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Ribulose Diphosphate Carboxylase from Freshly Ruptured Spinach Chloroplasts Having an *in Vivo* Km[CO_2][1]

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ABSTRACT

The properties of a form of ribulose diphosphate carboxylase having a high affinity for CO_2 have been studied. Its apparent Km(HCO_3^-) of 0.5 to 0.8 mM (pH 7.8) and calculated Km(CO_2) of 11 to 18 μM are comparable to the values exhibited by intact chloroplasts during photosynthesis. This form of the enzyme was released from chloroplasts in hypotonic media and was unstable, rapidly converting to a form having a high Km(HCO_3^-) of 20 to 25 mM similar to that for the purified enzyme. Incubation of the enzyme with $MgCl_2$ and HCO_3^- yielded a third form with an intermediate Km(HCO_3^-) of 2.5 to 3.0 mM.

The low Km form had sufficient activity both at air levels of CO_2 and at saturating CO_2 to account for the rates of photosynthesis by intact chloroplasts. The low Km form could be stabilized in the presence of ribose 5-phosphate, adenosine triphosphate, and $MgCl_2$, at low temperatures for up to 2 hours.

The isolation and purification of ribulose 1,5-diphosphate carboxylase (EC 4.1.1.39) was first reported by Weissbach, Horecker, and Hurwitz (20). More recently Paulsen and Lane (13) described a preparation of RuDP[2] carboxylase that was homogeneous by ultracentrifugation criteria. The majority of studies of the enzyme, including those on control of photosynthetic CO_2 fixation, have used similar preparations (1–3, 11–15, 17, 20). The Km(HCO_3^-) at 7.7 to 7.8 for the purified enzyme is 22 mM (13). However, for light-driven CO_2 fixation by intact spinach chloroplasts the apparent Km(HCO_3^-) at pH 7.6 is 0.6 mM (9). It is clear that the properties of the isolated carboxylase cannot be simply extended to the *in vivo* situation. Because of this discrepancy in the Km(HCO_3^-) values, a suggestion has been made that a CO_2-concentrating mechanism might exist in the chloroplast outer membrane (7).

Upon examination of the carboxylase activity released by intact spinach chloroplasts placed in hypotonic buffer in the dark, Jensen (8) observed that the apparent Km(HCO_3^-) was 0.6 mM (pH 7.8). This result suggested that the *in vivo* properties of the carboxylase could be investigated upon breakage of chloroplasts in hypotonic media.

This paper reports some of the kinetic properties of RuDP carboxylase as observed upon lysis of chloroplasts. The carboxylase was released in a form having a low Km(CO_2), similar to that observed *in vivo*, which was unstable and converted to a high Km(CO_2) form, typical of the purified enzyme. By proper incubation conditions, a third intermediate Km(CO_2) form was also identified.

METHODS

Chloroplasts were prepared by the method of Jensen and Bassham as modified by Jensen (8) from 5- to 6-week old spinach leaves (*Spinacia oleracea* L., var. Viroflay) grown in an environmental chamber. The chloroplasts were about 70% class I by phase contrast microscopy (16) and capable of light-driven fixation at rates up to 100 μmoles (mg Chl·hr)$^{-1}$.

Light-driven fixation was measured by adding chloroplasts to isotonic medium containing 0.33 M sorbitol, 50 mM HEPES, pH 7.8 with NaOH, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 2 mM sodium isoascorbate, 5 mM sodium pyrophosphate, and $NaH^{14}CO_3$ (5.5 μc/μmole) as indicated. Light intensity was 2000 ft-c, temperature, 25 C.

RuDP carboxylase activity was measured in the dark upon lysis of intact chloroplasts in hypotonic medium containing HEPES buffer, pH 7.8 with NaOH, 5 mM DTE, 25 mM $MgCl_2$, RuDP or R5P plus ATP, and $NaH^{14}CO_3$ (5.5 μc/μmole) at 25 C. The concentrations of the components varied with the experiment and are indicated in the appropriate figure legends. Within the range used, there was no effect of chloroplast concentration on the Km or stability properties of the carboxylase. DTE, required for maximal activity of phosphoribulokinase, had no effect on the activity of RuDP carboxylase. It was included routinely in these experiments to permit direct comparison between RuDP and R5P plus ATP as substrates. In both light and dark fixation experiments the media were bubbled with CO_2-free N_2 in 2-ml serum-capped plastic beakers prior to addition of $NaH^{14}CO_3$. Aliquots (25–100 μl) were removed at specified intervals, added to two volumes of formic acid, and dried on a planchet; and the acid-stable ^{14}C was determined by a planchet counter. Chlorophyll was assayed by the method of Vernon (19).

RuDP (Sigma Chemical Co.) was assayed by allowing complete conversion to ^{14}C-glycerate-3-P with excess $NaH^{14}CO_3$ and spinach chloroplasts in hypotonic buffer. Identical results were obtained using purified RuDP carboxylase in place of spinach chloroplasts.

Cooper *et al.* (3) have shown that the true substrate for RuDP carboxylase is CO_2, not HCO_3^-. In this paper we use "HCO_3^-" to refer to the total carbonate species added to the reaction vessel, of which HCO_3^- is the predominant form at pH 7.8. The concentration of CO_2 has been calculated on the assumption of equilibrium between CO_2 and HCO_3^-, and using the known liquid and gas volumes of the reaction vessels and the constants given by Umbreit *et al.* (18).

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[2] Abbreviations: RuDP: ribulose 1,5-diphosphate; R5P: ribose 5-phosphate; DTE: dithioerythritol.

RESULTS

CO_2 Fixation after Lysis of Chloroplasts. Intact chloroplasts, although capable of light-dependent CO_2 fixation, fix CO_2 at extremely low rates in the dark when supplied with RuDP, $MgCl_2$, and HCO_3^- (8, 10). However, upon lysis of chloroplasts in a hypotonic medium containing $MgCl_2$, RuDP, and HCO_3^-, CO_2 was incorporated without lag at rates greater than 50 μmoles(mg Chl·hr)$^{-1}$ in the dark. The major product was D-glycerate-3-P (>95%) with small amounts of D-glycerate-2-P, P-enolpyruvate, and aspartate (8). Without addition of RuDP or other Calvin cycle intermediates to the hypotonic medium, the lysed chloroplasts fixed CO_2 at a rate of less than 1.0 μmole(mg Chl·hr)$^{-1}$ up to a total amount of 0.02 to 0.04 μmole(mg Chl)$^{-1}$. Phosphoenolpyruvate carboxylase activity in these chloroplast preparations was less than 0.7 μmole(mg Chl·hr)$^{-1}$ with 2.5 mM P-enolpyruvate.

Low $Km(CO_2)$ Form of the Carboxylase. The $Km(HCO_3^-)$ of the carboxylase was determined upon lysis of the chloroplasts in a hypotonic medium containing $MgCl_2$, RuDP (both at rate-saturating concentrations), and varying HCO_3^- concentrations. The apparent $Km(HCO_3^-)$ was 0.59 mM (Fig. 1), and the calculated $Km(CO_2)$ was 13 μM, values comparable to those observed during light-dependent CO_2 fixation (5, 9). A Hill plot of the kinetic data (inset, Fig. 1) gives a slope of 0.99, suggesting noncooperativity between CO_2 binding sites in contrast to some previous reports for the purified carboxylase (12, 17). The values of $Km(HCO_3^-)$ from many chloroplast preparations in hypotonic media have ranged between 0.5 and 0.8 mM (pH 7.8).

The low $Km(CO_2)$ form of RuDP carboxylase did not require chlorophyll-containing plastid membranes for activity. After removal of the membranes by centrifugation, the supernatant from lysed chloroplasts contained greater than 80% of the low Km activity. Alternatively, low $Km(CO_2)$ carboxylase was obtained by filtration of intact chloroplasts through two 25-mm glass fiber filters, Gelman type A, and a 0.4-μm Nucleopore membrane filter at 0 to 4 C. This filtering technique allowed for rapid removal of plastid membranes, leaving a colorless filtrate containing the unstable low $Km(CO_2)$ enzyme.

The low $Km(CO_2)$ form of RuDP carboxylase also exhibited a high affinity for RuDP. The apparent Km(RuDP) was 0.03

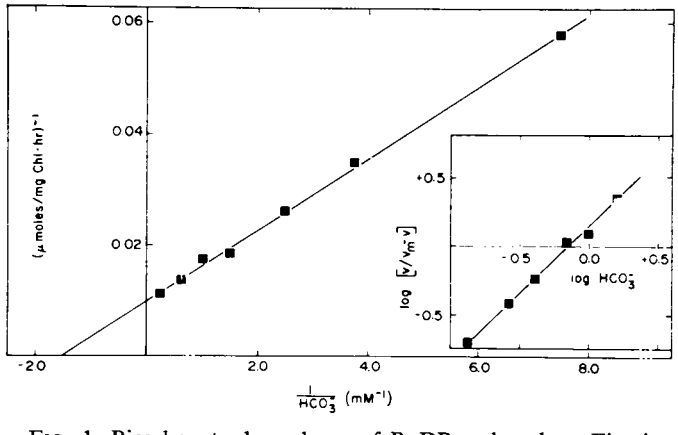

FIG. 1. Bicarbonate dependence of RuDP carboxylase. Fixation was initiated by addition of chloroplasts (15.3 μg Chl) to hypotonic medium containing 25 mM HEPES, pH 7.8, 25 mM $MgCl_2$, 5 mM DTE, 0.20 mM RuDP and $NaHCO_3$ in a total volume of 0.60 ml at 25 C in the dark. Samples were removed by a syringe for counting at 15, 30, and 45 sec after addition of chloroplasts. Linear rates of fixation were observed during this period. The maximal velocity was 105 μmoles (mg Chl·hr)$^{-1}$. The inset represents a Hill plot of the same data.

Table I. *Effect of Carbonic Anhydrase on CO_2 Fixation in the Dark*

Chloroplasts (9.0 μg Chl) were added to hypotonic assay medium containing 25 mM HEPES, pH 7.8; 25 mM $MgCl_2$; 5.0 mM DTE; 0.3 mM RuDP; and HCO_3^- as indicated in 0.30 ml. Carbonic anhydrase (1000 units, bovine erythrocyte, Sigma Chem.) was present where indicated. Fixation was assayed as in Figure 1.

HCO_3^- Concn	Rate of Fixation	
	Control	Plus carbonic anhydrase
mM	μmoles (mg Chl·hr)$^{-1}$	
0.4	38	38
0.8	56	58
1.6	83	77
6.4	96	96

mM, which is considerably lower than the 0.12 mM observed by Paulsen and Lane (13) with purified carboxylase.

Role of Carbonic Anhydrase. Given the amount of chloroplasts and reaction volumes used in these experiments, the rate of incorporation of CO_2 into products was as high as 0.1 mmole min^{-1}. The maximal rate of the uncatalyzed conversion of HCO_3^- to CO_2 can be calculated from the rate constants of Gibbons and Edsall (6). At pH 7.8, 0.8 mM HCO_3^-, and 25 C, this maximal rate is only 52 μmoles min^{-1}. As CO_2 and not HCO_3^- is the substrate, the formation of CO_2 would be expected to limit the observed rate, especially at low HCO_3^- concentrations, in the absence of carbonic anhydrase.

Everson (4) has reported substantial activities of carbonic anhydrase in spinach chloroplast preparations. Indicative of this, we noticed that the fixation rate at 0.8 mM HCO_3^- increased linearly with amount of chloroplasts added, to more than several times the maximal rate of the uncatalyzed conversion of HCO_3^- to CO_2. Furthermore, no increase in rate was observed at several HCO_3^- concentrations when excess purified carbonic anhydrase was added to the fixation assay (Table I). The conversion of HCO_3^- to CO_2 was not rate-limiting in these carboxylase assays.

Biphasic Kinetics of CO_2 Fixation. A biphasic pattern was observed in the kinetics of CO_2 fixation by chloroplasts following lysis in a hypotonic assay medium. The initial phase, on which the results of Figure 1 are based, lasted about 2 min and was followed by a slower second phase (Fig. 2). The reduced rate during the second phase was not due to the buildup of an inhibitory product or to exhaustion of substrates, since the addition of a fresh amount of chloroplasts to suspension already in the second phase gave a burst of fixation characteristic of the faster initial phase.

Significantly, the biphasic character of the kinetics was less evident if the assay was done at higher bicarbonate concentrations (Fig. 2, top curve). These biphasic kinetics resulted from a transition of the carboxylase from the low $Km(CO_2)$ form to a much higher $Km(CO_2)$ form. Analysis of the HCO_3^- dependence of Figure 2, plus other HCO_3^- concentrations, showed that during the initial phase the $Km(HCO_3^-)$ was 0.8 mM [$Km(CO_2)$ = 18 μM] and the maximal velocity was 50 μmoles (mg Chl·hr)$^{-1}$. During the second phase (4–10 min) the maximal velocity of the low Km form dropped to 6.5 μmoles(mg Chl·hr)$^{-1}$, while a form with high $Km(HCO_3^-)$ of 20 to 25 mM [$Km(CO_2)$ = 450–560 μM] appeared.

Effects of Incubation. Changes in activity of the low $Km(CO_2)$ carboxylase were observed after incubation of the chloroplasts in a hypotonic medium lacking one or several of the necessary components for fixation. Fixation was started at

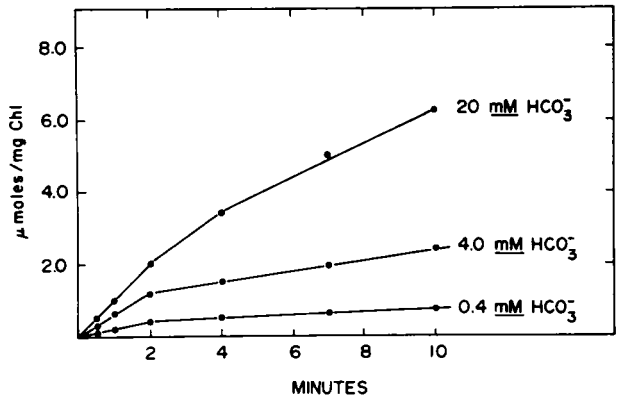

FIG. 2. Effect of bicarbonate concentration on biphasic kinetics of RuDP carboxylase. Fixation was initiated by addition of chloroplasts (4.9 μg Chl) to hypotonic medium containing 85 mM HEPES, pH 7.8, 25 mM $MgCl_2$, 4 mM DTE, 0.23 mM RuDP, and $NaHCO_3$ as indicated in a total volume of 0.6 ml. Aliquots were removed for counting at times indicated.

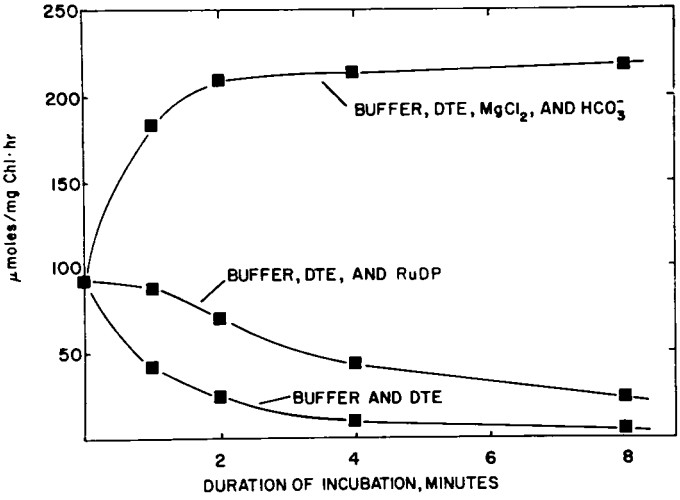

FIG. 3. Effect of incubation of RuDP carboxylase under various conditions on subsequent activity measured at 4.0 mM $NaHCO_3$. Chloroplasts (9.6 μg Chl) were added to 25 mM HEPES, pH 7.8, 8.4 mM DTE and, where indicated, 0.30 mM RuDP, 25 mM $MgCl_2$ and/or 4.0 mM $NaHCO_3$ in a total volume of 0.20 ml. After the indicated incubation period was complete the substrates not present during incubation were added to initiate fixation. The rate of fixation into acid-stable products was calculated after 30 sec. In the case of incubation with $H^{14}CO_3^-$, a correction was made for the small amount of incorporation during the incubation period.

various times by adding the missing components. The assay was carried out with 4 mM HCO_3^-, which is nearly saturating for the low $Km(CO_2)$ carboxylase but gives only minor activity with the higher *Km* form. In buffer and DTE, there was a loss of activity of the low *Km* form (Fig. 3). This loss was the result of a transition from a low $Km(CO_2)$ form to a high $Km(CO_2)$ form. This is shown by comparison of the HCO_3^- dependence prior to incubation to that after 5 min of incubation in buffer (Fig. 4). The nonincubated enzyme had an apparent $Km(HCO_3^-)$ of 0.77 mM [$Km(CO_2)$ = 17 μM] with a maximal velocity of 85 μmoles(mg Chl·hr)$^{-1}$. After 5 min of incubation, the maximal velocity of the low $Km(CO_2)$ form dropped to 13 μmoles (mg Chl·hr)$^{-1}$, while a high $Km(CO_2)$ form appeared with a maximal velocity of 52 μmoles(mg Chl·hr)$^{-1}$. The $Km(HCO_3^-)$ for the high *Km* form was 22 mM [$Km(CO_2)$ = 500 μM] (insert, Fig. 4), comparable to the purified enzyme (13).

This transition from a low *Km* form to a high *Km* form of the carboxylase was the same as that occurring during CO_2 fixation after a 2-min delay (Fig. 2). This delay was probably due to stabilization of the low *Km* form of the enzyme by RuDP during fixation, as suggested by a similar delay in loss of activity when chloroplasts were incubated in buffer plus RuDP prior to CO_2 fixation. (Fig. 3).

These observations identify two distinct kinetic forms of the carboxylase. The low *Km* form is observed immediately upon breakage of chloroplasts in hypotonic media but is unstable and changes into the higher *Km* form after a few minutes.

Incubation with $MgCl_2$ and HCO_3^-. Pon, Rabin, and Calvin (14) reported an activation of the carboxylase by incubation with $MgCl_2$ and HCO_3^- prior to assay. A similar activation of the rate was observed by incubation of chloroplasts in hypotonic medium containing $MgCl_2$ and HCO_3^- (Fig. 3). The $MgCl_2$ plus HCO_3^--activated carboxylase represents yet a third kinetic form of the enzyme characterized by an intermediate value for $Km(HCO_3^-)$ of 2.5 to 3.0 mM. Figure 5 shows an experiment where, after incubation of the chloroplasts in hypotonic media with 25 mM $MgCl_2$ and 4 mM HCO_3^- for 4 min (conditions sufficient for full activation), a $Km(HCO_3^-)$ of 2.5 mM [$Km(CO_2)$ = 55 μM] was obtained. The maximal velocity was 380 μmoles(mg Chl·hr)$^{-1}$. No activation was observed with $MgCl_2$ or HCO_3^- alone.

Although Figure 5 indicates that the low *Km* carboxylase was converted to an intermediate *Km* carboxylase, the high *Km* form could also be converted to the same intermediate *Km* form by incubation with $MgCl_2$ and HCO_3^-. In Figure 6,

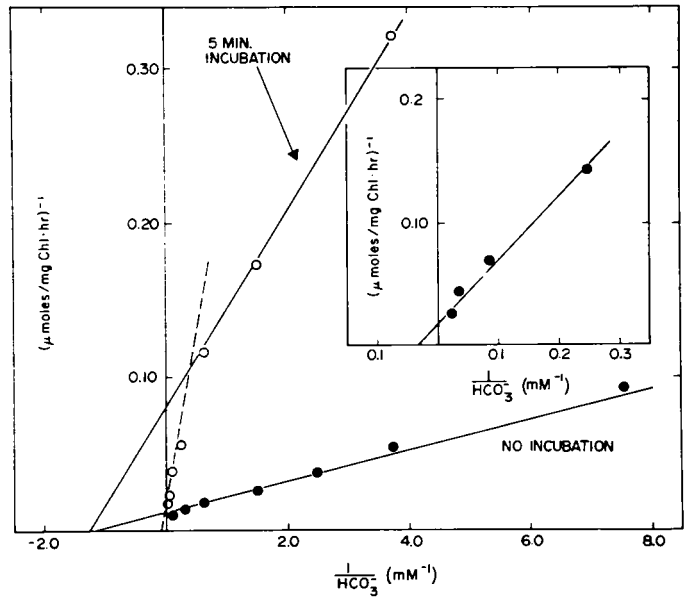

FIG. 4. Effect of incubation of RuDP carboxylase in hypotonic buffer on the $Km(HCO_3^-)$. Lower curve: chloroplasts (7.6 μg Chl) were added last to medium containing 0.1 M HEPES, pH 7.8, 25 mM $MgCl_2$, 4.2 mM DTE, 0.18 mM RuDP and various concentrations of $NaHCO_3$ in a total volume of 0.60 ml. Upper curve: chloroplasts (15.2 μg Chl) from the same preparation were incubated for 5 min in 0.1 M HEPES, pH 7.8, plus 4.2 mM DTE. Fixation was initiated by addition of 0.18 mM RuDP, 25 mM $MgCl_2$, and various HCO_3^- concentrations simultaneously to a total volume of 0.60 ml. Rates were determined as explained in Fig. 1. After correction for the contribution due to low *Km* carboxylase, the high HCO_3^- concentration data were plotted to give the *Km* for the high *Km* carboxylase (inset).

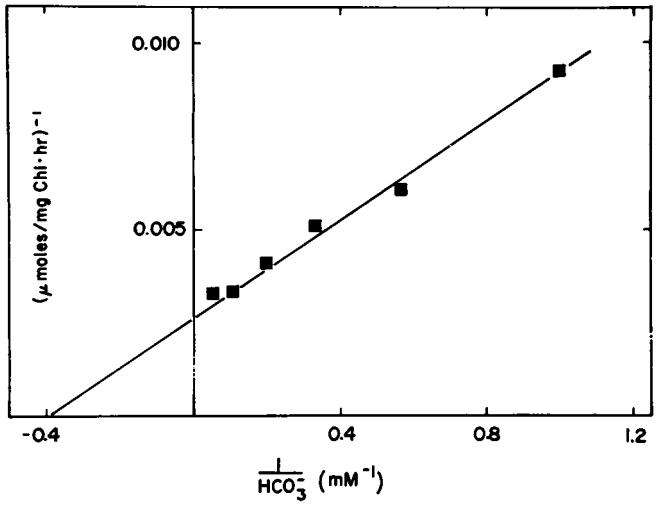

FIG. 5. Bicarbonate dependence of an intermediate $Km(CO_2)$ form of RuDP carboxylase. Chloroplasts were incubated at 25 C in 35 mM HEPES, pH 7.8, 5.6 mM DTE, 25 mM $MgCl_2$ and 4.0 mM $NaHCO_3$ in a total volume of 0.3 ml for 4 min. Fixation was initiated by diluting 0.075 ml of the incubation mixture (4.7 μg Chl) to a final volume of 0.3 ml with assay medium. The final assay medium contained 33 mM HEPES, pH 7.8, 5.6 mM DTE, 25 mM $MgCl_2$, 0.38 mM RuDP, and $NaHCO_3$ as indicated. Initial rates were determined as in Fig. 1.

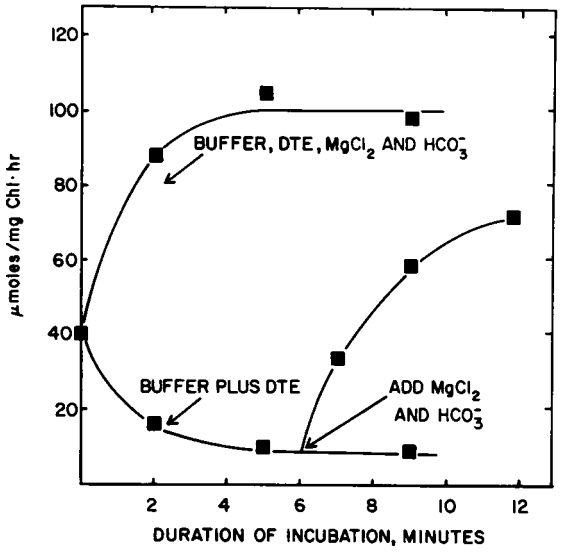

FIG. 6. $MgCl_2$ plus bicarbonate activation of the high Km form of RuDP carboxylase. Lower curve: chloroplasts (9.6 μg Chl) were incubated in 25 mM HEPES, pH 7.8, and 5.6 mM DTE in a total volume of 0.30 ml for the times indicated. Fixation was initiated by addition of 25 mM $MgCl_2$, 2.7 mM $NaHCO_3$, 3.3 mM ATP, and 2.2 mM R5P simultaneously. Middle curve: chloroplasts (9.6 μg Chl) were preincubated for 6 min as in lower curve, then 25 mM $MgCl_2$ and 2.7 mM $NaHCO_3$ were added. After additional incubation, fixation was initiated by addition of 3.3 mM ATP and 2.2 mM R5P. Upper curve: chloroplasts (9.6 μg Chl) were preincubated in 25 mM HEPES, pH 7.8, 5.6 mM DTE, 25 mM $MgCl_2$, and 2.7 mM $NaHCO_3$ in a total volume of 0.30 ml for the times indicated. Fixation was initiated by addition of 3.3 mM ATP and 2.2 mM R5P. In all three curves, rates were measured between 30 sec and 2 min.

chloroplasts were incubated in buffer for 6 min to convert the low Km enzyme to predominantly the high Km enzyme, and then $MgCl_2$ and HCO_3^- were added. Further incubation produced the intermediate form of the enzyme. The effects of continued incubation in buffer alone or of incubation with $MgCl_2$ and HCO_3^- from the start are shown for comparison.

Like the low Km form, the intermediate Km form is unstable and converts to the high Km form during CO_2 fixation. When fixation was initiated by addition of RuDP to the intermediate Km enzyme, a biphasic kinetic pattern was observed with the fast initial phase lasting 2 to 3 min.

Stabilization of the Low $Km(CO_2)$ Carboxylase. When fixation was measured using R5P plus ATP in place of RuDP, and the chloroplasts were added to the assay medium last, a linear rate was obtained for up to 8 min at 25 C. This is in contrast to the results with RuDP (Fig. 2) where the linear rate lasted about 2 min. This observation led to the discovery of the stabilization of the low Km form of the carboxylase by R5P, ATP, and $MgCl_2$ as components of an incubation medium (Fig. 7). At 25 C the stabilization lasted 6 to 8 min while at 0 C, stabilization up to 2 hr was observed. The low Km carboxylase from filtered chloroplasts responded in like manner to the enzyme from lysed chloroplasts.

Form of RuDP Carboxylase *in vivo*. Photosynthesis by plants in air occurs at CO_2 concentrations of about 0.03% (9.5 μM CO_2 [aqueous] at 25 C, 710 mm Hg). To indicate which of the three forms of the carboxylase could be involved in *in vivo* fixation at air levels of CO_2, we compared their activities in the dark to the rate of fixation by intact chloroplasts in the light at 9.0 μM CO_2 (Table II). Both the low Km and the intermediate Km forms of RuDP carboxylase possessed sufficient activity at air levels of CO_2 to account for the rate of light-driven fixation. Even though the Km of the intermediate form is higher than the Km of the low form, its maximal velocity is also increased so that similar fixation rates were observed.

The possibility that exposure of intact chloroplasts to light or to light plus CO_2, might have led to alterations in the kinetic properties of the carboxylase was investigated. Intact chloroplasts were illuminated at 25 C with HCO_3^- (0.4 mM;

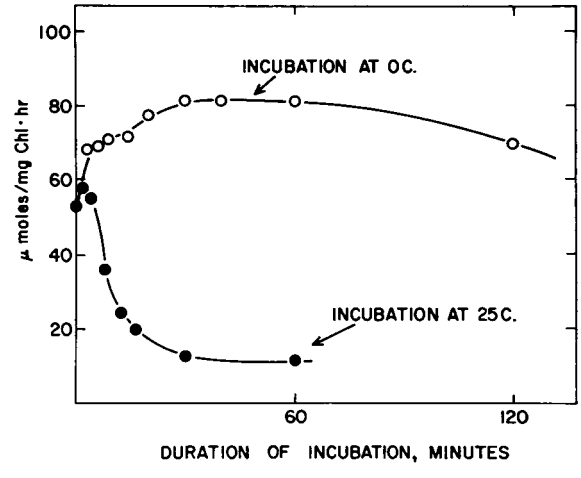

FIG. 7. Stabilization of the low $Km(CO_2)$ form of RuDP carboxylase. At 25 C, chloroplasts (10.5 μg Chl) were lysed and incubated in buffer containing 25 mM HEPES, pH 7.8, 25 mM $MgCl_2$, 2.2 mM R5P, 3.3 mM ATP, and 5.6 mM DTE for the times indicated. Fixation was initiated by addition of 2.7 mM $NaHCO_3$. At 0 C, a separate preparation of chloroplasts was used. Chloroplasts were lysed and incubated at ice temperatures in 25 mM HEPES, pH 7.8, 25 mM $MgCl_2$, 5.1 mM DTE, 3.0 mM ATP, and 2.5 mM R5P. Aliquots containing 6.9 μg Chl removed at indicated times and fixation measured at 25 C in 0.30 ml containing 25 mM HEPES, pH 7.8, 5.6 mM DTE, 2.7 mM $NaHCO_3$, 25 mM $MgCl_2$, and 0.36 mM RuDP. Initial rates were assayed during the first 30 sec.

Table II. *Activity of the Various Kinetic Forms of RuDP Carboxylase at Air Levels (9.0 μM) of CO_2*

Fixation by intact chloroplasts (14.0 μg Chl) in the light was assayed as described in "Methods." Dark fixation was assayed, after appropriate incubation treatments as explained in "Results," in medium containing 25 mM HEPES, pH 7.8; 25 mM $MgCl_2$; 4.4 mM DTE; 0.23 mM RuDP; and 0.40 mM $NaHCO_3$ in a volume of 0.60 ml. Experiments were done on the same preparation of chloroplasts.

Form of the Carboxylase	Observed Rate
	μmoles (mg Chl·hr)$^{-1}$
Intact chloroplasts in light	20
Low K*m* carboxylase	24
Intermediate K*m* carboxylase	27
High K*m* carboxylase	1

Table III. *Summary of Kinetic Parameters of RuDP Carboxylase*

The data summarize the results of numerous preparations of spinach chloroplasts. The maximal velocity of the low K*m* form varied between 50 and 110 μmoles (mg Chl·hr)$^{-1}$. The lower rates appeared to be due to leakage of enzyme into the isolation medium during chloroplast preparation. The maximal velocities are compared to that of the low K*m* form.

Kinetic Form	$K_m(HCO_3^-)$, pH 7.8	$K_m(CO_2)$, calculated	Relative Maximal Velocities
	mM	*μM*	
Low K*m*	0.5–0.8	11–18	1.0
Intermediate K*m*	2.5–3.0	56–67	3.5–4.0
High K*m*	20–25	450–560	0.8–0.9

or 2.0 mM) and without HCO_3^- in the isotonic medium for photosynthesis for 3 min prior to transfer to a hypotonic assay medium in the dark. (Three minutes is approximately the time required to achieve steady rates of fixation by intact chloroplasts.) The carboxylase that was released upon lysis of the illuminated chloroplasts had the same low K*m* and the same maximal velocity, whether or not fixation occurred in the light, as that obtained from chloroplasts adapted to the dark.

DISCUSSION

The experiments reported in this paper identify three kinetically different forms of RuDP carboxylase (Table III). The low K*m* form was observed on breaking intact chloroplasts in the light or dark in hypotonic media. Its maximal rate approached 110 μmoles (mg Chl·hr)$^{-1}$. The low K*m* form was unstable outside of the chloroplast and underwent spontaneous transition to a high K*m* form.

Both the low and high K*m* forms were converted to an intermediate K*m* form by incubation with $MgCl_2$ and HCO_3^- in the absence of RuDP. The maximal activity of the intermediate K*m* form was 3- to 4-fold greater than either the low or high K*m* forms.

The high K*m* form is equivalent in terms of $Km(HCO_3^-)$ to the purified enzyme studied by Weissbach *et al.* (20), Racker (15), and Paulsen and Lane (13). A purified carboxylase having properties similar to the intermediate K*m* form was reported by Murai and Akazawa (12) and by Andrews and Hatch (1). In both cases incubation with $MgCl_2$ plus HCO_3^- gave a $Km(HCO_3^-)$ of about 5 mM and decreasing activity after the first few minutes of assay. Recently Buchanan and Schurman (2) reported a similar transition of purified carboxylase from a $Km(HCO_3^-)$ of 25 mM to a $Km(HCO_3^-)$ of 4 mM. It was reported that this transition was induced by fructose-6-P. Ziegler (21) recently reported studies using sonicated spinach chloroplasts as a source of carboxylase and, after incubation with $MgCl_2$ and HCO_3^-, found a $Km(HCO_3^-)$ of 4.2 mM at pH 7.9. These results are consistent with our observation that a transition from a high K*m* enzyme to an intermediate K*m* enzyme can be effected upon incubation with $MgCl_2$ and HCO_3^- and that the intermediate K*m* form is unstable in the presence of RuDP during assay of carboxylation.

The existence of several kinetic forms of RuDP carboxylase requires that attention be paid to the assay methods of this enzyme. Since the low and intermediate K*m* forms are unstable during assay, assays of fixation based on incubation of enzyme with substrates for a single fixed time period may result in measurement of average kinetic parameters of more than one kinetic form. For example, in several of the studies discussed above (1, 12) the $Km(HCO_3^-)$ for the intermediate K*m* form is higher than our value. This could have arisen from the conversion of some of the enzyme back to the high K*m* form during assay.

Although the evidence is circumstantial, we suggest that the low K*m* form reported here is the form in which the carboxylase exists in the stroma region of intact chloroplasts. The low $Km(HCO_3^-)$ is similar to the $Km(HCO_3^-)$ observed during photosynthesis with intact chloroplasts (8, 9), and it is the form of the carboxylase which always appeared upon lysis of them. The low K*m* carboxylase would therefore seem to be a better choice for *in vitro* studies designed to elucidate mechanisms of control in photosynthetic CO_2 fixation. It also makes the proposal of a CO_2 concentrating mechanism across the chloroplast outer membrane unnecessary.

The stabilizing effect of R5P, ATP, and $MgCl_2$ on the low K*m* form of the carboxylase offers encouragement that this form might be preserved during purification procedures. The optimal concentrations for and nature of this stabilization are under investigation. It is not known whether this or some other method of stabilization acts to keep the carboxylase in the low K*m* form in the intact chloroplast.

ADDENDUM

Since submitting this paper, we have examined the CO_2 affinity properties of tobacco (*Nicotiana tabacum*, var. Samsun) RuDP carboxylase. Lysis of intact tobacco chloroplasts released a low $Km(CO_2)$ carboxylase [$Km(CO_2)=16\mu M$]. Preliminary experiments, similar to those described above, indicated that this form of the enzyme changes its CO_2 affinity during incubation and assay analogously to the low K*m* enzyme from spinach.

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