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Effects of pH on Activity and Activation of Ribulose 1,5-Bisphosphate Carboxylase at Air Level CO_2[1]

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KEITH A. MOTT*[2] AND JOSEPH A. BERRY
Carnegie Institution of Washington, Department of Plant Biology, Stanford, California 94305

ABSTRACT

The effects of pH on catalysis and activation characteristics of spinach ribulose 1,5-bisphosphate (RuBP) carboxylase were examined at air level of CO_2. Catalysis at limiting CO_2 was independent of pH over the range of pH 8.2 to 8.8. However, the kinetics of activation and the apparent equilibrium between the activated and inactivated forms of the enzyme were strongly dependent upon the pH and the presence or absence of the substrate RuBP. When incubated at air level of CO_2 at pH 8.2 in the absence of RuBP, the enzyme activation state was approximately 75% of that achieved with saturating CO_2 at that pH. The extent of activation increased with pH reaching 100% at pH values of 8.6 or higher. Adding RuBP to the activation medium after equilibrium activation state had been established decreased the apparent equilibrium activation level at pH values below 8.6. This effect was reversed at pH values above 8.6. Activation of inactive enzyme by CO_2 and Mg^{2+} was inhibited dramatically at pH values below 8.6 and less so at pH values above 8.6. Studies showed that binding of RuBP to the inactive form of the enzyme was pH dependent with tighter binding occurring at lower pH values. It is suggested that the tight binding of RuBP to the inactive enzyme tends to decrease the equilibrium concentration of the activated form at pH values less than 8.6. These studies indicate that stromal pH could have a strong effect on the activation state of this enzyme *in vivo*, and possible feedback interactions which might adjust the apparent V_{max} to match the rate of RuBP regeneration are discussed.

The primary CO_2 fixing reaction of photosynthetic carbon reduction is the carboxylation of the five carbon sugar RuBP[3] to form two molecules of phosphoglyceric acid. This reaction is catalyzed by the enzyme RuBP carboxylase. While the absolute amount of RuBP carboxylase in a given tissue probably changes only over a developmental time scale, short-term changes in the activity of the enzyme present in the tissue have been documented (14). The enzyme is known to be readily interconvertible between a catalytically competent (activated) and a catalytically incompetent (deactivated) form *in vitro*. The activation process involves the formation of a carbamate by the addition of one CO_2 and one Mg^{2+} per active site. The apparent V_{max} of the enzyme *in vitro* can be controlled by the concentrations of CO_2 and Mg^{2+} in the solution (see 12 for a review).

The activation state of RuBP carboxylase *in vivo* appears to vary with environmental conditions, particularly light intensity. Perchorowicz and Jensen (15) have shown that activation of RuBP carboxylase in intact leaves varied in parallel with photosynthetic rate as light intensity was varied. RuBP concentrations were high except at very low light intensities in these experiments, and they suggested that the rate of carboxylation *in vivo* was controlled by the activation of RuBP carboxylase. However, recent studies of Mott *et al.* (13) have suggested that the activity of RuBP carboxylase may be adjusted by a control mechanism such that the RuBP-saturated rate of reaction at steady state equals the light limited rate of RuBP synthesis. Evidence for this was obtained from studies on the kinetics of changes in photosynthesis and RuBP concentrations following a step decrease in light intensity provided to intact leaves. RuBP concentrations and photosynthesis dropped abruptly with a decrease in light intensity and were linearly related to each other for a short time after the decrease in light intensity. This was followed by a slow deactivation of the carboxylase and a corresponding increase in RuBP concentration with no significant decrease in photosynthetic rate.

No mechanism for feedback regulation of activation state has been suggested in the literature. Furthermore, there remain several other problems in attempting to relate RuBP carboxylase activation characteristics *in vitro* to the apparent activity of the enzyme *in vivo*. First, the carboxylase is not fully activated *in vitro* under conditions thought to exist in the stroma of an illuminated intact spinach chloroplast (air level CO_2 and pH 8.2). Yet, it is necessary to postulate full activation to explain photosynthetic rates observed with intact leaves at high light and limiting CO_2 (4, 16). Second, inactive spinach carboxylase *in vitro* will not activate in the presence of RuBP (7, 9). Yet, conditions when a portion of the total carboxylase is inactive and must activate in the presence of RuBP will occur *in vivo*, particularly following a step increase in light intensity. Third, spinach carboxylase which has been pre-activated in the absence of RuBP will tend to deactivate upon the addition of RuBP (9). This deactivation shows up as gradual decline in activity when a carboxylase assay is begun by bringing the preactivated enzyme and the substrate RuBP together.

In this paper we report studies on the effect of RuBP and pH on the activity and activation of spinach RuBP carboxylase. Our studies are based on the hypothesis that the balance between the steady state RuBP carboxylase activity and RuBP regeneration capacity would affect the stromal pH. For example, decreasing the light to a rate limiting fluence should lead to an imbalance where the transient rate of RuBP carboxylation exceeds the rate of PGA reduction. Accumulation of PGA and the dissipation of the thylakoid pH gradient should cause the stroma to become more acid. Conversely, increasing the light fluence may cause a transient decrease in PGA, an accumulation of RuBP, and an increase in the pH gradient; causing the stroma to alkalinize. Indeed, Lorimer *et al.* (10) show that alkalinization tends to increase the equilibrium activation state of RuBP carboxylase at air level CO_2 *in vitro*.

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[2] Current address: Department of Biology UMC 45, Utah State University, Logan, UT 84322-4500.

[3] Abbreviations: RuBP, ribulose 1,5-bisphosphate; TNS, 2-*p*-toluidinylnaphthylene-6-sulfonate; PGA, glycerate-3-phosphate; CABP, carboxyarabinatol bisphosphate.

In this study we confirm that the activation characteristics of RuBP carboxylase at air level CO_2 are strongly pH dependent, and we further show that the inhibition of the rate of activation by RuBP is reduced at alkaline pH. For these *in vitro* studies a pH of 8.6 was sufficient to attain high steady state activation and activity of RuBP carboxylase at air level CO_2 in the presence of RuBP. The effect of pH on the noncatalytic binding of RuBP to the deactivated form of the enzyme provides an explanation for the effects of pH and RuBP on activation. Furthermore, feedback regulation of RuBP carboxylase activity by stromal pH could be invoked to explain activation state changes with changes in light intensity observed with intact leaves.

MATERIALS AND METHODS

RuBP carboxylase was extracted from leaves of spinach (*Spinacia oleracea*) and purified as described by Hall and Tolbert (5). In some caes the final enzyme solution was further purified with a Sepharose column, but no differences in characteristics or activity were noticed as a result of this step. The purified carboxylase was stored as a precipitate in 50% $NH_4(SO_4)_2$. To resuspend the enzyme in the appropriate buffer for a particular experiment, 5 to 10 ml of the $NH_4(SO_4)_2$ suspension were centrifuged for 10 min at 10,000*g* at 4°C. The supernatant was discarded and the enzyme pellet resuspended in the appropriate buffer (see below for specific buffers used in each experiment). To remove any remaining $NH_4(SO_4)_2$ the enzyme solution was passed through a small Sephadex G-25 column which had been preequilibrated with the particular buffer. In cases where the initial [CO_2] was to be zero, the column was equilibrated with CO_2-free buffer under N_2.

Air level CO_2 was taken as 10 μM and was produced by adding the appropriate concentration of $NaHCO_3$ at the particular pH, calculated using a pK_a for bicarbonate of 6.11. Since this work has been completed Yokota and Kitaoka (18) have suggested that the actual pK_a for bicarbonate at the ionic concentrations used in this study is 6.06. Using this value for the pK_a of bicarbonate would increase the CO_2 concentrations reported in this study by approximately 10%. CO_2-free buffers were made by bubbling acidified water with N_2 for 30 min, adding the buffer under N_2, and using a saturated NaOH solution spun to remove carbonate to adjust pH.

Carboxylase activity was assayed by adding 10 μl of an enzyme solution to a 500 μl assay mixture at 25°C and stopping the reaction after 30 s with 500 μl of 2 N HCl. The assay mixture consisted of 100 mM Bicine pH 8.2, 5 mM DTT, and 20 mM $MgCl_2$. Approximately 0.5 mM RuBP was generated in the assay mixture less than 30 min before use with ATP and ribose 5-P (Sigma) using phosphoriboisomerase (Sigma) and ribulose 5-P kinase purified from spinach. After stopping the reaction with acid, the solution was evaporated, 0.5 ml of water and 5 ml of scintillation fluid added, and the remaining (acid stable) radioactivity determined by liquid scintillation. The concentration of RuBP carboxylase was determined using [^{14}C]CABP and antibody raised against the RuBP carboxylase (3).

Catalysis *versus* pH. Purified RuBP carboxylase (4 mg/ml) in the assay buffer was activated by adding 150 μM $^{14}CO_2$. The reactions were started by injection of 5 μl aliquots of enzyme to closed vials flushed with N_2 and containing 500 μl of assay buffer at pH 8.2, 8.4, 8.6, and 8.8 containing HCO_3^- to give final free CO_2 concentrations of 4, 6, 8, 10, and 12 μM. Reaction mixtures contained 2 Wilbur Anderson units of bovine erythrocyte carbonic anhydrase. The reactions were stopped after 30 s.

Mg^{2+} Requirement for Activation *versus* pH. Purified RuBP carboxylase was equilibrated at 10 μM CO_2 at different Mg^{2+} concentrations at three different pH values. RuBP (0.5 mM) was added to start the reaction and steady state activity was determined 2 to 3 min after the addition of RuBP.

Activation State *versus* pH. Purified RuBP carboxylase was completely deactivated by resuspending it in CO_2-free 100 mM Bicine, 5 mM DTT at pH 8.2, and kept under N_2. Enzyme prepared in this manner typically showed less than 10% of full activity and could not be activated significantly by the addition of CO_2 or Mg^{2+} alone. Aliquots of the inactive enzyme solution were added to solutions of 100 mM Bicine, 5 mM DTT at various pH values with 10 μM CO_2, and 10 mM $MgCl_2$ to activate the enzyme. Preliminary experiments showed that equilibrium activation levels were reached in 1 to 2 min. Activation levels are expressed relative to that achieved in control experiments conducted with the same enzyme preparation incubated at 150 μM CO_2, 10 mM Mg^{2+}, and pH 8.2. After activation was started, aliquots of the activating enzyme were removed and assayed for activity in a standard activity assay.

Activation Rate in the Presence of RuBP. Aliquots of the deactivated carboxylase solution were added to solutions as above, with or without 20 mM RuBP. Ten μl aliquots were removed at various times after adding the enzyme to the activating buffer and assayed in a standard activity assay.

Activity of RuBP Carboxylase as Influenced by pH and RuBP. RuBP carboxylase was incubated in 100 mM Bicine, 5 mM DTT at varying pH values with 10 μM CO_2, and 10 mM $MgCl_2$. RuBP was added to the mixture to start the reaction and samples were removed and killed with acid at various times after adding the RuBP to assess the progress of the reaction.

RuBP Binding to Inactive RuBP Carboxylase. Fluorescence spectroscopy of the RuBP carboxylase-TNS complex was used to determine the pH dependency of RuBP binding to inactive carboxylase. For these experiments RuBP carboxylase was added to a solution of 100 mM Bicine, 5 mM DTT, and 0.1 mM TNS. Fluorescence at 427 nM was measured using a fluorescence spectrophotometer and an excitation wavelength of 366 nm. The solution was then titrated with RuBP (Sigma) and fluorescence measured after each increment in RuBP concentration. The entire procedure was repeated at pH 7.8, 8.2, 8.4, 8.6, 8.8, and 9.0. The concentration of RuBP carboxylase was determined by [^{14}C]CABP binding as in other experiments, and the concentration of binding sites calculated assuming 8 sites per holoenzyme.

RESULTS

Activity of RuBP Carboxylase *versus* pH. The effect of pH on the catalysis rate of carboxylation of RuBP at air level CO_2 and low O_2 was determined by activating carboxylase at saturating CO_2 and pH 8.2 and assaying activity at various pH values over a range of CO_2 concentrations near ambient (Fig. 1). Analysis of variance using the Michaelis-Menten equation showed an error of about 5% with no significant differences among different pH values over the range 8.2 to 8.8 (Fig 1).

Effect of pH on Mg^{2+} Requirement for Activation. The effect of Mg^{2+} on steady state activity of the carboxylase at air level CO_2 and 0.5 mM RuBP is shown for pH 8.4 and 8.8 in Figure 2. Over this range the concentration necessary to saturate the activation reaction with Mg^{2+} was constant at approximately 5 mM. Differences in the steady state activity with pH were not due to an interaction with Mg^{2+} requirement for activation.

Activation State *versus* pH. The equilibrium activity reached at air level CO_2 over the pH range from 7.8 to 9.2 is shown in Figure 3. Activity was assayed at a constant pH, but differences in activity reflect differences in the activation state reached at the preincubation pH. As reported by Lorimer *et al.* (10) the activation state of the carboxylase at air level CO_2 was strongly dependent on the pH of the medium. The carboxylase is only approximately 75% activated at air level of CO_2 and pH 8.2 (Fig 3). More important, at pH 8.6 and above the carboxylase was fully activated at this CO_2 concentration. These studies were, however, conducted in the absence of RuBP, and high concentrations of RuBP would occur in the chloroplast stroma (13).

Effect of RuBP on the Activation Equilibrium. In these exper-

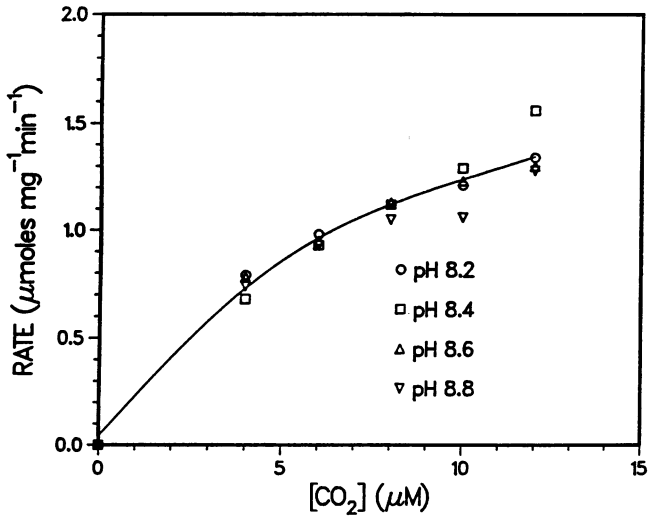

FIG. 1. Activity of fully activated RuBP carboxylase as a function of CO_2 concentration at different pH values. The complete data set was fit to a Michaelis-Menten function. The mean error (predicted rate − measured rate) at each pH was 0.014, 0.006, −0.009, and −0.032 at pH 8.2, 8.4, 8.6, and 8.8, respectively. For the preparation of spinach enzyme used in these studies K_m = 8.4 μM and V_{max} = 2.26 μmol (mg-min)$^{-1}$.

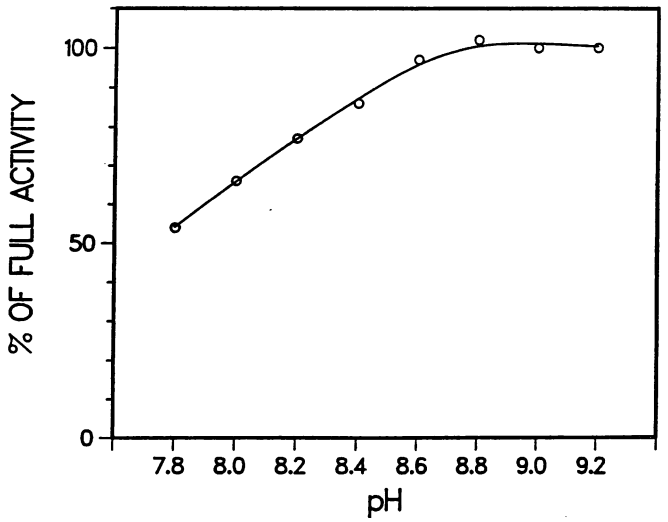

FIG. 3. Activation of RuBP carboxylase at 10 μM CO_2, 10 mM $MgCl_2$ as a function of pH. The inactive enzyme was activated at 10 μM CO_2, 10 mM $MgCl_2$ at each pH, and assayed at pH 8.2, 150 μM CO_2, 10 mM $MgCl_2$, and 0.5 mM RuBP. The data are presented as the percent of full activation (at saturating CO_2 and Mg^{2+}, pH 8.2) achieved at each pH.

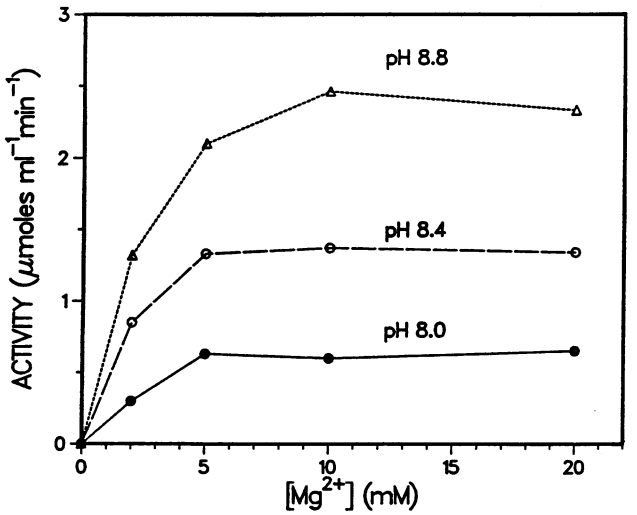

FIG. 2. Effect of pH on Mg^{2+} requirement for activation. Steady state activity of RuBP carboxylase in the presence at 10 μM CO_2, 0.5 mM RuBP as a function of Mg^{2+} concentration at different pH values. Experiments were performed by equilibrating the enzyme with 10 μM CO_2 and the indicated Mg^{2+} concentration at each pH, starting the reaction with RuBP, and assaying activity over time. The activity plotted is the steady state rate.

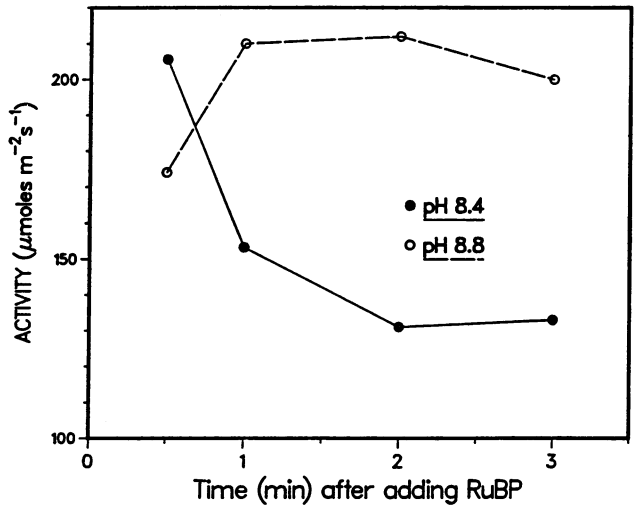

FIG. 4. Time course of the carboxylation reaction at air level CO_2 after adding RuBP at pH 8.4 and 8.8. The enzyme was equilibrated with 10 μM CO_2 and 10 mM Mg^{2+} at the indicated pH before the reaction was started with 0.5 mM RuBP.

iments we examined the effect of adding RuBP to carboxylase which was preactivated at air level CO_2. The time course of the carboxylation reaction was followed for several min after the addition of RuBP (Fig. 4). In these experiments the initial activity (over the first 30 s) reflects the equilibrium activation state reached at that pH during the preincubation. A new steady state activity was reached within 2 min, which presumably reflects a change in the number of activated enzyme molecules. At pH 8.4 initial activity over the first 30 s was greater than the steady state activity after 2 to 3 min. At pH 8.8 the initial activity was lower than the steady state activity. Similar experiments were done at 0.2 pH intervals between pH 7.8 and 9.0 (Fig. 5). At low pH values (<8.6) RuBP reduced the equilibrium activity of the preactivated enzyme substantially, but at pH values above 8.6 RuBP increased the equilibrium activity of the enzyme.

Activation Rate in the Presence of RuBP. The above studies of the steady state rate of carboxylation as a function of pH were all conducted by adding RuBP to previously activated enzyme. It is known, however, that addition of RuBP to deactivated enzyme dramatically slows the rate at which the enzyme activates (7, 9). We found that in the absence of RuBP, the carboxylase activated rapidly; typically so rapidly (reaching 70–90% of the equilibrium value in 1 min) that it was impossible to accurately determine the kinetics of activation at 25°C. However, in the presence of RuBP, activation kinetics were substantially slower (Fig. 6). At pH values below 8.6 the carboxylase activated at a rate of less than 3% of maximum activity per min. At pH values above 8.6 there was a marked increase in the rate of activation in the presence of RuBP.

Binding of RuBP to Inactive RuBP Carboxylase. Fluorescence of RuBP carboxylase-TNS complex was used to determine bind-

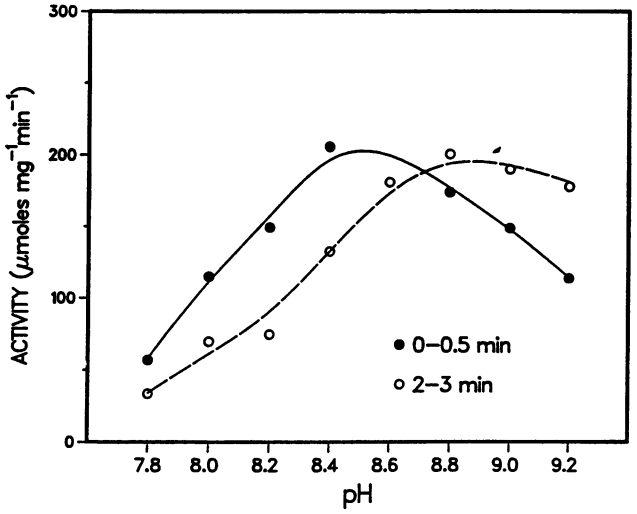

FIG. 5. Initial and steady state activity of RuBP carboxylase at physiological concentrations of CO_2 and RuBP as a function of pH. RuBP carboxylase was activated at 10 μM CO_2 at each pH for 5 min and the reaction started by adding RuBP to a concentration of 0.5 mM. The solid line indicates the activity during the first 0.5 min after adding RuBP, and the dashed line indicates the steady state rate achieved after 2 min.

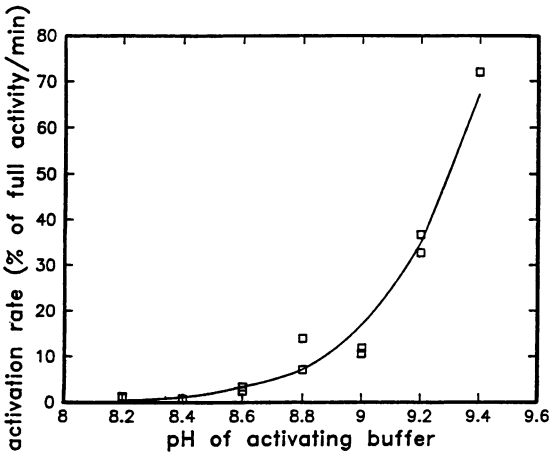

FIG. 6. Activation rate of RuBP carboxylase in the presence of 1 mM RuBP, 10 μM CO_2, and 10 mM $MgCl_2$ as a function of pH. Inactive enzyme was activated at 10 μM CO_2, 10 mM $MgCl_2$ in the presence of 1 mM RuBP at each pH. Activation state as a function of time was determined at each pH by assaying at pH 8.2, 150 μM CO_2, 10 mM $MgCl_2$, and 0.5 mM RuBP. The activation rate for the enzyme in the presence of RuBP was determined as a percentage of the maximum activity reached in the absence of RuBP at that pH.

ing of RuBP to the inactive form of RuBP carboxylase (8, 17). In the absence of RuBP carboxylase, the aqueous solution of TNS showed very little fluorescence at any wavelength with an excitation wavelength of 366 nm. Upon the addition of carboxylase a strong fluorescence peak appeared at 427 nm. The absolute fluorescence of the enzyme-TNS complex at 427 nm was independent of pH, and fluorescence at this wavelength decreased at all pH values with the addition of RuBP. Figure 7 shows the change in fluorescence (ΔF) of the RuBP carboxylase-TNS complex in the absence of CO_2 and Mg^{2+} as the solution was titrated with RuBP. The concentration of RuBP sites was determined by [^{14}C]CABP binding and is indicated on the figure. At low pH values fluorescence change was linear with RuBP concentration for RuBP concentrations below the concentration of binding sites on the carboxylase. Above the binding site

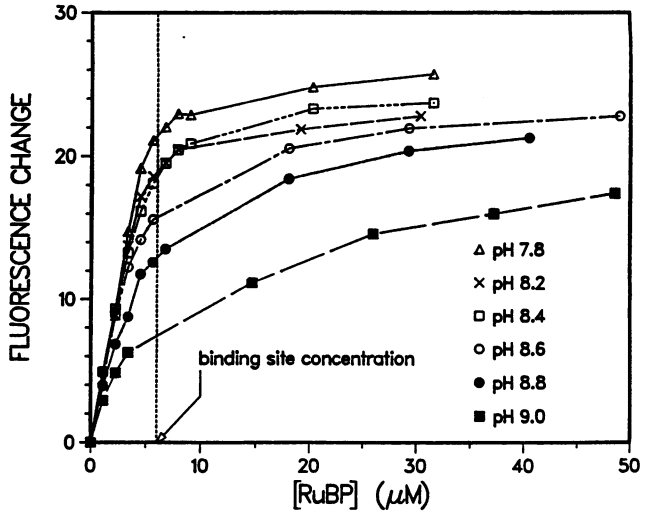

FIG. 7. Binding of RuBP to inactive RuBP carboxylase at different pH values as determined by enzyme-TNS fluorescence changes. TNS (0.1 mM) was incubated with inactive RuBP carboxylase with no CO_2 or Mg^{2+} present. Fluorescence was measured as the mixture was titrated with RuBP. The process was repeated as each pH. The concentration of binding sites for RuBP on the carboxylase is indicated.

concentration a slow rise in ΔF was observed. At higher pH values ΔF changed in more curvilinear fashion with added RuBP and significant changes in ΔF were observed well above the binding site concentration.

DISCUSSION

The effect of pH on the activity of RuBP carboxylase may be influenced by effects of pH on both catalysis and activation (see discussion in Ref. 10). Studies of Andrews *et al.* (1) resolved these effects and reported that the pH optimum for catalysis at rate-saturating CO_2 was near 8.2. In that study, however, catalysis declined only slightly at more alkaline pH, and it is possible that this decline was due to inhibition by the high ionic strength from $NaHCO_3$ required to maintain CO_2-saturation at high pH. We examined the pH profile of catalysis at CO_2 concentrations near air level (Fig. 1). At these low concentrations of CO_2 (and $NaHCO_3$), we could detect no decline in activity over the range pH 8.2 to 8.8. These results indicate that the catalytic activity at air level CO_2 is not substantially affected by changes in pH within this range. We also examined the Mg^{2+} requirement for activation as a function of pH at air level CO_2 and could find no effect of pH on the concentration of Mg^{2+} necessary to saturate the activation reaction. Effects of pH or other factors on the steady state activation level will, therefore, be the major determinant of activity.

In the absence of RuBP or any other effector, spinach carboxylase could be fully activated at air level CO_2, but only at pH values above 8.6. At lower pH values the activation state was strongly dependent on pH. This finding is in general agreement with the data of Lorimer *et al.* (10). However, in that study the equilibrium activation at air level CO_2 was not compared to that attained at saturating CO_2. Lorimer *et al.* (10) explained the effect of pH on activation of the carboxylase by proposing that activation involves the addition of CO_2 to an uncharged amino group whose pK_a is between 8 and 9. At more alkaline pH there is more of the uncharged form, so activation is favored at high pH. Recent data support this mechanism, and carbamate formation is thought to occur at the ϵ-amino group of lysine residue 201 (12). The marked dependence of equilibrium activation state at air level CO_2 on pH should not be confused with the rather

broad pH optimum around 8.2 for activation, observed at saturating CO_2 concentrations (15).

In accord with several other studies (7, 9), we report that RuBP is a strong inhibitor of the rate of activation of deactivated RuBP carboxylase. Activation rate was very slow at pH values below 8.6 in the presence of RuBP, but results presented here show that the rate of activation in the presence of RuBP is much faster above pH 8.6. Laing and Christeller (9) postulated that RuBP prevents activation by binding to inactive carboxylase. This was confirmed by Jordan and Chollet (7) who estimated the K_d for RuBP with inactive spinach carboxylase to be 0.021 μM at pH 8.0, and found that binding of RuBP to inactive enzyme prevented activation by excluding activator CO_2 from the catalytic sites. The pH dependency of activation rate in the presence of RuBP suggests that the binding of RuBP to the inactive enzyme is pH dependent. This possibility is discussed below.

It has often been observed that RuBP tends to inhibit the activity of RuBP carboxylase that has been activated in the absence of RuBP. This decline in activity shows up as a decline in reaction rate over the first 2 to 3 min of the reaction if the reaction is started by the addition of RuBP (9). At air level CO_2 we found that a significant decline in activity (20%) occurred over the first 2 to 3 min after RuBP was added to activated enzyme at pH values below 8.6 (Fig. 3). At pH values above 8.6, however, RuBP apparently improved activity. Laing and Christeller (9) postulated that the observed decline in activity was due to deactivation of RuBP carboxylase by RuBP and was caused by the formation of a complex of RuBP with the inactive carboxylase that is stronger than the catalytic complex of RuBP with the active carboxylase. If this is the case, then the positive effect of RuBP on activation at pH values greater than 8.6 could be due to an effect of pH on the relative binding affinities such that binding of RuBP to the inactive form is not favored at pH values higher than 8.6.

The pH dependency of binding of RuBP to inactive RuBP carboxylase was tested using the fluorescence of TNS-enzyme complex. TNS is known to fluoresce in the presence of certain organic solvents and proteins, and its fluorescence in the presence of proteins is directly related to the hydrophobicity of the protein (11). Because of this, it can be used to study conformational changes of enzymes which involve a change in the orientation of hydrophobic regions. It has been used several times to study the binding of RuBP to RuBP carboxylase (8, 17). Fluorescence of the enzyme-TNS complex at 427 nm was found to drop as the solution was titrated with RuBP in the absence of CO_2 and Mg^{2+} at various pH values. Since this change in fluorescence was presumably due to binding of RuBP to the inactive carboxylase a plot of fluorescence change as a function of RuBP yields a binding curve for RuBP to the inactive carboxylase.

The binding curve for RuBP to inactive RuBP carboxylase at pH 7.8 (Fig. 7) is typical of systems in which the binding constant is lower than the concentration of binding sites. In these cases very little of the ligand is in the free form if total ligand concentration is below binding site concentration. Therefore, additions of ligand yield virtually equal increases in enzyme-ligand complex until all binding sites are occupied. As pH is increased the binding curves indicate rising K_d until at pH 9.0 the curve resembles more typical binding data in which k_d exceeds the concentration of binding sites (6 μM in these experiments). The slow rise in fluorescence change observed at pH 7.8 for increases in RuBP concentration above the binding site concentration indicates either some direct effect of RuBP on fluorescence or an additional low affinity binding of RuBP to the enzyme. In either case it prevents accurate estimates of binding constants from these data. However, the largest apparent changes in K_d appear to occur above pH 8.6 in accordance with the response of activation rate and activation state to RuBP. These data support the hypothesis that RuBP binds tightly to the inactive form of RuBP carboxylase at low pH values, and show that this binding is less tight at higher pH values.

This strong effect of pH on the binding of RuBP to the inactive enzyme can be used to explain several of the observations reported here. Binding of RuBP to inactive RuBP carboxylase should prevent activation of the enzyme. This effect is very pronounced at pH values less than 8.6, and declines at pH values greater than 8.6. This is consistent with a change in binding affinity at this pH. Similarly, RuBP acts as a negative effector for preactivated carboxylase at low pH values. This could be due to preferential binding of RuBP to the inactive form of the carboxylase, shifting the equilibrium away from the activated form. At higher pH values the binding affinity of RuBP to the inactive form is reduced and this effect should diminish. In fact, RuBP appears to act as a positive effector at pH values greater than 8.6. This could occur if binding of RuBP to the activated enzyme is stronger than binding of RuBP to the inactivated enzyme. This concept of differential binding of effectors to the activated and inactivated enzyme was eloquently developed by Badger and Lorimer (2) who studied the effects of other phosphorylated metabolites on the activation state of RuBP carboxylase.

The mechanism behind the observed change in K_d for RuBP binding to inactive carboxylase is unclear. We speculate that the binding of the negatively charged RuBP to inactive carboxylase may involve the protonated (positively charged) form of a lysine residue; possibly, but not necessarily the lysine residue at which carbamate formation occurs during activation. At pH values below the pK_a of this group the protonated form would predominate, leading to a lower equlibrium activation level and to tight binding of RuBP to the inactive enzyme. Above the pK_a this group would be deprotonated. This would tend to favor the activation reaction and at the same time lower the affinity of the inactive enzyme for RuBP. The pK_a of free lysine is about 10, but it is well known that the local environment of the proton can alter the pK_a of lysine residues on proteins. In our assay system this occurred near pH 8.6. However, an effector or the high protein concentration of the stroma could effect the apparent pK_a of this group and the critical pH range *in vivo*.

Activation *in Vivo*. The data presented here lead us to suggest that several important problems concerning the activation and activity of RuBP carboxylase *in vivo* could be explained by postulating that the stromal pH may be sufficiently alkaline to deprotonate the active lysine residue discussed above. First, consider the problem of fully activating RuBP carboxylase at normal air level CO_2. Although high activities can be achieved at low pH values with high CO_2 concentrations, these high activities cannot be maintained at physiological concentrations of CO_2 and RuBP. Figure 5 shows the steady state activity achieved at physiological concentrations of RuBP and CO_2, and it is clear that high activities were reached at pH values above 8.6. Second, consider the problem of activation in the presence of RuBP. The data shown in Figure 6 confirm that this reaction can be prohibitively slow at pH values below 8.4. Our studies indicate that high activation rates in the presence of RuBP can be achieved at pH values above 8.8.

It should be noted that the detailed response of RuBP carboxylase to pH may differ among species. Different carboxylases may well have different pK_a values for the ϵ-amino group of the active lysine and would therefore exhibit the effects of pH on activity at different pH values. In support of this, Jordan and Chollet (7) found that RuBP did not inhibit activation of RuBP carboxylase from *Rhodospirillum rubrum*. We speculate that the effect of pH on the binding of RuBP to both the activated and inactivated forms of this enzyme may play a key role in the mechanism regulating its apparent V_{max} *in vivo*.

pH control of activation state *in vivo* is an attractive possibility because it provides a plausible feedback system to regulate acti-

vation state in parallel with RuBP regeneration rate. Under the appropriate conditions light intensity may be increased such that the potential to transport electrons temporarily exceeds the rate at which they can be used to produce ATP because of restriction on the turnover of ADP imposed by the rate of carboxylation. Under these conditions stromal pH will increase, activating the carboxylase (if it is not already fully active), and relieving the limitation of carboxylase activity for photosynthesis. On the other hand, if the RuBP saturated rate of carboxylation were to temporarily exceed the electron transport determined rate of RuBP regeneration, then carboxylation would consume RuBP, ATP and NADPH, and tend to collapse the pH gradient across the thylakoid membranes. This should cause the stroma to become more acid, resulting in deactivation of carboxylase. Feedback would occur because as the enzyme deactivated or activated in response to a pH change it would tend to counteract the change in pH. Therefore, the system should tend to adjust stromal pH and carboxylase activity such that carboxylation capacity and regeneration capacity are equal at steady state. As a result, there would be only small changes in the pool size of RuBP and, perhaps more importantly, in Pi with changes in light intensity. The concentration of Pi appears to play an important role in the regulation of processes such as starch and sucrose synthesis, and wide variations in the concentration of Pi could have far reaching effects.

Stromal pH of illuminated isolation chloroplasts has been measured to be approximately 8.2 (6); well below the value necessary to explain activation *in vivo*. This discrepancy is as yet unexplained, but it is possible that the stromal pH of chloroplasts in an intact leaf is somewhat higher than that of isolated chloroplasts. Alternatively, the high protein concentrations or an effector may shift the pK_a of the enzyme group(s) involved in RuBP binding and activation. Regardless of the exact mechanism it is clear from the data that pH could play an important role in the regulation of RuBP carboxylase activity.

SUMMARY

1. Catalysis of RuBP carboxylation by active RuBP carboxylase is independent of pH over the range 8.2 to 8.8 at air level CO_2.

2. The equilibrium activation state of RuBP carboxylase at air level CO_2 increases with increasing pH up to approximately 8.6. At pH values above 8.6 RuBP carboxylase activation state at air level CO_2 is equal to that achieved with saturating CO_2 at pH 8.2.

3. RuBP is a negative effector for activation state at pH values below 8.6 and a positive effector at pH values above 8.6.

4. The net effect of statements 1, 2, and 3 is that the activity of RuBP carboxylase at physiological substrate concentrations is strongly dependent on pH at pH values below 8.6 and is maximal at pH values above 8.6.

5. RuBP carboxylase does not activate at a significant rate in the presence of RuBP at low pH values. At pH values above 8.6, however, activation rate in the presence of RuBP increases dramatically with increasing pH.

6. The pH dependency of the effect of RuBP on activation rate and on activation state itself is due to the pH dependency of RuBP binding to the inactive form of RuBP carboxylase.

7. It is proposed that a stromal pH of 8.6 would be sufficient to attain full activation of spinach RuBP carboxylase at physiological substrate concentrations and that feedback of RuBP carboxylase activity on stromal pH would provide regulation of RuBP carboxylase activation state over a wide range of steady state activation levels.

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