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Activation State of Ribulose Bisphosphate Carboxylase in Soybean Leaves[1]

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ABSTRACT

Conditions for extraction and assay of ribulose-1,5-bisphophate carboxylase present in an *in vivo* active form (initial activity) and an inactive form able to be activated by Mg^{2+} and CO_2 (total activity) were examined in leaves of soybean, *Glycine max* (L.) Merr. cv Will. Total activity was highest after extracts had preincubated in $NaHCO_3$ (5 millimolar saturating) and Mg^{2+} (5 millimolar optimal) for 5 minutes at 25°C or 30 minutes at 0°C before assay. Initial activity was about 70% of total activity. K_{act} (Mg^{2+}) and K_{act} (CO_2) were approximately 0.3 millimolar and 36 micromolar, respectively. The carry-over of endogenous Mg^{2+} in the leaf extract was sufficient to support considerable catalytic activity. While Mg^{2+} was essential for both activation and catalysis, Mg^{2+} levels greater than 5 millimolar were increasingly inhibitory of catalysis. Similar inhibition by high Mg^{2+} was also observed in filtered, centrifuged, or desalted extracts and partially purified enzyme. Activities did not change upon storage of leaves for up to 4 hours in ice water or liquid nitrogen before homogenization, but were about 20% higher in the latter. Activities were also stable for up to 2 hours in leaf extracts stored at 0°C. Initial activity quickly deactivated at 25°C in the absence of high CO_2. Total activity slowly declined irreversibly upon storage of leaf homogenate at 25°C.

Purified RuBPCase[2] requires preincubation in Mg^{2+} and CO_2 before assay to attain maximal activity (for details, see reviews by Jensen and Bahr [6] and Lorimer [12]). The model for this activation (12) involves the stepwise addition of CO_2 and Mg^{2+} to the enzyme in the following sequence:

$$\text{Enzyme} + CO_2 \leftrightarrow \text{Enzyme-}CO_2 + Mg^{2+} \leftrightarrow \text{Enzyme-}CO_2\text{-}Mg^{2+}$$

(E form, inactive) (EC form, inactive) (ECM form, active)

The K_{act} for Mg^{2+} and CO_2 measured with purified enzyme are approximately 1 and 0.1 mM, respectively (4, 8). However, the concentrations of Mg^{2+} and CO_2 which are present in the chloroplast may be insufficient for complete activation (8, 20). A number of workers have estimated the amount of RuBPCase which is in an active state in leaves. Perchorowicz *et al.* (17–19) measured per cent activation, the quotient of initial and total activity, to estimate the fraction of the total RuBPCase which is in the active state. Initial activity was measured by the addition of a filtered leaf-extract, homogenized in a buffered medium without added Mg^{2+} and CO_2, to an assay medium containing saturating Mg^{2+} and CO_2 concentrations. Total activity was measured in a similar manner, but after homogenate had preincubated for 10 min in saturating Mg^{2+} and CO_2 to attain full activation. Other workers (2, 15) have measured per cent activation of RuBPCase in wheat leaves but have included Mg^{2+} in the homogenizing medium as a necessary precaution against deactivation of the active enzyme during extraction. The per cent activation of RuBPCase in wheat leaves is significantly influenced by irradiance level (15, 17, 18), CO_2 (2, 15, 17), temperature (15), and has been found to increase with increases in leaf photosynthesis rate (2, 15, 17, 18). For example, increasing the illumination of wheat leaves increased per cent activation from 20 to 60% and photosynthesis rate from 0 to 60 μmol CO_2 mg^{-1} Chl h^{-1} (17). Hence, the amount of active RuBPCase in the leaf could be an important factor in regulating the rate of photosynthetic CO_2 fixation, and its physiological significance and control need to be examined. Per cent activation varies considerably among different species of plants as well as the optimal conditions for measuring it (19). Before examining the influence of environmental factors on the per cent activation of RuBPCase in soybean leaves, we have established procedures for optimizing extraction and assay of initial and total enzyme activity in soybean leaves.

MATERIALS AND METHODS

Plant Material. Soybeans [*Glycine max* (L.) Merr. cv Will] were grown in vermiculite in chambers under a 12-h photoperiod (25°C day and 20°C night) and illuminated with 540 μE m^{-2} s^{-1} provided by a combination of fluorescent and incandescent lamps. Plants were watered daily with a balanced nutrient solution.

Leaf Homogenization. Fully mature leaves, generally from the third to fifth node, were sampled in the middle of the light period by either removing leaf discs (0.5 cm^2) with a cork borer or removing and quickly submerging the entire trifoliolate leaf into LN_2 or an ice water bath. Leaves stored in LN_2 were ground to a fine powder in an LN_2-chilled mortar. Samples (0.1 g) of LN_2-frozen leaf powder or leaf discs were homogenized by hand for 30 s with a Tenbrock glass homogenizer in 5 ml of ice-cold homogenizing medium (50 mM Tris-Cl, 5 mM DTT, 10 mM isoascorbic acid, and 0.1% [w/v] BSA, pH 8.0 at 0°C) and stored in an ice bath or immediately assayed. In some cases, either 5 mM $MgCl_2$ and/or $NaHCO_3$ was added to the homogenization medium as indicated in the text.

RuBPCase Assays. RuBPCase activity was measured in a medium containing (final concentration): homogenate (approximately 5 μg Chl), Tris-Cl (100 mM), pH 8.0 at 25°C, isoascorbic acid (10 mM), DTT (5 mM), BSA (0.1%, w/v), Mg^{2+} (concentration as indicated in text), $NaH^{14}CO_3$ (20 mM, 0.1 Ci mol^{-1}), and RuBP (0.4 mM) (total volume, 0.51 ml) in a stoppered 4-ml plastic vial. Vials were shaken reciprocally (3 s^{-1}) in a 25°C water

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[2] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; LN_2, liquid nitrogen.

bath. Initial activity was measured by the addition of 50 μl of extract by means of positive displacement micropipette (Sigma) to the assay medium and terminated after 1 min by the addition of 0.2 ml of 6 N formic acid. Total activity was measured in a similar manner except that the extract was added to the assay medium and incubated at 25°C with shaking for 5 min before initiating the assay by the addition of RuBP. After assay, vials were purged with air and dried overnight at 70°C. Acid-stable ^{14}C was determined by scintillation counting and corrected for quenching by external standard method. Ba_2RuBP (Sigma) was solubilized with excess Dowex-50-H^+ and after filtration the pH was taken to 6.5 with KOH. This solution was stored at 0°C and added immediately before assay. RuBP concentration was routinely assayed using crystalline tobacco RuBPCase and $NaH^{14}CO_3$. Chl was determined by the procedure of Wintermans and DeMot (23) and soluble protein using a dye-binding reagent (Bio-Rad) and a BSA (fraction V) standard. CO_2 concentration was calculated from bicarbonate concentration using a pK′ value of 6.38 (8). Corrections were made in the specific activity of assay $H^{14}CO_3^-$ due to carry-over of $H^{12}CO_3^-$ if added to the homogenizing medium. No corrections were made for the presence of atmospheric CO_2 in assay medium.

In this study, initial activity refers to activity measured immediately upon addition of the extract at 0°C to an assay medium containing RuBP. Final activity refers to activity measured after the extract had been preincubated for a period of time, usually 5 min at 25°C, before assay. Total activity was measured after extract had preincubated in 20 mM $NaHCO_3$ and 5 mM Mg^{2+} for 5 min before assay. All assays were linear for at least 3 min.

RESULTS

Optimum pH for Activation and Assay. Both catalysis and activation in 5 mM Mg^{2+} and 20 mM $NaHCO_3$ were optimal at pH 8.0. Initial and total activities were reduced about 30% at the extremes of pH range of 7.4 to 8.8 (data not shown).

Mg^{2+} Concentration and Time Course of Activation. RuBPCase activity increased with time upon preincubation of extract in an assay medium containing added Mg^{2+} (2–5 mM optimal) and 20 mM $NaHCO_3$ before addition of RuBP (Fig. 1). Activity remained constant between 5 and 20 min of preincubation. Incubation of extract in an assay medium without added Mg^{2+} resulted in no substantial loss of activity with time. The addition of Mg^{2+} (5 mM optimal) to the assay medium slightly increased the initial activity. The presence of an 'endogenous' initial activity (measured without added Mg^{2+}) has been reported earlier (19). In some of the experiments which follow we continued to measure this endogenous activity in the event that it might have significance. However, subsequent work (22) has shown that this activity is the result of carry-over of endogenous Mg^{2+} present in the leaf extract. Soybean leaf extracts were found to contain approximately 15 to 20 μmol Mg^{2+} mg Chl^{-1} (1.5–2.0 mM Mg^{2+}) as measured by atomic absorption spectroscopy. After a 10-fold dilution into assay medium, the final assay concentration of endogenous Mg^{2+} would be 0.15 to 0.2 mM Mg^{2+}. This endogenous Mg^{2+} is sufficient for maintaining considerable activity (Fig. 2). While the conversion of E to EC is considered to occur slowly (min) at assay temperature, the conversion of EC to ECM occurs very rapidly (13). We also observed that preincubation of extract in 20 mM $NaHCO_3$ followed by assay in 5 mM Mg^{2+} and 20 mM $NaHCO_3$ increased total activity, but the change in activity resulting from either increasing or decreasing assay Mg^{2+} concentration was so rapid that it could not be measured in an interval as short as 5 s.

Assay of initial and final activities at added Mg^{2+} levels greater than 5 mM was increasingly inhibitory (Figs. 1 and 2). This inhibition was unexpected in that 20 mM Mg^{2+} is optimal for activation and assay of purified spinach carboxylase (14) and 30 mM for filtered soybean leaf extracts (19). We then determined if this inhibition also occurred in filtered, centrifuged, and desalted extracts or partially purified enzyme, because Mg^{2+} has also been reported to bind RuBPCase to chloroplast membranes (16). High Mg^{2+} levels also inhibited final RuBPCase activities in homogenates which had been either filtered through a nylon net (10 μm opening), centrifuged for 1 min at 12,000 *g*, or centrifuged and then passed through a 5-ml syringe filled with Sephadex G-25 (equilibrated in homogenizing medium without BSA) by centrifugation at 300 *g* for 1 min (Fig. 2). About 20% of the final activity was lost by filtration, centrifugation, or desalting, based on the amount of Chl present in the original homogenate. Enzyme which was partially purified by precipitation by 20 mM $MgCl_2$ in the presence of 18% (w/v) PEG-4000 (3) and then passed through a small column of Sephadex G-25 equilibrated in homogenizing medium without BSA also showed a similar pattern of inhibition by Mg^{2+} concentrations greater than 5 mM. The $K_{act}(Mg^{2+})$ for partially purified enzyme was approximately 0.3 mM (Fig. 2). Of interest is the observation that the ratio of the activity in the presence of endogenous Mg^{2+} only

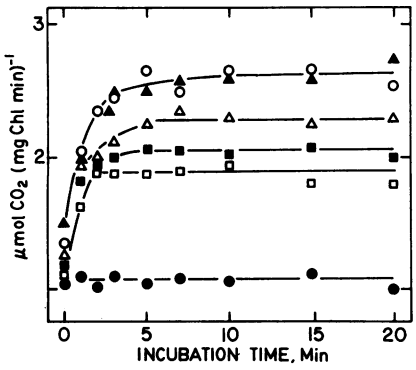

FIG. 1. Time course of activation of RuBPCase activity in 20 mM $NaHCO_3$ and increasing concentrations of Mg^{2+}. Leaf extracts were prepared from the same LN_2-frozen leaf powder at 0°C and added to assay medium containing 20 mM $NaHCO_3$ and either 0 (●), 2 (○), 5 (▲), 10 (△), 20 (■), or 30 (□) mM Mg^{2+} at 25°C. After the indicated time, RuBP was added to initiate the assay and terminated after 1 min.

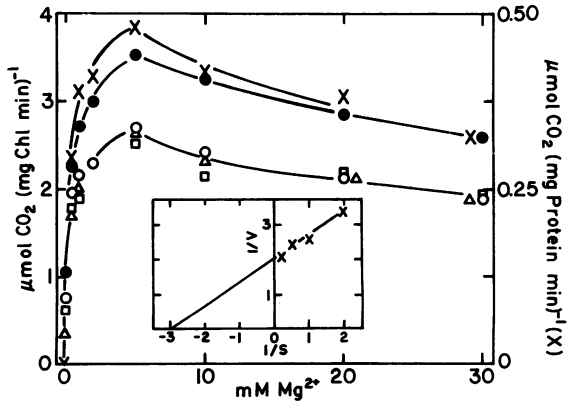

FIG. 2. Effect of Mg^{2+} concentration on final RuBPCase activity. Leaf extracts were prepared at 0°C from the same LN_2-frozen leaf powder and aliquots either added directly to assay medium (●) or added after either filtration (○), centrifugation (□), centrifugation and desalting (△), or partial purification (×) of the extract. After incubation for 5 min in assay medium containing Mg^{2+} (as indicated) and 20 mM $NaHCO_3$, assays were initiated by the addition of RuBP and terminated after 1 min. Means of triplicate assays are shown (avg. SD = ±4.4%). Inset: double reciprocal plot of Mg^{2+} concentration *versus* final activity of partially purified soybean RuBPCase.

(zero added Mg^{2+}) to maximal activity (5 mM added Mg^{2+}) was not reduced by filtration or centrifugation but was reduced about 50% by the centrifuge-desalting step and completely reduced by partial purification of the enzyme. Partial purification might be expected to remove all endogenous Mg^{2+} from the extract.

To determine whether Mg^{2+} was inhibiting activation, catalysis, or both, LN_2-frozen leaf powder was extracted at 25°C in homogenizing medium containing increasing Mg^{2+} concentrations (0 to 50 mM) and 20 mM $NaHCO_3$ and incubated for 5 min before assay. Similar activities were observed for all extracts if the assay medium were adjusted such that the final concentration of added Mg^{2+} in the assay was 5 mM (data not shown). However, when the homogenates were assayed in the same concentration of added Mg^{2+} as was present in the homogenizing medium, a similar inhibition of final activity was observed (Figs. 1 and 2). These data indicate that added Mg^{2+} levels above 5 mM inhibit catalysis directly and not activation, in that the inhibition is rapidly reversible upon lowering Mg^{2+} concentration to 5 mM.

CO_2 Concentration. CO_2 is required for the conversion of E to EC and also as substrate for the enzyme. In order to determine its effect on activation only, we extracted leaf powder in homogenizing medium containing increasing concentrations of HCO_3^- or HCO_3^- and 5 mM Mg^{2+} and assayed initial and final (after incubation in homogenizing medium for 5 min at 25°C) activities (Fig. 3). Increasing HCO_3^- concentration had little effect on initial activity measured immediately after extraction, except at very high concentrations. Initial activities assayed with 5 mM Mg^{2+} were much higher than activities assayed in the absence of added Mg^{2+}, as shown previously (Figs. 1 and 2). Final activities assayed in the presence of Mg^{2+} were reduced after incubation of extract at low HCO_3^- concentration and increased with increasing HCO_3^- concentration, saturating at approximately 5 mM. The enzyme did not completely deactivate in the absence of added HCO_3^- even though an effort was made to eliminate atmospheric CO_2 from both homogenization and assay media before conducting the experiment (14). This inability to deactivate the enzyme completely by eliminating CO_2 has been observed previously with purified enzyme (13) and is probably the result of endogenous Mg^{2+} stabilizing the EC complex. The amount of added HCO_3^- required to promote one-half maximal activation ($K_{act}[HCO_3^-]$) is approximately 2.2 mM (53 μM CO_2) in the presence of endogenous Mg^{2+}. If 5 mM Mg^{2+} were added to the homogenizating medium, initial activities were not different, but final activities were slightly higher and the $K_{act}(HCO_3^-)$ was reduced to 1.5 mM (36 μM CO_2). When leaf powder was homogenized and assayed without added Mg^{2+}, final activities were lower and did not substantially increase above initial levels at higher HCO_3^-.

RuBP Concentration. Initial and total activities increased with increasing RuBP concentration, but did not saturate even at 1 mM RuBP. Between 0 and 0.12 mM RuBP, both activities followed Michaelis-Menten kinetics (K_m[RuBP] = 31 μM) but deviated from these kinetics at higher RuBP levels (Fig. 4). The ratio of initial to total activity or per cent activation decreased about 20% with increasing RuBP concentration from 0 to 1 mM.

Other Compounds. The addition of 0.1% (w/v) BSA, 5 mM DTT, and 10 mM Na isoascorbate had little effect on both initial and total activities (data not shown), but these were added as a precaution against denaturing substances and proteases in the homogenate.

Enzyme Stability in the Leaf. Initial and final activities did not decrease significantly with time while leaves were stored in ice or LN_2 for periods of up to 4 h (Fig. 5). Leaves stored in ice had activities similar to those measured in a homogenate prepared from leaf discs removed from those leaves immediately before being added to ice water. However, both initial and final activities were about 20% higher when leaves were frozen and ground in LN_2 before extraction. In a following experiment, leaf discs were removed from the same leaflet and half were extracted immediately in a glass homogenizer. The other half was ground to a powder in LN_2 and then extracted. Total activities were measured before and after passage of each extract through a French pressure cell at 5000 p.s.i. to completely lyse any unbroken cells. Activities were highest in the extract of LN_2-frozen leaf powder and about 40% lower in the extract of fresh leaf tissue, but similar in both extracts after pressure-cell treatment (about 8% lower). Evidently, extraction of leaves directly in a glass homogenizer did not completely rupture all leaf cells. Microscopic examination of fresh leaf homogenates revealed many

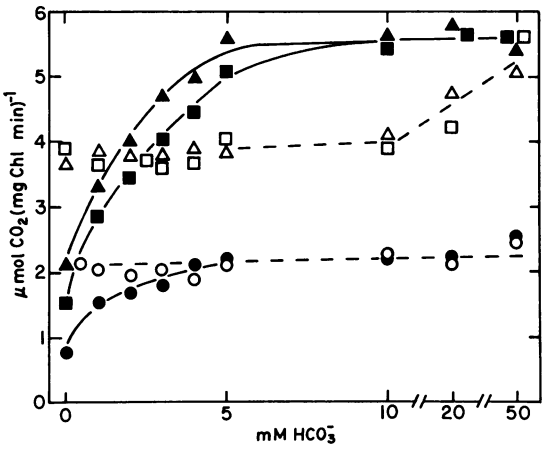

FIG. 3. Effect of HCO_3^- concentration during preincubation on initial and final RuBPCase activity. Leaf extracts were prepared from the same LN_2-frozen leaf powder at 0°C and varying concentration of HCO_3^- plus 0 mM Mg^{2+} (●, ○, ■, □) or 5 mM Mg^{2+} (▲, △) and assayed (single measurements) immediately (○, □, △, initial activity) or assayed after extracts had preincubated for 5 min at 25°C (●, ■, ▲, final activity). The assay medium contained 20 mM $NaHCO_3$ and either 0 mM Mg^{2+} (●, ○) or 5 mM Mg^{2+} (■, □, ▲, △). Mean of triplicate assays are shown (avg. SD = ±5%).

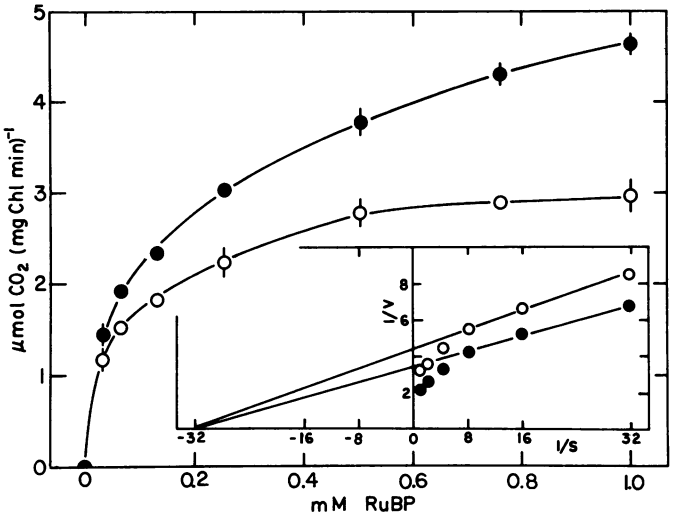

FIG. 4. RuBP response curve for initial and total RuBPCase activity. Extracts of LN_2-frozen leaf powder were assayed immediately in assay medium containing 5 mM Mg^{2+}, 20 mM $NaHCO_3$, and RuBP concentration as indicated (○, initial activity) or after preincubation in assay medium for 5 min before the addition of RuBP (●, total activity). Triplicate measurements were made from the same extract, and vertical bars represent SD. Inset, Double reciprocal plot of RuBP concentration *versus* initial and final activities.

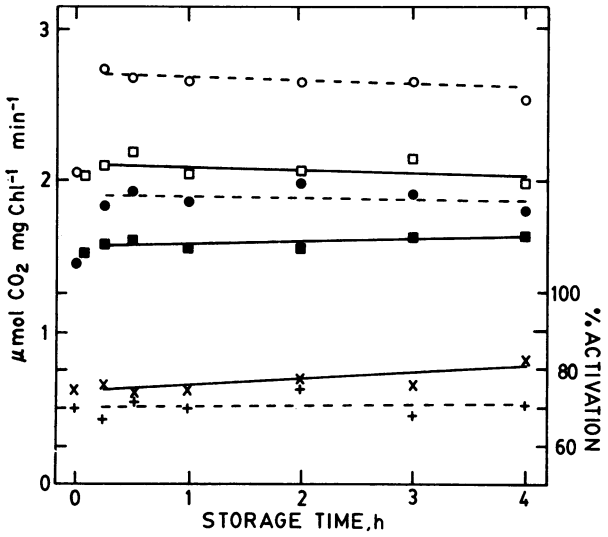

FIG. 5. Stability of RuBPCase activity in soybean leaves stored in ice water or LN_2. Leaf discs were removed from leaves and immediately homogenized at 0°C and aliquots added to assay medium at 25°C. These leaves were then submerged into LN_2 (●, ○, +) or ice-water (■, □, ×). At the times indicated, leaf tissue was removed and extracts prepared and assayed. Different leaves were used for the two treatments. Initial activities (●, ■) and final activities (○, □) were assayed in medium containing 5 mM Mg^{2+} and 20 mM $NaHCO_3$. Per cent activation (+, ×) was determined as (initial activity) (final activity)$^{-1}$ × 100%. Triplicate assays were measured for each extract.

cells with intact chloroplasts. Homogenates of LN_2-frozen leaf powder were observed to contain fewer cells, but chloroplasts in these cells were all ruptured.

Stability in the Homogenate. Initial activities of extracts prepared and stored at ice temperatures remained constant with time. (Fig. 6A). Initial activities were higher if 5 mM Mg^{2+} was included in the assay medium; however, initial activities were no different if Mg^{2+} was included in the homogenizing medium as well as the assay. Extracts prepared in medium containing HCO_3^- or Mg^{2+} and HCO_3^- and assayed in Mg^{2+} both increased in activity similarly with time and attained optimal activity after 30 min. The addition of HCO_3^- to the homogenizing medium had no effect on initial activity assayed without added Mg^{2+}, indicating that Mg^{2+} concentration was limiting for total activity. Total activities decreased about 20% over the 2-h period. Extracts prepared in medium without HCO_3^- and stored at 25°C quickly lost activity before reaching a stable but lower final activity (Fig. 6B). Activities were about 30% higher if 5 mM Mg^{2+} was included in the homogenizing and assay mediums but deactivation occurred none the less. Extracts prepared at 25°C in HCO_3^- or HCO_3^- and Mg^{2+} and assayed in Mg^{2+} increased in activity over the first 5 min before gradually decreasing with time. Activities of extracts prepared in HCO_3^- and assayed without Mg^{2+} remained constant for 15 min before also gradually losing activity. This loss of total activity at 25°C is apparently different from deactivation and is irreversible. Total activities, measured after 5 min incubation in saturating Mg^{2+} and CO_3, were similar in all extraction buffers and decreased about 38% after 2 h at 25°C. The irreversible loss of final activity appeared to be more rapid if the extracts were exposed to high HCO_3^- concentrations during preincubation (Fig. 6B).

DISCUSSION

RuBPCase activity in soybean leaf extracts showed many similarities to activity in purified enzyme from leaves of soybean and other species. Preincubation of homogenate in high CO_2 and

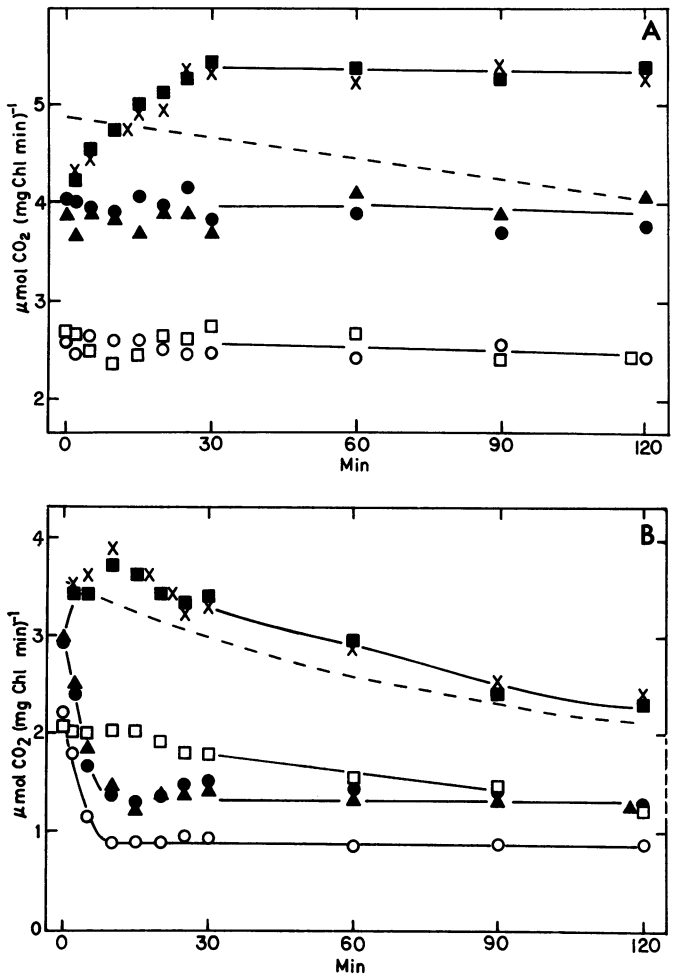

FIG. 6. Stability of RuBPCase activity in soybean leaf extracts stored at 0°C (A) or at 25°C (B). Extracts were prepared from the same LN_2-frozen leaf powder in homogenizing medium (○, ●), medium plus 20 mM $NaHCO_3$ (□, ■), medium plus 5 mM $MgCl_2$ (△), or medium plus 20 mM $NaHCO_3$ and 5 mM $MgCl_2$ (×) and assayed (single measurements) at the times indicated in medium plus 0 mM $MgCl_2$ (○, □) or 5 mM $MgCl_2$ (●, ■, ▲, ×). Different leaves were used in A and B. (- - -), Mean of total activity for all extracts.

Mg^{2+} increased enzyme activity above initial levels presumably by stabilizing enzyme already active and activating inactive enzyme. The $K_{act}(Mg^{2+})$ and $K_{act}(CO_2)$ are similar to those reported for purified enzyme measured under the same conditions (4, 7). The presence of endogenous Mg^{2+} in leaf extracts complicates the accurate measurement of the kinetic constants for activation. Following a 10-fold dilution of extract into the assay medium, the endogenous Mg^{2+} concentration is sufficiently high to support appreciable catalytic activity because $K_{act}(Mg^{2+})$ is comparatively low, approximately 0.3 mM measured with partially purified enzyme (Fig. 2).

Mg^{2+} concentrations above 5 mM were inhibitory to catalysis but not activation. This inhibition was similar in partially purified RuBPCase (Fig. 2), essentially free of all Chl, thus eliminating the possibility that inhibition may have occurred because of a binding of RuBPCase to chloroplast membranes (16). This inhibition of soybean RuBPCase by high Mg^{2+} is insignificant as regards assay because the enzyme is approximately 94% activated by 5 mM Mg^{2+} in the presence of 20 mM $NaHCO_3$ (Fig. 2). Laing *et al.* (10) found purified soybean RuBPCase to be inhibited about 85% by 46 mM Mg^{2+} compared to 6 mM Mg^{2+}. Recently, Bahr *et al.* (1) also reported that purified tobacco enzyme was

increasingly inhibited by increasing Mg^{2+} concentrations above 2.5 mM, while the spinach enzyme showed maximal activity with 25 mM Mg^{2+}. Such differences among species reaffirms the necessity for optimizing activation and assay conditions for a particular species (19).

The K_m (RuBP) measured for soybean leaf homogenates is identical to that reported for purified soybean enzyme (8). We also observed a deviation from Michaelis-Menten kinetics with RuBP concentrations above 0.12 mM. Laing and Christeller (8) explained the increased activity with higher levels of RuBP as substrate activation of enzyme which was already fully activated by CO_2 and Mg^{2+}. Total activities (measured after incubation in optimal CO_2 and Mg^{2+} for activation) increased at a greater rate than initial activities resulting in a slight decrease in per cent activation with increasing RuBP concentration (Fig. 4).

Both initial and final activities were stable in leaves stored in an ice water bath (19; Fig. 5) or stored frozen in LN_2 (Fig. 5). Activities of leaf tissue previously frozen and ground in LN_2 are higher than activities measured in leaves extracted directly without freezing because of a complete rupturing of the leaf cells. Evidently not all of the RuBPCase is released from the cells by the LN_2 treatment because filtering or centrifuging removed about 20% of the total activity (Fig. 2). An attempt to increase total activity by passing an extract of LN_2-frozen leaf powder through a French pressure cell did not increase activity indicating that all of the enzyme in an extract of LN_2 frozen leaf tissue is accessible to RuBP.

Both initial and total activity were very stable for at least 2 h if extracts were stored at ice temperature (Fig. 6A). The inclusion of 5 mM Mg^{2+} in the extraction medium did not increase initial activity, but the inclusion of 20 mM bicarbonate either alone or with Mg^{2+} resulted in enzyme activation both at 0°C (Fig. 6A) and 25°C (Fig. 6B). At the latter temperature, activation was much faster. The observed stability of initial activity in leaf extracts prepared in the absence of added Mg^{2+} and CO_2 is in contrast to the rapid deactivation which occurs in purified spinach enzyme upon removing Mg^{2+} by dilution (7) or gel filtration (11) at 0°C. Further investigation has shown that reducing the endogenous Mg^{2+} concentration in leaf extracts by either dilution or gel filtration (22) or the addition of excess Na_2EDTA (Servaites, unpublished) results in a rapid loss of initial activity at 0°C. While removing Mg^{2+} deactivates the enzyme, the addition of Mg^{2+}, above 2 mM, does not appear to further activate the enzyme, but rather to hold the active enzyme in a nonequilibrium state. Homogenizing leaf tissue at 25°C (Fig. 6B) in the absence of added Mg^{2+} and CO_2 or warming a cold extract (data not shown) results in a rapid loss of initial activity to a new equilibrium dependent upon the Mg^{2+} and CO_2 level.

Upon incubation of extract at 25°C, total activity is slowly and irreversibly lost (Fig. 6B). However, the loss of total activity was essentially eliminated upon a 10-fold dilution of extract into assay medium (Fig. 1). Removal of low mol wt compounds from the extract by gel filtration had no effect upon final activities (Fig. 2). This irreversible loss of activity with time is probably the result of protease action rather than progressive inhibition by small mol wt compounds present in the leaf extract. Extracts of soybean leaves are known to contain a number of endoproteases which can rapidly degrade the large subunit RuBPCase (21) which presumably results in a loss of activity. For an unknown reason, loss of activity was more rapid when leaves were extracted and stored at 25°C in medium containing HCO_3^-. Mächler and Nösberger (15) have also reported a higher loss of initial activity of wheat leaf homogenates stored in the presence of HCO_3^-. They attributed this loss of activity as a direct inhibition of the enzyme by HCO_3^-. This loss of total activity upon warming the homogenate is unfortunate in that the activation by CO_2 is more rapid at higher temperatures and is usually conducted immediately before assay of total activity. It may be advisable if critical measurements of total activity (E, EC, and ECM forms) are being made to allow diluted extract to activate at ice temperatures for 30 to 60 min in a medium containing bicarbonate and Mg^{2+} before measuring total activity.

Much evidence indicates that photosynthesis rate at low CO_2 concentrations is correlated with RuBPCase activity (5, 6, 18). However, when measurements of RuBPCase activity and photosynthesis rate are made on the same leaf, RuBPCase activity substantially exceeds photosynthesis rate (5, 18). There are at least two reasons for this apparent inconsistency. First, photosynthesis rate is measured at air level of CO_2 (10 μM CO_2), while RuBPCase activity is measured at saturating CO_2 concentration (*i.e.* greater than 100 μM CO_2). Second, RuBPCase activity is measured after conversion of E and EC to ECM form by activation with CO_2 and Mg^{2+}, while the true rate of *in vivo* carboxylation is probably a function of only the ECM form. If our measurements of initial activity are a true reflection of *in vivo* carboxylation rates, then only 70% of the total carboxylase (E, EC, ECM) is in the active form (ECM) in the soybean leaves we examined and this activity may better correlate to rates of photosynthesis than total RuBPCase activity.

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LITERATURE CITED

1. BAHR JT, S JOHAL, M CAPEL, DP BOURQUE 1981 High specific activity ribulose 1,5-biphosphate carboxylase-oxygenase from *Nicotiana tabacum*. Photosynth Res 2: 235–242
2. BOYLE FA, AJ KEYS 1982 Regulation of RuBP carboxylase activity associated with photo-inhibition of wheat. Photosynth Res 3: 105–111
3. HALL NP, NE TOLBERT 1978 A rapid procedure for the isolation of ribulose bisphosphate carboxylase/oxygenase from spinach leaves. FEBS Lett 96: 167–169
4. HATCH AL, RG JENSEN 1980 Regulation of ribulose-1,5-biphosphate carboxylase from tobacco: changes in pH response and affinity for CO_2 and Mg^{2+} induced by chloroplast intermediates. Arch Biochem Biophys 205: 587–594
5. HESKETH JD, WL OGREN, ME HAGEMAN, DB PETERS 1981 Correlations among leaf CO_2-exchange rates, areas and enzyme activities among soybean cultivars. Photosynth Res 2: 21–30
6. JENSEN RG, JT BAHR 1977 Ribulose 1,5-biphosphate carboxylase-oxygenase. Annu Rev Plant Physiol 28: 379–400
7. JORDAN DB, R CHOLLET, WL OGREN 1983 Binding of phosphorylated effectors by active and inactive forms of ribulose-1,5-bisphosphate carboxylase. Biochemistry 22: 3410–3418
8. LAING WA, JT CHRISTELLER 1976 A model for the kinetics of activation and catalysis of ribulose 1,5-bisphosphate carboxylase. Biochem J 159: 563–570
9. LAING WA, JT CHRISTELLER 1980 A steady-state kinetic study on the catalytic mechanism of ribulose bisphosphate carboxylase from soybean. Arch Biochem Biophys 202: 592–600
10. LAING WA, WL OGREN, RH HAGEMAN 1975 Bicarbonate stabilization of ribulose 1,5-diphosphate carboxylase. Biochemistry 14: 2269–2275
11. LORIMER GH 1979 Evidence for the existence of discrete activator and substrate sites for CO_2 on ribulose-1,5-bisphosphate carboxylase. J Biol Chem 254: 5599–5601
12. LORIMER GH 1981 The carboxylation and oxygenation of ribulose 1,5-bisphosphate: the primary events in photosynthesis and photorespiration. Annu Rev Plant Physiol 32: 349–383
13. LORIMER GH, MR BADGER, TJ ANDREWS 1976 The activation of ribulose-1,5-bisphosphate carboxylase by carbon dioxide and magnesium ions. Equilibria, kinetics, a suggested mechanism, and physiological implications. Biochemistry 15: 529–536
14. LORIMER GH, MR BADGER, TJ ANDREWS 1977 D-Ribulose-1,5-bisphosphate carboxylase-oxygenase: improved methods for the activation and assay of catalytic activities. Anal Biochem 78: 66–75
15. MÄCHLER F, J NÖSBERGER 1980 Regulation of ribulose bisphosphate carboxylase activity in intact wheat leaves by light, CO_2, and temperature. J Exp Bot 31: 1485–1491
16. MCNEIL PH, DA WALKER 1981 The effect of magnesium and other ions on the distribution of ribulose 1,5-bisphosphate carboxylase in chloroplast extracts. Arch Biochem Biophys 208: 184–188
17. PERCHOROWICZ JT, RG JENSEN 1983 Photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings: regulation by CO_2 and O_2. Plant Physiol 71: 955–960
18. PERCHOROWICZ JT, DA RAYNES, RG JENSEN 1981 Light limitation of photosynthesis and activation of ribulose bisphosphate carboxylase in wheat seedlings. Proc Natl Acad Sci USA 78: 2985–2989

19. PERCHOROWICZ JT, DA RAYNES, RG JENSEN 1982 Measurement and preservation of the *in vivo* activation of ribulose 1,5-bisphosphate carboxylase in leaf extracts. Plant Physiol 69: 1165–1168
20. PORTIS JR AR, HW HELDT 1976 Light-dependent changes of the Mg^{2+} concentration in the stroma in relation to the Mg^{2+} dependency of CO_2 fixation in intact chloroplasts. Biochim Biophys Acta 449: 434–446
21. RAGSTER LE, MJ CHRISPEELS 1981 Autodigestion in crude extracts of soybean leaves and isolated chloroplasts as a measure of proteolytic activity. Plant Physiol 67: 104–109
22. SERVAITES JC 1984 Maintenance of ribulose bisphosphate carboxylase activity by endogenous Mg^{2+} in soybean leaf extracts. *In* C. Sybesma, ed, Proceedings of the Sixth International Congress on Photosynthesis. In press
23. WINTERMANS JFGM, A DE MOTS 1965 Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim Biophys Acta 109: 448–453