Determinant of Apparent $K_m$ Values for Ribulose 1,5-
Bisphosphate Carboxylase/Oxygenase (Rubisco) Activase
Using the Spectrophotometric Assay of Rubisco Activity

Yun Lan and Keith A. Mott*

Biology Department, Utah State University, Logan, Utah 84322

ABSTRACT

The spectrophotometric assay for ribulose 1,5-bisphosphate
carboxylase/oxygenase (Rubisco) was used to determine the
rate of increase in Rubisco activity over time in the presence
or absence of Rubisco activase. Polynomial approximations to the
raw data were used to smooth out minor fluctuations in the
spectrophotometer readings, and Rubisco activase activity was
expressed as nanomoles of activated Rubisco per minute. This
assay was used to examine the effects of CO$_2$ and the inactive-
Rubisco:ribulose 1,5-bisphosphate complex (ER) on the activase-
catalyzed activation reaction. Double-reciprocal plots of activase
activity and ER at several concentrations of CO$_2$ were consistent
with two-substrate Michaelis-Menten kinetics, and the apparent
$K_m$(CO$_2$) and $K_m$(ER) were determined to be 53 and 2.7 micromolar,
respectively. These data do not prove that ER and CO$_2$ are
substrates for the reaction catalyzed by activase, but they may
be important to our understanding of the activation process in vivo.
The implications of these data and their relation to previously
published data on the effects of ER and CO$_2$ on activase are
discussed.

In many plants, extractable Rubisco$^2$ activity is low for
leaves in low light, but increases when the leaf is transferred
to a higher light intensity. This increase in activity occurs over
a period of minutes and can limit the rate of photosynthesis
during the nonsteady-state following an increase in light
intensity (16, 21). For plants without the tight-binding inhibitor
carboxyarabinitol 1-P, the increase in Rubisco activity
associated with higher light intensities is the result of covalent
modification of the catalytic site by CO$_2$ and Mg$^{2+}$. This
reaction involves the formation of a carbamate at a specific
lysine residue, which is then stabilized by Mg$^{2+}$, to form the
catalytically competent enzyme (9). The thermodynamic
equilibrium of the activation reaction, however, favors the
noncarbamylated (inactivated) form of the enzyme under the
conditions thought to exist in the stroma of an illuminated
chloroplast. Tight binding of the substrate RuBP to the inactivated
form of the enzyme further increases the proportion of the enzyme
in the inactivated form (7), and the $k_{off}$ for this binding restricts the rate at which active enzyme can be
formed from the ER in vitro (10). Inactivated enzyme has
been shown to bind to RuBP in intact leaves (1, 3), and it is
therefore impossible to explain the relatively rapid activation
rates and high steady-state activation states that are observed
in leaves based on the activation process as studied in vitro.

The discovery of the enzyme Rubisco activase (15), which
apparently catalyzes some aspect of the activation reaction,
has provided an explanation for the rapid activation rates
observed in intact leaves. The discovery that the activase-
catalyzed reaction also dephosphorylates ATP (18) has
explained how a substantial portion of Rubisco can exist in the
activated form in intact leaves despite the unfavorable equili-
rium of the noncatalyzed reaction. A hypothetical reaction
for Rubisco activase, along with the uncatalyzed activation
reactions, is shown below, where E is Rubisco, R is RuBP, C
is CO$_2$, and M is Mg$^{2+}$:

$$
\begin{align*}
  R & \quad R \\
  & \quad + \\
  M + C + E & \quad \rightleftharpoons \quad ECM \\
  \uparrow & \quad \uparrow \\
  \text{ATP} + M + C + ER & \quad \rightleftharpoons \quad \text{ECMR} + \text{ADP} + \Pi \\
  \text{activase} & \\
\end{align*}
$$

Since the increase in Rubisco activity following an increase
in photon flux density has been shown to limit the approach
of photosynthesis to steady-state following an increase in
photon flux density (16, 21), the kinetics of the activase-
catalyzed reaction may be important for the total carbon gain
of plants growing in fluctuating-light conditions. In this study,
we used the spectrophotometric assay of Rubisco activity (8)
to monitor increases in Rubisco activity over time in the
presence of Rubisco activase. The dependence of activase
activity on CO$_2$ and ER was examined and found to be
consistent with two-substrate, Michaelis-Menten kinetics.

MATERIALS AND METHODS

Plant Material

Spinach (Spinacea oleracea L.), used for extraction of Rub-
isco and Rubisco activase, was grown hydroponically in aer-
ated, half-strength Hoagland's solution that was changed
weekly. Controlled environment growth chambers were used
to produce a 10-h photoperiod with day and night tempera-
tures of 25°C and 18°C, respectively. Light intensity was approxi-
mately 350 $\mu$mol-m$^{-2}$-s$^{-1}$.

---

$^1$ Supported by U.S. Department of Agriculture/Cooperative State
Research Service grant 89-37130-4741.

$^2$ Abbreviations: Rubisco, ribulose 1,5-bisphosphate carboxylase/
oxygenase; RuBP, ribulose 1,5-bisphosphate; ER, inactive-Rubi-
isco:RuBP complex; ECM, activated Rubisco.
Enzymes and Chemicals

Rubisco was purified as described previously (5) with some minor modifications. The purified enzyme was precipitated with 50% ammonium sulfate and stored in liquid nitrogen until use. Rubisco activase was purified as described by Robinson et al. (14), except that standard ion-exchange was used instead of fast protein liquid chromatography and the enzyme was concentrated and desalted using microfiltration (Centri-prep-30; Amicon). The purified enzyme was stored in liquid nitrogen with 0.2 mM ATP. RuBP was produced from ribose-5-phosphate and ATP using phosphoribulokinase and phosphoribulokinase. RuBP generated in this manner was then purified as described by Horecker et al. (6). NADH was obtained from Boehringer Mannheim. All other chemicals and enzymes were obtained from Sigma.

Rubisco Assays

Purified Rubisco was desalted by gel filtration and then activated at 15 mM Mg2+ and 130 μM CO2 that was produced with the appropriate concentration of NaHCO3 (pKα = 6.11). Each assay (total volume, 0.5 mL) contained 50 mM Bicine (pH 8.0), 15 mM MgCl2, 10 mM NaCl, 5 mM DTT, 5 mM phosphocreatine, 5 mM ATP, 5 mM phosphoenolpyruvate, and the appropriate amount of NaHCO3. This mixture was prepared before each set of experiments using distilled water that had been bubbled with N2 for 30 min. Five minutes before initiating the reaction with activated Rubisco, stock solutions were added to produce final concentrations as follows: 1 mM RuBP, 0.3 mM NADH, 10 units mL-1 glyceraldehyde-3-phosphate dehydrogenase, 10 units mL-1 3-phosphoglycerate kinase, 20 units mL-1 pyruvate kinase, and 2 units mL-1 creatine phosphokinase. Following the addition of Rubisco, NADH concentration was measured spectrophotometrically (λ = 340 nm) over time for 5 min. Amax was measured at 3-s intervals and stored digitally in computer files. Absorbance values were converted to NADH concentrations using an extinction coefficient of 6.22 mM-1.

Radiometric assays of Rubisco activity were performed as described by Cardon and Mott (3). The concentration of Rubisco was determined either spectrophotometrically, using the equation A280 × 0.61 = mg mL-1, or from the maximum activity of the enzyme. In the latter case, Rubisco was fully activated and then assayed at saturating CO2 and Mg2+ concentrations using both the radiometric and spectrophotometric techniques. The number of catalytically competent sites was determined from this activity assuming a turnover number of 3.3 sites s-1.

Rubisco activase concentration was determined with the dye-binding assay (17) using BSA as a standard.

Rubisco Activase Assays

Purified Rubisco was desalted and inactivated with Sephadex G-25 column that had been pre-equilibrated with CO2-free, Mg2+-free buffer. The inactivated enzyme was collected and stored under nitrogen, and RuBP was added (final concentration = 2 mM) to form the ER complex. Rubisco that had been prepared in this manner showed less than 5% of the fully activated activity, so [ER] was assumed to be equal to the concentration of enzyme sites. The assay mixture for Rubisco activase was identical to that used for spectrophotometric Rubisco assays except that the appropriate concentration of activase was added to the mixture 1 min before initiating the reaction, and the reaction was initiated with the ER form of Rubisco. NADH concentration was measured over time as described above.

RESULTS

Determination of Rubisco and Rubisco Activase Activities

The circles in Figures 1a, 2a, and 3a show NADH versus time for Rubisco or activase assays. Despite the smooth appearance of these data, determination of the first and second derivatives of the curves by subtraction yielded a fair amount of scatter. To remove this scatter and yet retain the information contained in the data, NADH versus time curves for each experiment were fitted to polynomial functions. To obtain the best fit, the order of the polynomial was increased until there was no improvement in residuals and the second derivative versus time was stable. In all cases, a fourth-order polynomial satisfied both of these conditions (i.e. neither the residuals nor the second derivative of the fifth-order polyno-

Figure 1. Rubisco activity at 130 μM CO2. Rubisco (0.05 mg mL-1) was activated at 130 μM CO2, 15 mM Mg2+, and assayed at 130 μM CO2. The symbols in panel a show NADH oxidized over time as measured spectrophotometrically at 340 nm (see "Materials and Methods" for assay description). The line shows the fourth-order polynomial approximation of the data that was used to calculate the first derivative. Panel b shows Rubisco activity over time as calculated from the first derivative of the polynomial shown in panel a, assuming two NADH oxidized per RuBP carboxylated.
Rubisco activity was calculated from the first derivative of NADH oxidation (Fig. 2a), assuming the presence of Rubisco activase. The activity of Rubisco was determined by measuring NADH oxidation over time using a spectrophotometric assay. The first derivative of NADH oxidation was calculated using polynomial approximations (lines, Figs. 1a, 2a, 3a) with the actual absorbance data (symbols, Figs. 1a, 2a, 3a) showing that the polynomials accurately described the data.

Rubisco activity was calculated from the first derivative of NADH oxidation (Fig. 2a), assuming the presence of Rubisco activase. The activity of Rubisco was determined by measuring NADH oxidation over time using a spectrophotometric assay. The first derivative of NADH oxidation was calculated using polynomial approximations (lines, Figs. 1a, 2a, 3a) with the actual absorbance data (symbols, Figs. 1a, 2a, 3a) showing that the polynomials accurately described the data.

Rubisco activity was calculated from the first derivative of NADH oxidation (Fig. 2a), assuming the presence of Rubisco activase. The activity of Rubisco was determined by measuring NADH oxidation over time using a spectrophotometric assay. The first derivative of NADH oxidation was calculated using polynomial approximations (lines, Figs. 1a, 2a, 3a) with the actual absorbance data (symbols, Figs. 1a, 2a, 3a) showing that the polynomials accurately described the data.

Rubisco activity was calculated from the first derivative of NADH oxidation (Fig. 2a), assuming the presence of Rubisco activase. The activity of Rubisco was determined by measuring NADH oxidation over time using a spectrophotometric assay. The first derivative of NADH oxidation was calculated using polynomial approximations (lines, Figs. 1a, 2a, 3a) with the actual absorbance data (symbols, Figs. 1a, 2a, 3a) showing that the polynomials accurately described the data.

Rubisco activity was calculated from the first derivative of NADH oxidation (Fig. 2a), assuming the presence of Rubisco activase. The activity of Rubisco was determined by measuring NADH oxidation over time using a spectrophotometric assay. The first derivative of NADH oxidation was calculated using polynomial approximations (lines, Figs. 1a, 2a, 3a) with the actual absorbance data (symbols, Figs. 1a, 2a, 3a) showing that the polynomials accurately described the data.
from the rate of activation in the presence of activase (solid line, Fig. 3b), thereby correcting for the rate of noncatalyzed activation. The initial activase activity was taken as representative of the starting conditions, and this value is reported in the experiments described below.

As an initial test of the Rubisco activase assay procedure, the effect of activase concentration on activase activity was determined. Activase activity was linearly related to activase concentration for three concentrations of ER (Fig. 4).

To determine the effects of CO2 and ER on Rubisco activase activity, assays were performed at many concentrations of each. Since CO2 is also a substrate for Rubisco, it was necessary to correct for the influence of CO2 on Rubisco activity to convert activation rate data to ECM min\(^{-1}\). This was accomplished by adjusting the turnover number for each active site using a \(K_m\) (CO2) of 9 \(\mu\)M (19). A double-reciprocal plot of the data for various concentrations of ER and CO2 revealed a pattern consistent with an ordered or random ternary-complex, two-substrate reaction mechanism involving ER and CO2 (Fig. 5). In these experiments, Rubisco extractions of differing specific activities (open and closed symbols, Fig. 5) gave consistent data for Rubisco activase activity if ER was determined from the fully activated specific activity of the Rubisco extraction (see "Materials and Methods"). This was not true if ER was determined by A\(_{280}\).

To determine the kinetic constants for ER and CO2 from the double-reciprocal plot, two secondary plots were used. Figure 6 shows a plot of 1/[ER] versus the ordinate intercept of Figure 5. The ordinate intercept of this plot equals \(1/V_{\text{max}}\), and \(V_{\text{max}}\) was determined to be 64 nmol ER min\(^{-1}\) mg protein\(^{-1}\). The slope of the line equals \(K_m(\text{ER})/V_{\text{max}}\), and \(K_m(\text{ER})\) was determined to be 2.7 \(\mu\)M. The specific activity of the Rubisco was 2.0 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (open circles) or 2.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (solid circles).

Figure 7 shows a plot of 1/[ER] versus the double-reciprocal slopes (Fig. 5). The slope of this line equals \(K_m(\text{CO}_2)/V_{\text{max}}\), and \(K_m(\text{CO}_2)\) was determined to be 53 \(\mu\)M. These data were also consistent with a two substrate mechanism involving ER and CO2.

Figure 4. Activase activity as a function of activase concentration. Activase activity at different activase concentrations was assayed at 10 \(\mu\)M CO2 and three different ER concentrations.

Figure 5. Double-reciprocal plots of activase activity (V0) and CO2 concentration at different ER concentrations. The concentration of Rubisco activase was 15 \(\mu\)g mL\(^{-1}\). The specific activity of the Rubisco was 2.0 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\).

Figure 6. Ordinate intercepts of two double-reciprocal plots (Fig. 5 and one not shown) as a function of the reciprocal of ER. The ordinate intercept of this plot equals \(1/V_{\text{max}}\), and \(V_{\text{max}}\) was determined to be 64 nmol ER min\(^{-1}\) mg protein\(^{-1}\). The slope of the line equals \(K_m(\text{ER})/V_{\text{max}}\), and \(K_m(\text{ER})\) was determined to be 2.7 \(\mu\)M. The specific activity of the Rubisco was 2.0 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (open circles) or 2.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (solid circles).

Figure 7. Slopes of two double-reciprocal plots (Fig. 5 and one not shown) as a function of the reciprocal of ER concentration. The ordinate intercept of this plot is equal to \(K_m(\text{CO}_2)/V_{\text{max}}\), and \(K_m(\text{CO}_2)\) was determined to be 53 \(\mu\)M. The specific activity of the Rubisco was 2.0 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (open circles) or 2.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\) (closed circles).
ordinate intercept of this plot is equal to $K_m(CO_2)/V_{max}$, and $K_m(CO_2)$ was determined to be 53 μM.

**DISCUSSION**

Agreement between the spectrophotometric and the radiometric assays for Rubisco has been previously documented (8, 19) and is confirmed in this study. We present data for spectrophotometric Rubisco assays that demonstrate that this assay yields data for activity at low CO$_2$ and for activity over time that are consistent with the known properties of Rubisco. The initial rate of carboxylation at 10 μM CO$_2$ was approximately 60% of that obtained at 130 μM CO$_2$ (Figs. 1, 2), as would be expected based on a $K_m(CO_2)$ of 9 μM (20). Since the assay mix was prepared from N$_2$-bubbled water, the O$_2$ concentration was presumably low, and little oxygenase activity would be expected. Activity of Rubisco at 130 μM CO$_2$ was relatively constant over the first minute of the assay, but at 10 μM, CO$_2$ activity declined by almost 50% over the first minute. This decline may have been caused by deactivation of the enzyme (i.e., loss of activator CO$_2$ and Mg$^{++}$), since 10 μM CO$_2$ is well below saturating for activation. It may also have been caused by the binding of a phosphorylated inhibitor to the active site (13), and the data of Edmondson et al. (4), which show no loss of activator CO$_2$ at low CO$_2$, would support this interpretation.

Polynomial approximation proved a useful technique for smoothing the raw data and determining the first and second derivatives, from which Rubisco activity and the rate of increase in Rubisco activity were calculated. These values could also have been calculated by subtraction, as has been done in studies of Rubisco activase utilizing the radiometric assay of Rubisco (e.g., ref. 14). However, small fluctuations in the spectrophotometer readings produced a fair amount of scatter in the absorbance differences, and polynomial approximation removed this scatter. Visual comparison of the polynomial curves and the actual data (Figs. 1a, 2a, 3a) confirms that the curves were good representations of the data, as is suggested by the small residuals.

Using the techniques described above, it was possible to calculate the rate of Rubisco activation as a function of time (e.g., Fig. 3c). This rate generally decreased with time, probably because of substrate (CO$_2$ or ER) disappearance. Rigorous analysis of these time courses was not attempted because as the proportion of activated and inactivated enzyme changed over time, so also would the rate (and possibly the direction) of the uncatalyzed interconversions between the two forms. For this reason, only the initial rate of activation could be accurately corrected for the uncatalyzed background reaction, and only the initial rate was used to calculate activase activity.

In contrast with previous work, Rubisco activase activity was expressed in units of nmol ECM min$^{-1}$ in this study. These units were suggested by Robinson et al. (14), but not used because they require knowledge of the maximum activity of the Rubisco used in the assay, and because they require assumptions regarding the rate of Rubisco deactivation. By using the spectrophotometric assay to document changes in Rubisco activity, we were able to determine activation rates over much shorter time intervals (our readings were 3 s apart) than is possible using the radiometric assay, thus minimizing the problem with Rubisco deactivation (because it is more accurately corrected for by the measured background). Although the units used in previous studies have the advantage that they are directly comparable with increases in Rubisco activity in leaves and other systems, units of ECM min$^{-1}$ are “true” enzyme units and can more easily be used in kinetic analyses.

To determine the rate of ECM production from the increase in Rubisco activity, it was necessary to correct both for the effect of CO$_2$ on Rubisco activity and for differences in the total activity among Rubisco preparations. To correct for the influence of CO$_2$ on Rubisco activity, the turnover number was adjusted to reflect the lower CO$_2$ using a $K_m(CO_2)$ of 9 μM. This allowed computation of activation rate in units of ECM min$^{-1}$ despite nonsaturating CO$_2$ concentrations. To correct for differing specific activities among Rubisco preparations, the concentrations of catalytically competent sites was calculated based on the fully activated, CO$_2$-saturated activity of the preparation and a turnover number of 3.3 s$^{-1}$.

The linear relationship between activase activity and activase concentration (Fig. 4) agrees with the data of Robinson et al. (14) and is good evidence that the spectrophotometric procedure is an effective assay for Rubisco activase. The spectrophotometric assay has several disadvantages. The most important of these is the need to maintain a high ATP:ADP ratio for the coupling enzyme phosphoglyceric acid kinase. Other disadvantages include the presence of NADP in the reaction mixture, which could affect activase activity, and the need for an optically clear solution, which prevents assessment of the effect of thylakoids on activase activity. Despite these disadvantages, the assay is quite useful for certain studies of activase. It is inexpensive, quick, and provides estimates of Rubisco activation rate over shorter time scales than is possible with radiometric assay.

The dependence of activase activity on ER and CO$_2$ is consistent with two-substrate Michaelis-Menton kinetics. The data indicate that ER and CO$_2$ could be substrates for the reaction catalyzed by Rubisco activase and, if so, must be present simultaneously (ternary-complex formation) at the active site for product formation to occur. However, additional experiments are necessary to conclusively establish ER and CO$_2$ as substrates for the reaction and to determine the order of binding, etc.

Although the data presented in this study do not prove that ER and CO$_2$ are substrates for Rubisco activase, they are consistent with previously published properties of activase and may provide insight into the kinetics of activation in vivo. The dependence of activase activity on ER found in this study seems inconsistent with the data of Robinson et al. (14), who showed that Rubisco activase activity declined slightly as ER was increased. They, however, defined a unit of activase activity as an increase in the specific activity of Rubisco of 1 μmol min$^{-1}$ mg$^{-1}$ Rubisco/min. If one assumes a constant maximum specific activity of Rubisco, their units can be made proportional to ECM min$^{-1}$, and the resultant data are consistent with a Michaelis-Menton relationship between ER and activase activity.

The apparent $K_m$(ER) at 10 μM CO$_2$ will be quite low compared with the concentration of Rubisco-active sites in the stroma, which has been estimated to be in the mm range.
(9). Thus, even when Rubisco is substantially in the activated form, the concentration of ER should be well above the \( K_m \) value. The rate of activase-catalyzed Rubisco activation should therefore be independent of the concentration of ER in the stroma. This is not consistent with the data of Woodrow and Mott (21), which show apparent first-order rate kinetics with respect to ER for activation in intact leaves. This inconsistency suggests that some other factor, such as modulation of activase activity or the rate of the uncatalyzed deactivation reaction, is strongly influencing the rate of activation \textit{in vivo}.

The apparent \( K_m(CO_2) \) for activase-catalyzed activation is quite high compared with the \( CO_2 \) concentration necessary for half-maximal activation of Rubisco, determined by Portis \textit{et al.} (11). However, the latter number presumably reflects the relative rates of activation and deactivation and their dependence on \( CO_2 \), while the former describes the \( CO_2 \) dependence of activase-catalyzed activation. At 53 \( \mu \)M, the apparent \( K_m(CO_2) \) is well above the concentration of \( CO_2 \) in air, so the rate of activase-catalyzed activation should be sensitive to \( CO_2 \) concentration in this range. This agrees with unpublished data of Woodrow and Mott that show that Rubisco activation rate in intact leaves is dependent on \( CO_2 \) concentration.

In summary, we present evidence that the spectrophotometric assay of Rubisco can be adapted to assay Rubisco activase, and we use this assay to determine apparent \( K_m \) values for ER and \( CO_2 \) in the activase reaction. Several other factors may also influence the rate of activation \textit{in vivo}. For example, the data of Streusand and Portis (18) indicate that activase activity is strongly influenced by ATP:ADP, and those of Robinson and Portis (12) show that Rubisco activation state is correlated with ATP in chloroplasts. Also, the activity of Rubisco activase has recently been shown to be stimulated by illuminated thylakoids (2). Additional studies are necessary to quantify the effects of these factors on the kinetic properties of activase. These types of data will clarify the processes that determine the rate of Rubisco activation \textit{in vivo}, and may ultimately improve our understanding of the response of photosynthesis to fluctuating light conditions.

**ACKNOWLEDGMENTS**

We thank Thomas Sharkey for helpful discussions concerning the spectrophotometric assay of Rubisco, and Odette Michaelson for technical assistance. We also thank Ian Woodrow and Joe Berry for helpful comments on the manuscript.

**LITERATURE CITED**

10. Mott KA, Berry JA (1986) Effects of \( pH \) on activity and activation of ribulose-1,5-bisphosphate carboxylase at air level \( CO_2 \). Plant Physiol 82: 77–82