pH Dependence of the Km(\text{CO}_2) of Ribulose 1,5-Diphosphate Carboxylase

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GEORGE BOWES
Department of Botany, University of Florida, Gainesville, Florida 32611

WILLIAM L. OGREN
United States Regional Soybean Laboratory, Agricultural Research Service, United States Department of Agriculture, Urbana, Illinois 61801

RICHARD H. HAGEMAN
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

ABSTRACT

The Km(\text{CO}_2) values of ribulose 1,5-diphosphate carboxylase in freshly ruptured spinach (Spinacia oleracea L.) chloroplasts and in the purified form isolated from spinach leaves were found to be pH dependent. Raising the pH of the assay solution produced a substantial decrease in the Km(\text{CO}_2) of both enzyme systems. In freshly ruptured chloroplasts at pH 7.2 the Km(\text{CO}_2) was 25 \, \mu\text{M}, at pH 8 it decreased to 19 \, \mu\text{M}, and at pH 8.8 a further decrease to 7 \, \mu\text{M} was found. With the purified enzyme at pH 7.2 the Km(\text{CO}_2) was 147 \, \mu\text{M}, while the corresponding Km values for pH 8 and 8.8 were 34 and 15 \, \mu\text{M} CO_2, respectively. The latter figure approximates the physiological Km(\text{CO}_2) of 10 \, \mu\text{M} estimated for photosynthesizing leaves and intact chloroplasts. The maximum velocity for both enzyme systems at optimum substrate levels was at pH 8, but the highest calculated rate of CO_2 uptake at atmospheric CO_2 levels occurred at pH 8.8. These results support the proposal that the light-induced efflux of protons out of the chloroplast stroma may be a major factor involved with the reported in vivo light activation of ribulose 1,5-diphosphate carboxylase.

Ribulose 1,5-diP carboxylase (EC 4.1.1.39) is a bifunctional chloroplast enzyme which catalyzes both the primary carboxylation reaction of C₅ photosynthesis (15) and the primary oxygenation reaction of photorespiration (4, 5). In view of the central role of RuDP³ carboxylase in photosynthesis and photorespiration, it might be expected that its activity would be subject to various regulatory factors. Light seems to be one of the major factors affecting the in vivo carboxylation activity of RuDP carboxylase. Long term (6) and very rapid (15) stimulatory effects of light upon the in vivo activity of this enzyme have been reported. The mechanism mediating the rapid light stimulation has been the subject of speculation, with both direct enzyme activation and indirect mechanisms being postulated to account for this rapid in vivo response (15).

Within the chloroplast, an event that occurs rapidly in response to irradiation is the efflux of protons out of the stromal region and into the thylakoids (18). This proton movement probably causes a pH change in the stromal region of the chloroplast (18), which is the region where RuDP carboxylase seems to be located (19). Several workers have shown that pH changes can affect the activity of isolated RuDP carboxylase (3, 13, 14), although in these studies high Km(HCO₃⁻) forms of the enzyme were used. A recent report indicated that, in freshly ruptured chloroplasts, RuDP carboxylase can exist in a transitory, low-Km(HCO₃⁻) form (2). The kinetic properties of this form seem to resemble most closely the in vivo carboxylation system. In view of the stromal pH changes that probably occur upon chloroplast irradiation, we compared the effects of three H⁺ ion concentrations upon the kinetic properties of RuDP carboxylase in freshly ruptured chloroplasts and in the purified form.

MATERIALS AND METHODS

Chloroplast Extraction. Intact chloroplasts, 50 to 70% Class I as determined by phase-contrast microscopy, were isolated from spinach (Spinacia oleracea L., var. Bloomsdale) grown in vermiculite and Hoagland's solution under a 12 hr, 22 C day, at 1000 \mu\text{einsteins/m}^2\text{sec} (400–700 nm) and an 18 C night. Fourteen grams of the ribbed leaves were sliced with razor blades in 50 ml of ice-cold extraction buffer identical to buffer A of Jensen and Bassham (11), except at pH 6.5. The resultant suspension was filtered through eight layers of Miracloth and a 20 \mu\text{m} mesh nylon cloth, centrifuged at 800g for 90 sec, and the pellet was resuspended in 20 ml of extraction buffer. After further centrifugation the washed pellet was resuspended in 5 ml of extraction buffer and was used in measurements of ^14\text{CO}_2 incorporation. The photosynthetic rate of the intact chloroplasts was 45 \mu\text{mole} of CO₂/mg of Chl/hr at saturating bicarbonate concentrations. Chl concentration was determined by the method of Arnon (1).

^14\text{CO}_2 Fixation by Ruptured Chloroplasts. Chloroplasts were ruptured and assayed for RuDP carboxylase activity by injection into a hypotonie assay solution. The assay solution contained in 1 ml: 25 mM tris-HCl buffer (prepared and stored CO₂-free) at pH 7.2, 8, or 8.8; 10 mM MgCl₂; 0.1 mM EDTA; 5 mM DTT; 0.4 mM RuDP; and NaH¹⁴CO₃ (0.25 \mu\text{Ci}/µ\text{mole}) at concentrations ranging from 0.4 to 20 mM, prepared with CO₂-free dis-
injected into carboxylase method was enzyme species, approximately (C02-free) assay the a cultured equilibrium of described for chromatography step removed with N2 gas to remove dissolved CO2 and O2. The assay flasks were then sealed and the remaining components were injected through a serum cap. The chloroplast suspension, containing approximately 30 µg of Chl, was used to initiate the reaction in the dark at 25 C. All assays were performed under N2 with the flasks being gently shaken. After 1 min, 0.1 ml of 3 N HCl was added to stop the reaction, aliquots were placed in scintillation vials, dried at 90 C, and the radioactivity was determined by scintillation spectroscopy. For time course studies, 0.1-ml aliquots were removed at 30-sec intervals from 2 ml of assay solution and injected into 0.1 ml of 3 N HCl and 0.3 ml methanol. The ruptured chloroplasts, with RuDP and NaH14CO3 as substrates, incorporated 80 µmoles of CO2/mg of Chl-hr in the dark.

**Purification and Assay of RuDP Carboxylase.** RuDP carboxylase was purified from spinach leaves using the procedure described previously (4), but with the final hydroxylapatite chromatography step omitted. Before the assay, the precipitated enzyme was dissolved in 50 mM tris-HCl, pH 8; 0.1 mM EDTA; 10 mM MgCl2; 5 mM DTT; and was held in ice for 4 hr to attain maximum activity (4). The assay procedure was similar to that described for the ruptured chloroplasts except 50 mM tris-HCl buffer (CO2-free) was used, and the reaction was initiated with approximately 25 µg of protein and stopped after 3 min with 0.1 ml of 3 N HCl. Protein concentration was determined by the method of Warburg and Christian (16). At pH 8 and 25 C, the specific activity of purified RuDP carboxylase was 1.1 µmoles of CO2/mg of protein-min.

As used in this paper, HCO3- refers to the total carbonate species, of which HCO3- ions predominate at alkaline pH values. Free CO2, as opposed to CO32-, is the substrate used by RuDP carboxylase (9) and Km(CO2) values were calculated on the basis of published equilibrium constants (10).

**RESULTS**

**Carbon Dioxide Fixation by Ruptured Chloroplasts.** Bahr and Jensen (2) reported that the Km(HCO3-) for RuDP carboxylase in freshly ruptured spinach chloroplasts was approximately 0.8 mM at pH 7.8, and that this value increased after about 2 min incubation in the hypotonic reaction mixture. At pH 8 we observed similar biphasic kinetics for RuDP carboxylase activity in freshly ruptured chloroplasts incubated in the dark with RuDP (Fig. 1). At HCO3- concentrations below 20 mM, the initial carboxylation rate was constant for 3 to 4 min and then declined to a lower, constant rate. Calculations of the Km (HCO3-) at the initial and later rate indicated that the apparent Km increased from an initial value of 1.1 mM to about 3 mM HCO3-. This confirms the suggestion that the kinetic properties of RuDP carboxylase in ruptured chloroplasts change with time in a discrete step, although the difference we observed is not as great as that reported by Bahr and Jensen (2).

The effect of pH on the apparent Km(HCO3-) for RuDP carboxylase in the freshly ruptured chloroplasts was also determined (Fig. 2). An assay period of 1 min was used in order to examine the initial form of the enzyme. As the pH increased from 7.2 to 8.8, the apparent Km expressed as HCO3-, showed an 11-fold increase, from 0.3 to 3.3 mM (Table I). When the Km was expressed as CO2 concentration, its value decreased from 25 µM at pH 7.2 to 7 µM at pH 8.8 (Table I). The Vmax for enzyme activity was highest at pH 8, but the calculated rate of CO2 fixation at atmospheric CO2 concentration (10 µM) was highest at pH 8.8 (Table I).

**Carbon Dioxide Fixation by Purified RuDP Carboxylase.** The apparent Km(HCO3-) of purified RuDP carboxylase was also found to be pH dependent. The Km(HCO3-) was determined over a 3-min assay period with saturating levels of Mg2+ and RuDP at three H+ ion concentrations (Fig. 3). As with ruptured chloroplasts, the apparent Km, on a HCO3- basis, increased as the pH was raised from 7.2 to 8.8, although the increase was only about 4-fold (Table II). However, the Km(CO2) decreased over this pH range, from 147 µM CO2 at pH 7.2 to 15 µM CO2.
FIG. 2. Double reciprocal plot of rate versus bicarbonate concentration for RuDP carboxylase in freshly ruptured chloroplasts at pH 7.2, 8, and 8.8.

Table I. Effect of pH on $K_m$ ($HCO_3^-$ and $CO_2$), $V_{max}$, and Calculated $CO_2$ Fixation Velocity ($v$) at Atmospheric $CO_2$

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ (mM $HCO_3^-$)</th>
<th>$V_{max}$ (µmoles $CO_2$/mg Chl/hr)</th>
<th>$v$ (µmoles $CO_2$/mg Chl-hr)</th>
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<tr>
<td>7.2</td>
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<td>6</td>
</tr>
<tr>
<td>8.0</td>
<td>1.3</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>8.8</td>
<td>3.3</td>
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<td>50</td>
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</tbody>
</table>

1 Values for $v$ were calculated from the standard enzyme equation $v = VS/(K_m + S)$, where $S = 10$ µM $CO_2$.

FIG. 3. Double reciprocal plot of rate versus bicarbonate concentration for purified RuDP carboxylase at pH 7.2, 8, and 8.8.

Table II. Effect of pH on $K_m$ ($HCO_3^-$ and $CO_2$), $V_{max}$, and Calculated $CO_2$ Fixation Velocity ($v$) at Atmospheric $CO_2$

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_m$ (mM $HCO_3^-$)</th>
<th>$V_{max}$ (µmoles $CO_2$/mg protein-min)</th>
<th>$v$ (µmoles $CO_2$/mg protein-min)</th>
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<tr>
<td>7.2</td>
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<td>8.0</td>
<td>2.3</td>
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<td>1.05</td>
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<tr>
<td>8.8</td>
<td>6.7</td>
<td>15</td>
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</tbody>
</table>

1 Values for $v$ were calculated from the standard enzyme equation $v = VS/(K_m + S)$, where $S = 10$ µM $CO_2$. 

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at pH 8.8. As with the ruptured chloroplast system, the highest measured \( V_{\text{max}} \) occurred at pH 8, but the highest rate of CO\(_2\) fixation calculated for atmospheric CO\(_2\) concentration occurred at pH 8.8 (Table II).

**DISCUSSION**

Weissbach *et al.* (17), studying purified spinach RuDP carboxylase with a Km(HCO\(_3^-\)) of 11 mm, found maximum activity at pH 8. Basham *et al.* (3) reported that the pH optimum could be shifted to 8.6 by reducing the Mg\(^{2+}\) concentration. However, these assays were conducted at a pH below the Km of the enzyme, which probably resulted in severe substrate-limited reactions. At limiting Mg\(^{2+}\) levels, increasing the pH to 8.8 has been shown to decrease the Km(HCO\(_3^-\)) from 29 to 13.4 mm (14). Similarly, the Km(CO\(_2\)) of a crude, G-25 Sephadex-treated, chloroplast extract also decreased as the pH of the reaction was raised to 9 (13). Ribose-5-P and ATP were used as substrates, in place of RuDP, and it was postulated that increasing the pH activated a CO\(_2\)-fixing enzyme complex (13). The results presented here indicate that RuDP carboxylase itself is capable of pH-dependent variations in Km(CO\(_2\)); thus, it would seem unnecessary to propose that an enzyme complex is involved.

Because the pH effect upon the Km(CO\(_2\)) of RuDP carboxylase has been demonstrated with a crude chloroplast extract (13), with freshly ruptured chloroplasts, and with the purified enzyme, it appears likely that the phenomenon has physiological significance. The light-induced efflux of protons from the chloroplast stroma could be envisaged as resulting in a rapid decrease in the Km(CO\(_2\)) of RuDP carboxylase. Such a system could be involved in the rapid, light activation of RuDP carboxylase, if, as has been suggested (18), high pH values can be achieved in vivo within the chloroplast stroma. Several other factors, including levels of HCO\(_3^-\) (8, 12), Mg\(^{2+}\), and sugar phosphates (7, 8, 15), have been shown to influence the activity of isolated RuDP carboxylase, and have been implicated in the light-activation phenomenon (15). Some, or all, of these compounds may have physiologically significant roles in the *in vivo* regulation of RuDP carboxylase.

The apparent differences in the Km(CO\(_2\)) of RuDP carboxylase when determined *in vitro* and *in vivo* have been attributed to an increase in the Km(CO\(_2\)) upon removal of the enzyme from the chloroplast environment (2). The results in Figure 1 are in accord with this hypothesis. At high pH, little difference exists between the Km(CO\(_2\)) for the purified enzyme and the enzyme in freshly ruptured chloroplasts (Tables I and II). At low HCO\(_3^-\) levels, the activity of purified soybean RuDP carboxylase declined continuously during assay (12). The apparent Km(CO\(_2\)) after a 3-min assay period, as used in the present study, was about twice the Km(CO\(_2\)) calculated for zero time (12). When this time-related increase in the apparent Km(CO\(_2\)) of the purified enzyme is taken into account, then the data in Tables I and II for pH 8.8 indicate that the purified enzyme has a very similar Km(CO\(_2\)) to the enzyme within the ruptured chloroplasts. As the apparent Km(CO\(_2\)) for intact leaf photosynthesis and intact chloroplast CO\(_2\) fixation is about 10 \(\mu\)M (11, 15), the data in Tables I and II indicate that at high pH little difference exists in Km(CO\(_2\)) between the intact system and either the ruptured chloroplasts or the purified enzyme.

**LITERATURE CITED**