). Continuous addition of RuBP into the reaction mixture containing 40 \( \mu \)M initially), addition of saturating RuBP returned only 54% of the initial activity. However, when the saturating addition was delayed

Inactivation of Rubisco at Subsaturating \( \alpha \)-Ribulose-1,5-BisP

---

**Copyright © 1995 American Society of Plant Biologists. All rights reserved.**

---

Downloaded from on October 14, 2017 - Published by [www.plantphysiol.org](http://www.plantphysiol.org)
the outset. For some of the time points have been omitted to improve legibility. Quickly after RuBP exhaustion, whereupon the activity re-enhanced inactivation occurring on exposure to subsaturating CO₂ that 2.5 μmol mL⁻¹ was used when 1000 nmol of RuBP was added at the outset. 1000 nmol of RuBP added at 30 s: 80 nmol of RuBP added at 30 s followed by a further 1000 nmol at 500 s; 80 nmol of RuBP added at 30 s followed by a further 1000 nmol at 810 s: 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 270 s. 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 970 s. Data for some of the time points have been omitted to improve legibility.

RuBP is thus a transitory phenomenon, which disappears quickly after RuBP exhaustion, whereupon the activity returns to approximately the level that would have been observed following a similar period of exposure to saturating RuBP. On the other hand, Edmondson et al. (1990a) showed that the inactivation occurring while RuBP is saturating persisted for hours after RuBP exhaustion, and even complete removal of small molecules from the enzyme solution by gel filtration induced only a limited recovery. For complete recovery, exposure to a high salt concentration during gel filtration or dialysis was required.

Figure 5. Time courses of RuBP concentration (A) and carboxylation rate (B) at saturating CO₂ (10 mM HCO₃⁻) following a single addition of 1000 or 80 nmol of RuBP or continuous addition of 80 nmol over 240 s, followed by a single addition of 1000 nmol, either immediately or after the original 80 nmol had been completely consumed. All reaction mixtures contained 10 μg mL⁻¹ spinach Rubisco, except that 2.5 μg mL⁻¹ was used when 1000 nmol of RuBP was added at the outset. 1000 nmol of RuBP added at 30 s; 80 nmol of RuBP added at 30 s followed by a further 1000 nmol at 500 s; 80 nmol of RuBP added at 30 s followed by a further 1000 nmol at 810 s; 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 270 s; 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 970 s. Data for some of the time points have been omitted to improve legibility.

Results at Limiting CO₂

Subsaturating CO₂ concentration reduced both the degree of carbamylation and the activity of the carbamylated enzyme; therefore, all rates were considerably lower than when CO₂ was saturating (Fig. 6). Furthermore, the inactivation seen with saturating RuBP proceeded faster (calculated t₁/₂ = 3 min), in agreement with previous observations (Edmondson et al., 1990a). Significantly, the enhancement of inactivation caused by RuBP limitation was markedly increased. Indeed, the extent of inactivation was so large that a constant rate of consumption could not be maintained during continuous RuBP addition. The rates peaked approximately 60 s after addition commenced and declined slowly thereafter, despite large progressive increases in RuBP concentration. This indicates that the decline in Vm must have been so great that even saturating RuBP could a further 690 s until the RuBP was completely exhausted, it restored the activity to 84% of the initial activity. The enhanced inactivation occurring on exposure to subsaturating RuBP is thus a transitory phenomenon, which disappears quickly after RuBP exhaustion, whereupon the activity returns to approximately the level that would have been observed following a similar period of exposure to saturating RuBP. On the other hand, Edmondson et al. (1990c) showed that the inactivation occurring while RuBP is saturating persisted for hours after RuBP exhaustion, and even complete removal of small molecules from the enzyme solution by gel filtration induced only a limited recovery. For complete recovery, exposure to a high salt concentration during gel filtration or dialysis was required.

Figure 6. Time courses of RuBP concentration (A) and consumption rate (B) at limiting CO₂ (using 1.1 mM NaHCO₃) after a single addition of 900 nmol of RuBP (○) or during continuous addition of RuBP at 20 (○) or 40 (●) nmol min⁻¹ followed by the subsequent addition of a further 750 nmol to estimate Vm. RuBP additions commenced at 32 s, and further single additions occurred at either 402 s (○) or 432 s (●) as indicated. Rubisco (tobacco) concentration was 40 μg mL⁻¹ (○, ●) or 10 μg mL⁻¹ (●). The data obtained following the single initial addition of RuBP (●) were fitted to Equation 3 giving the following parameter estimates: k = 0.00389 s⁻¹ (t₁/₂ = 3 min), Vmₘₐₓ = 960 nmol min⁻¹ mg⁻¹, and Vₘₐ₈ = 290 nmol min⁻¹ mg⁻¹. The decay of Vm during continuous RuBP addition was modeled (dotted line) using the same Vmₘₐₓ estimate but increasing k to 0.00693 s⁻¹ (t₁/₂ = 1.67 min) and decreasing Vₘₐ₈ to 180 nmol min⁻¹ mg⁻¹. These parameter estimates were obtained by fitting Equation 3 to the time course of Vm calculated from the measured rates and calculated RuBP concentrations during continuous addition of RuBP (40 nmol min⁻¹), assuming a hyperbolic response to RuBP concentration and a Kₘ(RuBP) of 25 μM (data not shown). Inj. injection.
not increase the activity to match the rate of RuBP addition, and this was confirmed by the barely significant increases in consumption rate induced by a saturating addition of RuBP after 400 s. The restored \( V_{\text{m}} \) was only 20 to 24% of the initial \( V_{\text{m}} \), whereas the continuous presence of saturating RuBP had maintained 47% of the initial \( V_{\text{m}} \). The estimated \( t_{1/2} \) for the decline in \( V_{\text{m}} \) under RuBP- and CO\(_2\)-limiting conditions was only 1.7 min.

An experiment analogous to that shown in Figure 5 was also performed under CO\(_2\)-limiting conditions (with a higher enzyme concentration to accommodate the reduced carboxylation and activity caused by CO\(_2\) limitation) (Fig. 7). Here again, the \( V_{\text{m}} \) seen on addition of a saturating concentration of RuBP, following exposure to subsaturating RuBP for several minutes (30% of initial \( V_{\text{m}} \)), was substantially less than that of enzyme maintained at saturating RuBP (53% of initial), and the \( V_{\text{m}} \) was restored to levels slightly above those of the saturating-RuBP control by delaying the saturating-RuBP addition until complete exhaustion of the RuBP had occurred.

**Effect of Rubisco Activase**

When reaction mixtures with subsaturating concentrations of both RuBP and CO\(_2\) were supplemented with activase, the enhancement of inactivation above that seen in the saturating-RuBP control was prevented (Fig. 8). The \( V_{\text{m}} \) seen after addition of saturating RuBP was dependent on the activase concentration, and at the higher concentration of activase, it was greater than the \( V_{\text{m}} \) seen in the saturating-RuBP control at that time. It is probable that, had the concentrations of both Rubisco and activase been high enough, the reduction in \( V_{\text{m}} \) would have been prevented completely, as demonstrated previously by Robinson and Portis (1989a). The demands that activase makes on the ATP-regenerating system and other constraints inherent in the spectrophotometric assay prevented the use of higher Rubisco and activase concentrations in this study. Activase-induced retardation of the decline in \( V_{\text{m}} \) was also reflected in the maintenance of a constant consumption rate at, ultimately, lower RuBP concentrations than seen in the absence of activase.

Another feature is apparent in the data for reactions containing activase (Fig. 8). In the period soon after RuBP addition was started, the rate of increase in activity was reduced as the activase concentration increased. Furthermore, this reduction was accompanied by a faster rate of increase in RuBP concentration as the activase concentra-

---

**Figure 7.** Time courses of RuBP concentration (A) and consumption rate (B) at limiting CO\(_2\) (1.1 mM HCO\(_3^-\)) following a single addition of 1000 or 80 nmol of RuBP or continuous addition of 80 nmol over 240 s followed by a single addition of 1000 nmol, either immediately or after the original 80 nmol had been completely consumed. All reaction mixtures contained 40 μg mL\(^{-1}\) spinach Rubisco. ■. 1000 nmol of RuBP added at 30 s; △, 80 nmol of RuBP added at 30 s followed by a further 1000 nmol at 500 s; ○, 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 270 s; ●, 80 nmol of RuBP added continuously from 30 to 270 s followed by a further 1000 nmol at 970 s. Data for some of the time points have been omitted to improve legibility.

---

**Figure 8.** Effect of Rubisco activase on the time courses of RuBP concentration (A) and consumption rate (B) at limiting CO\(_2\) (1.1 mM HCO\(_3^-\)) with continuous addition of RuBP at 20 nmol min\(^{-1}\) starting at 32 s. Tobacco Rubisco was present at 40 μg mL\(^{-1}\) without (■) and with 100 (○) or 200 (△) μg mL\(^{-1}\) tobacco Rubisco activase. Saturating amounts of RuBP (750 nmol) were added at 390 or 430 s as indicated. For comparison, data following a single addition of 900 nmol of RuBP at 32 s to a reaction without Rubisco activase are also shown (solid line).
tion increased. Therefore, the ratio of consumption rate to RuBP concentration was substantially lower with activase, to an extent dependent on the activase concentration. Control experiments (not shown) with continuous addition of PGA in the presence and absence of activase showed that this effect was not caused by the increased demands that the ATPase activity of activase makes on the ATP-regenerating system. We conclude that activase may increase Rubisco's $K_m$ (RuBP). This phenomenon is worthy of further investigation.

**Measurement of Rubisco Carbamylation**

The decline in Rubisco activity that occurs in the presence of saturating RuBP at pH 8.3 and saturating or limiting CO$_2$ concentrations is not a result of decarbamylation (Edmondson et al., 1990b). However, significant decarbamylation does occur at pH 7.3 to 7.5 (Edmondson et al., 1990b; Zhu and Jensen, 1991b). We measured the carbamylation status of the enzyme during the decline in activity while CO$_2$ and RuBP were both limiting. Before exposure to RuBP, carbamylation under subsaturating CO$_2$ was one-third of that at saturating CO$_2$, as expected (Table I). However, under limiting CO$_2$ conditions, exposure to saturating RuBP for 7 min caused carbamylation to decrease to 64% of that seen before addition of RuBP. This result differs from those of Edmondson et al. (1990b), who observed little or no RuBP-induced decline at limiting CO$_2$ and pH 8.3, and probably reflects the lower pH (8.0) used in the present experiments. This observation establishes that, at pH 8.0 and saturating RuBP, decarbamylation contributes to the enhancement of inactivation caused by lowering the CO$_2$ concentration into the subsaturating range (cf. Figs. 4 and 6). The production of inhibitory catalytic by-products is also likely to be increased by CO$_2$ limitation (Edmondson et al., 1990c). Exposure to subsaturating RuBP caused more extensive decarbamylation. After 4 and 7 min of exposure, carbamylation was 33 and 28%, respectively, of that seen before exposure to RuBP. This enhancement in decarbamylation is sufficient to account for the enhancement in inactivation caused by RuBP limitation. The ratio between the $V_m$ values for limiting (addition at 20 nmol min$^{-1}$) and saturating RuBP at 420 s (0.43, Fig. 6) closely matches the ratio between the carbamylation under comparable conditions at this time (0.44, Table I).

**DISCUSSION**

Andrews et al. (1995) described a model for the function of activase in physical terms. This model proposes that activase recognizes the particular conformation that Rubisco adopts when several mobile loops close over a ligand to bind it tightly to the active site. When primed into a tense conformation by the hydrolysis of ATP, activase binds selectively to this closed form of Rubisco and, in so doing, causes the active site to open and release the resident ligand as well as activase, now in its relaxed, inactive conformation. Closure of the active site occurs when a ligand binds tightly to either the carbamylated or the uncarbamylated form of the enzyme site, but carbamylation or decarbamylation cannot occur while a ligand occupies the site. The function of activase is the same regardless of carbamylation status or of the identity of the ligand; primed activase simply binds to closed complexes, causing them to open. When the ligand is the substrate, RuBP, catalytic conversion to loosely binding, monophosphorylated products provides an alternative rapid means for the carbamylated enzyme to escape from the closed complex without the assistance of activase. However, this route is not available to the uncarbamylated enzyme, and the activase-mediated route assumes major importance. Mate et al. (1996) mathematically formulated this model for the case when the ligand is RuBP. This formulation is summarized in Figure 9. The model predicts that, when activase is absent or inactive, all finite RuBP concentrations should induce extensive decarbamylation; however, when activase is sufficiently active, carbamylation responds positively to RuBP concentration so that, at saturating RuBP, nearly full carbamylation is achieved even at subsaturating CO$_2$ concentrations. Carbamylation thus depends on both the RuBP concentration and the activity of activase but is rather unresponsive to CO$_2$ concentration, particularly at saturating RuBP concentrations.

The model is consistent with a wide variety of observations about activase (Mate et al., 1996). However, the prediction that RuBP should always promote decarbamylation when activase is absent or inactive is not consistent with observations at saturating RuBP. Although the predicted decarbamylation occurs slowly at pH 8 and below, particularly if the CO$_2$ is subsaturating, little if any decarbamylation is observed during extended assay periods above pH 8, regardless of CO$_2$ concentration (Table I; Edmondson et al., 1990b; Zhu and Jensen, 1991b). There may be a kinetic basis for this apparent inconsistency. When carbamylated enzyme is exposed to a saturating RuBP concentration in the absence of activase—circumstances in which the model

<table>
<thead>
<tr>
<th>Table 1. Carbamylation status of Rubisco</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition</td>
</tr>
<tr>
<td>10 mm NaHCO$_3$ (pH 8)</td>
</tr>
<tr>
<td>1.1 mm NaHCO$_3$ (pH 8)</td>
</tr>
<tr>
<td>7 min after a single addition of RuBP to 1 mm</td>
</tr>
<tr>
<td>After 4 min of exposure to subsaturating RuBP</td>
</tr>
<tr>
<td>After 7 min of exposure to subsaturating RuBP</td>
</tr>
</tbody>
</table>

Copyright © 1995 American Society of Plant Biologists. All rights reserved.
Inactivation of Rubisco at Subsaturating d-Ribulose-1,5-BisP

Figure 9. A model describing the influence of RuBP concentration on the carbamylation of Rubisco. The equation is an expression for the fraction of Rubisco carbamylated in the steady state. A detailed description of the model and a derivation of the equation is given by Mate et al. (1996). C, O, M, and R represent CO₂, O₂, Mg²⁺, and RuBP, respectively. Lowercase symbols designate the concentrations of the various species. The RuBP concentration, r, is that of the free, unchelated species. ĝ is the total concentration of all forms of enzyme and ecmr* designates the total concentration of all forms of ECM that have RuBP, or any of the catalytic intermediates of both carboxylation and oxygenation, bound to them. Kᵢ and K₆ are the dissociation constants of the EC and ECM species, respectively. Kᵢ (= e/er) is the apparent dissociation constant of the ER complex in the absence of activase. Kᵦ becomes the equilibrium dissociation constant. Similarly, Kᵦ'( = ecm-ecmr*) is the apparent Kᵦ of ECM for RuBP at a particular CO₂ and O₂ concentration and activase activity. Since, for simplicity, the ionization of the ε-amino group of the lysyl residue that becomes carbamylated is not represented in the model, some of these constants will be pH dependent. Activase, when activated by ATP hydrolysis, accelerates the dissociation of ER and perhaps also of ECMR. However, since catalysis provides another rapid means of dissociation of ECM, this acceleration will induce a much larger increase in Kᵢ than in Kᵦ'. The graphs show the predicted influence of total RuBP concentration and activase activity on the steady-state carbamylation of Rubisco. The results of the model are calculated for the conditions of the experiments described in Figures 6A (low CO₂) and 4B (high CO₂). The Mg²⁺ concentration, m (12 mM), is assumed to be so large that sequestration of metal by RuBP or enzyme may be neglected and that the term Kᵦ/m is insignificant compared with unity. The product KᵦKᵦ₆ is assumed to be 1.6 × 10⁵ μM² (Laing and Christeller, 1976; Lorimer et al., 1976), and ĝ is assumed to be insignificant compared with the RuBP concentration. The concentration of unchelated RuBP was calculated from the total RuBP concentration using a dissociation constant for the Mg-RuBP complex of 1.6 mM (von Caemmerer and Edmondson, 1986). The assumed value for Kᵦ (2 μM in the absence of activase) was similarly adjusted for the effects of Mg²⁺ chelation and is expressed in terms of unchelated RuBP. Since the Kᵦ value in the absence of activase was determined in the absence of metal (Jordan and Chollet, 1983), it does not need similar adjustment. The solid lines represent the condition in which activase is inactive or absent. We assume arbitrarily that activase was determined at more acidic pH, rather than in Kᵦ', as might be concluded from the data in Figure 8 (see “Results”).
of RuBP (Figs. 5 and 7), and it occurs in concert with decarbamylation of the active site (Table I). Furthermore, the enhancement of inactivation correlates with the extent of decarbamylation. The enhanced inactivation occurs at all CO\textsubscript{2} concentrations but is increased by undersaturation with CO\textsubscript{2} (Figs. 4 and 6). All of these characteristics are consistent with the enhanced inactivation induced by undersaturation with RuBP being a result of the decarbamylation that is predicted by the model to occur when RuBP is present but activase is absent or inactive (Fig. 9).

We do not suppose that complete adjustment to the carbamylation level predicted by the model was attained during the few minutes that subsaturating RuBP concentrations were sustained during our experiments (Figs. 4–7). Even subsaturating RuBP concentrations will still reduce the concentration of ECM available and thus retard decarbamylation. High concentrations of Mg\textsuperscript{2+} and/or CO\textsubscript{2} will exacerbate this retardation. Thus, the difference in the extent of inactivation between high (Fig. 4) and low (Fig. 6) CO\textsubscript{2} concentrations may be more a reflection of differences in the rates of decarbamylation than differences in the position of the ultimate steady states. Indeed, the exact positions of the steady states predicted by the model must be treated with some caution because of uncertainties in the estimations of the various dissociation constants. For example, the values for $K_r$ (Jordan and Chollet, 1983) and $K_{r,0}$ (Laing and Christaller, 1976; Lorimer et al., 1976) were determined at lower temperatures than are usual for the determination of $K_r'$. Nevertheless, the trends predicted by the model will not be compromised by such uncertainties.

It seems unlikely that the enhancement of inactivation induced by undersaturation with RuBP can be caused by an increase in the rate at which inhibitory catalytic by-products are produced. These by-products are derived from the 2,3-enediol form of RuBP, which is an intermediate in the catalytic process (Pierce et al., 1986), and undersaturation with RuBP could only reduce the steady-state concentration of this intermediate, not increase it. Furthermore, the decarbamylation that accompanies the inactivation induced by undersaturation with RuBP (Table I) is sufficient, by itself, to account for the inactivation. No further inhibitory mechanism (Yokota, 1991) is required to explain the data.

Activase prevented the enhancement of inactivation induced by undersaturation with RuBP (Fig. 8) in accordance with the mechanism of activase proposed by the model. High molar ratios of activase to Rubisco were required as has been noticed previously but, in vivo, at Rubisco concentrations nearly 4 orders of magnitude higher than those used in our experiments, much lower activase/Rubisco ratios would be needed (Robinson et al., 1988).

The conclusions we draw from this study have important physiological implications. Following a reduction in irradiance, two conditions must be met before Rubisco's activity can be reduced to match the reduced rate of RuBP supply: (a) The activity of activase must be reduced so that the $K_r' / K_r$ ratio is increased to the level required to sustain the new, lower steady-state carbamylation. The manner in which this is achieved in vivo in not completely clear, but modulations in activase activity in response to the ATP/ADP ratio (Robinson and Portis, 1989b) and the rate of electron transport through PSI (Campbell and Ogren, 1990) have been observed. (b) The RuBP concentration must decrease into the subsaturating region to open a kinetic path for the new steady-state carbamylation to be achieved in a reasonable time. Once Rubisco's carbamylation has been reduced to the level appropriate to the reduced irradiance, the RuBP concentration will increase again. Reduced carbamylation of Rubisco will be accompanied by an increase in the concentration of the ER complex. This is consistent with the observation of Brooks and Portis (1988) that a reduction of irradiance was accompanied by the formation of a complex between Rubisco and RuBP that was tight enough to survive gel filtration.

Received June 26, 1995; accepted September 8, 1995.

Copyright Clearance Center: 0032-0889/95/109/1441/11.

LITERATURE CITED


Edmondson DL, Badger MR, Andrews TJ (1990c) Slow inactivation of ribulosebisphosphate carboxylase during catalysis is caused by accumulation of a slow, tight-binding inhibitor at the catalytic site. Plant Physiol 93: 1390–1397


transgenic tobacco suggests a simple model of activase action. Plant Physiol 76: 968–971


