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Regulation of Ribulose Bisphosphate Carboxylase Activity in Intact Wheat Leaves by Light, CO_2, and Temperature

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ABSTRACT

The activity of the enzyme ribulose bisphosphate carboxylase (RuBPCase) was estimated after rapidly extracting it from intact wheat leaves pretreated under different light and CO_2 levels. No HCO_3^- was added to the extraction buffer since it is shown to inhibit RuBPCase. The activity increased as light intensity or CO_2 concentration during pretreatment was increased. Enzyme activity increased as temperature during pretreatment was decreased. Light activation did not affect the affinity of RuBPCase for CO_2. A K_m of 30 μM CO_2 under air level O_2 was determined. CO_2, light and temperature are three main limiting factors of photosynthesis. It seems that the activity of RuBPCase is regulated by these factors according to the requirements for CO_2 fixation.

INTRODUCTION

Ribulose-1,5-bisphosphate carboxylase (RuBPCase) is an enzyme of the photosynthetic carbon reduction cycle and catalyses the reaction of CO_2 with RuBP to form 3-phosphoglyceric acid. Purified enzyme from wheat leaves shows different degrees of activation depending on the concentrations of CO_2 and Mg^{2+}, pH, and temperature. Kinetic analyses have indicated that the enzyme is activated in three steps according to the equation $E^* \rightleftarrows E + CO_2 \rightleftarrows E\text{–}CO_2 + Mg^{2+} \rightleftarrows E\text{–}CO_2\text{–}Mg^{2+}$ (Lorimer, Badger, and Andrews, 1976; Laing and Christeller, 1976; Chollet and Anderson, 1977; Mächler, Keys, and Cornelius, 1980). The first step may be a conformational change of the protein and is influenced by temperature. Activation of RuBPCase can be shown not only *in vitro* but also *in vivo*. The degree of activation of enzyme in intact chloroplasts is shown to depend on CO_2 and light (Bahr and Jensen, 1978). Part of the light activation is most likely due to increased Mg^{2+} and pH in the stroma (Werdan, Heldt, and Milovancev, 1975; Portis and Heldt, 1976). This paper reports experiments in which intact wheat leaves were treated under different CO_2 and light levels before RuBPCase was extracted rapidly for estimation of activity.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* var. Kolibri) was grown in soil in cabinets at 20 °C/15 °C day/night temperatures and a light intensity of 300 μE m^{-2} s^{-1} with a day length of 16 h. Seedlings 10 d old were pretreated in glass vessels with air of different CO_2 concentrations and with different light intensities. For preparation of gas mixtures Wösthoff pumps were used. The light source was a 400 W sodium vapour lamp separated from the plants by a 6 cm water layer.

For rapid extraction of enzyme the leaves of five seedlings were cut into pieces of about 2 cm in length and homogenized in a mortar with 3 g sand and 4 ml 20 mM Tris–HCl (pH 8·2), 20 mM $MgCl_2$, and 10 mM mercaptoethanol. The homogenate was diluted with 25 ml of the same solution and filtered through a nylon net (20 μm mesh opening) by placing an open-ended rubber cylinder with the net attached to the bottom end into the homogenate. 20 μl of the filtrate which rose up inside the cylinder were tested for carboxylase activity at 10 °C. Mortar, sand, and solutions were precooled in ice. The entire extraction procedure was completed in less than 1 min.

To study the influence of pH and HCO_3^- concentration on the extracted enzyme 4 g freshly cut leaves were homogenized with 6 g sand and 8 ml 20 mM Tris–HCl (pH 8·2), 20 mM $MgCl_2$, and 10 mM mercaptoethanol. The homogenate was filtered through two layers of muslin and centrifuged at 40 000 *g* for 20 min at 4 °C. The extract was used within 2 h. 0·1 ml extract was added to 0·9 ml of either 500 mM Tris–HCl (pH 8·3) or 250 mM HEPES (pH 6·8) with 20 mM $MgCl_2$, 10 mM mercaptoethanol, and different $NaHCO_3$ concentrations in 50 ml Erlenmeyer flasks. The flasks were placed in a water bath of 20 °C and gassed with air of different CO_2 content, which was in equilibrium with the HCO_3^- concentrations of the solutions (calculation of the concentrations according to Umbreit, Burris, and Stauffer, 1972). After 10 min 10 μl solution were tested for carboxylase activity at 25 °C.

The enzyme activity was assayed by the procedure of Lorimer *et al.* (1977) using 0·5 ml 84 mM Tris–HCl (pH 8·2), 10 mM mercaptoethanol, 0·4 mM RuBP, 20 mM $MgCl_2$, and 20 mM $NaH^{14}CO_3$ (1 Ci mol^{-1}). For determination of the kinetic constants the reaction mixtures were prepared under CO_2-free air. The reaction was started by adding the enzyme and stopped after 30 s by adding 0·1 ml 2 N HCl. Of this mixture 0·4 ml was transferred to a scintillation vial and dried at 90 °C. The residue was dissolved in 1 ml water, 10 ml scintillator solution (Patterson and Greene, 1965) were added, and the amount of ^{14}C present was measured using a scintillation spectrometer. Soluble protein was estimated according to Bensadoun and Weinstein (1976).

RESULTS AND DISCUSSION

The activation of RuBPCase in intact leaves by light and CO_2

The purpose of the experiments was to examine the degree of activation of RuBPCase in intact leaves. Therefore the amount of the active complex E–CO_2–Mg^{2+} had to be kept as constant as possible during extraction. This was achieved by adding Mg^{2+} in excess to the extraction medium in order to inhibit the dissociation of the active complex, and by keeping the CO_2 concentration low in order to avoid the activation of the inactive enzyme.

The activity of RuBPCase, rapidly extracted from intact leaves, was dependent upon light intensity and CO_2 concentration during preincubation. The activity increased as CO_2 concentration (Fig. 1) or light intensity was increased (Fig. 2).

Temperature showed a marked effect on the activity of RuBPCase in intact leaves. Preincubation at 7 °C at a light intensity of 300 μE m^{-2} s^{-1} under air level CO_2 resulted in an activity of 0·13 μmol CO_2 min^{-1} mg^{-1} protein as compared with 0·07 μmol CO_2 min^{-1} mg^{-1} protein for preincubation at 25 °C. The assay temperature was 10 °C. This is obviously not a direct effect of temperature on the protein, since studies with purified enzyme show that RuBPCase activity decreases as temperature is decreased (Mächler *et al.* 1980). In intact leaves temperature seems to affect enzyme activity by changing the level of regulatory metabolites.

As has already been shown with protoplasts (Robinson, McNeil, and Walker,

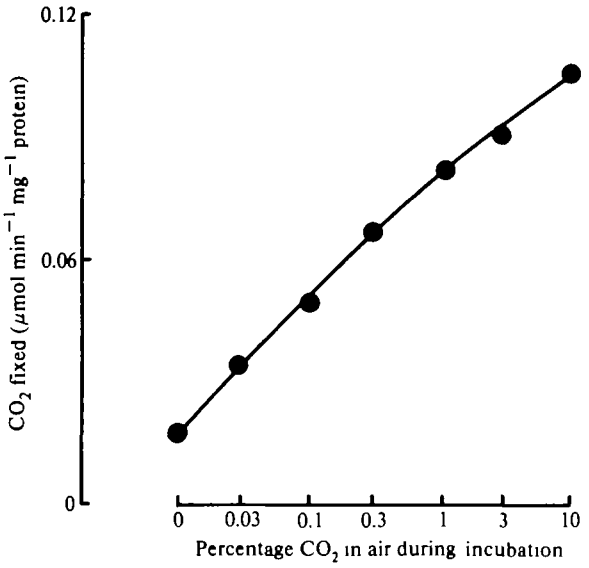

FIG. 1. Influence of percentage CO_2 in air during 2 h incubation of wheat seedlings in the dark at room temperature on activity of rapidly extracted RuBPCase. The enzyme was tested at 10 °C. For details see Materials and Methods.

1979), dark incubation does not inactivate RuBPCase completely. Leaves incubated in the dark in 10% CO_2 attained activities similar to leaves which had been illuminated at 800 μE m^{-2} s^{-1} under air level CO_2. Enzyme in leaves incubated in the dark in 10% CO_2 was activated relatively slowly, requiring 1 h. This may be due to very low levels of Mg^{2+} present in the stroma when it is dark (Portis and Heldt, 1976).

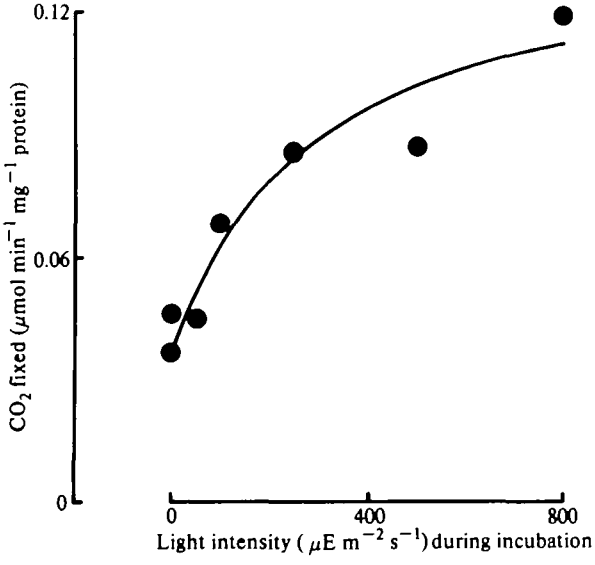

FIG. 2. Influence of light intensity during 2 h incubation of wheat seedlings in air at room temperature on the activity of rapidly extracted RuBPCase. The activity of enzyme was tested at 10 °C. For details see Materials and Methods.

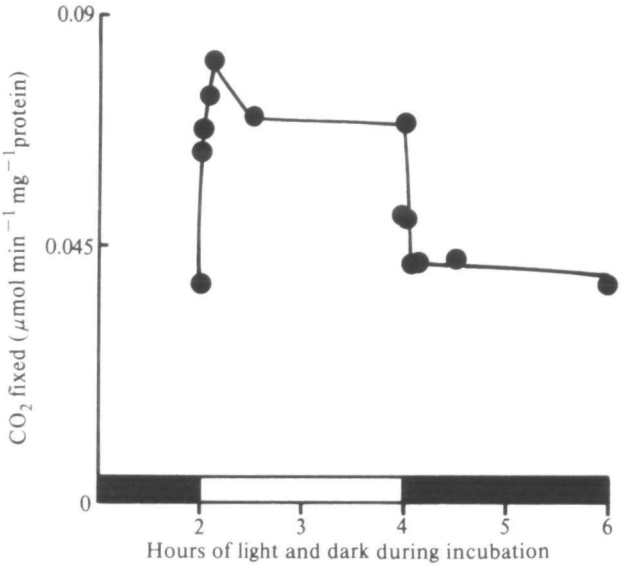

FIG. 3. Influence of light (100 μE m^{-2} s^{-1}) and dark during incubation of wheat seedlings in air at room temperature on the activity of rapidly extracted RuBPCase. The activity of enzyme was tested at 10 °C. For details see Materials and Methods.

The induction of photosynthesis by illumination was accompanied by a rapid increase of enzyme activity followed by a decline and levelling off (Fig. 3). The decline can be explained by a decrease in CO_2 and an increase in RuBP concentration, the latter being known to inhibit RuBPCase (Sicher and Jensen, 1979; Laing and Christeller, 1976).

In contrast to Bahr and Jensen (1978) no difference in affinity of RuBPCase for CO_2 was found between light and dark treatments (Fig. 4). In both cases a K_m of 30 μM CO_2 under air level O_2 was determined.

The concentration of the substrate CO_2 and the intensity of light as source of energy are two of the main limiting factors of photosynthesis. The activity of RuBPCase seems to be regulated by the degree to which these factors are present. Temperature is another factor affecting CO_2 fixation. The carboxylation of RuBP *in vitro* shows a sharp decrease with temperature (Mächler *et al.*, 1980). *In vivo* this decrease can be partly compensated for by an increased solubility of the substrate CO_2 at low temperatures and an increased activation of RuBPCase.

The stability of enzyme activity during extraction in the absence and presence of HCO_3^-

RuBPCase extracted from intact leaves in the absence of HCO_3^- changed its activity only slowly within the next few minutes (Fig. 5). If 20 mM $NaHCO_3$ was present in the extraction medium, the activity changed rapidly. Relatively inactive enzyme from leaves incubated in CO_2–free air was activated, whereas highly active enzyme from leaves incubated in 10% CO_2 was inactivated by $NaHCO_3$. Four minutes after extraction both enzymes had attained the same level of activity regardless of how the leaves had been preincubated. Extracted enzyme is known to

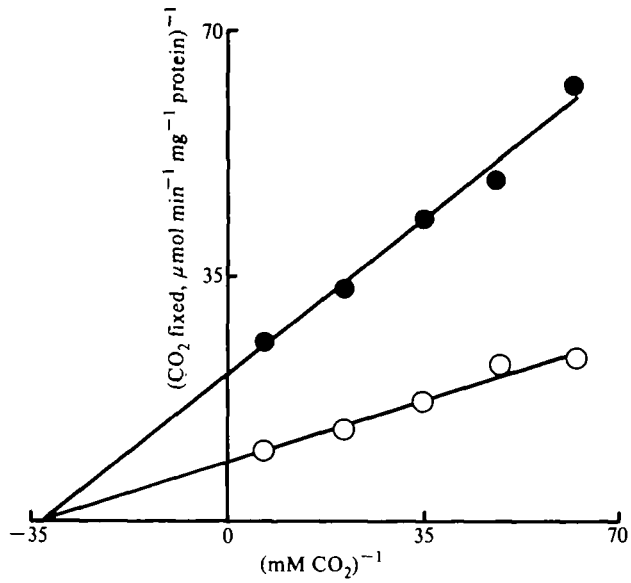

FIG. 4. Double reciprocal plots of RuBPCase activity as a function of CO_2 concentration with air level O_2 in the assay medium. RuBPCase was extracted rapidly from leaves incubated for 30 min in the dark (●) or at a light intensity of 800 μE m^{-2} s^{-1} (○) both at room temperature and 300 parts 10^{-6} CO_2. The activity of enzyme was tested at 10 °C. For details see Materials and Methods.

be activated by CO_2, not by HCO_3^- (Lorimer *et al.*, 1976). Figure 5 suggests that HCO_3^- inhibits the enzyme. This suggestion is supported by the interaction of CO_2 and pH on the activity of extracted RuBPCase (Fig. 6). The combination of an increased CO_2 concentration with a high pH inactivates RuBPCase, probably due

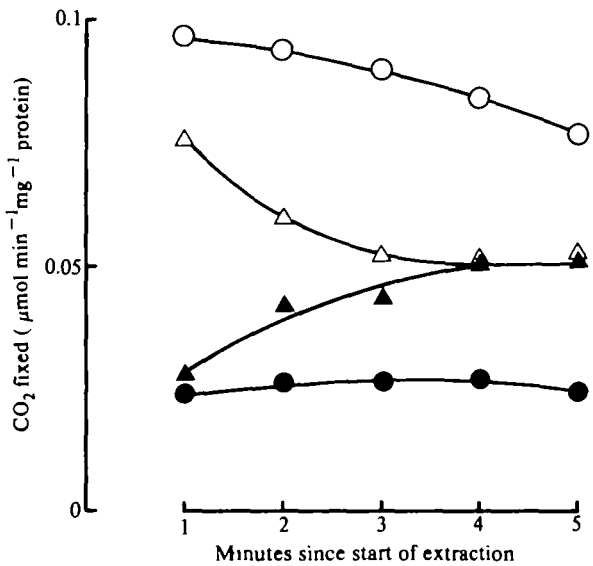

FIG. 5. Influence of presence (△, ▲) and absence (○, ●) of 20 mM $NaHCO_3$ in the extraction medium on the activity of RuBPCase. The enzyme was extracted rapidly from wheat leaves preincubated for 2 h in CO_2 free air (▲, ●) or 10% CO_2 (△, ○) in the dark at room temperature. The enzyme was tested at 10 °C. For details see Materials and Methods.

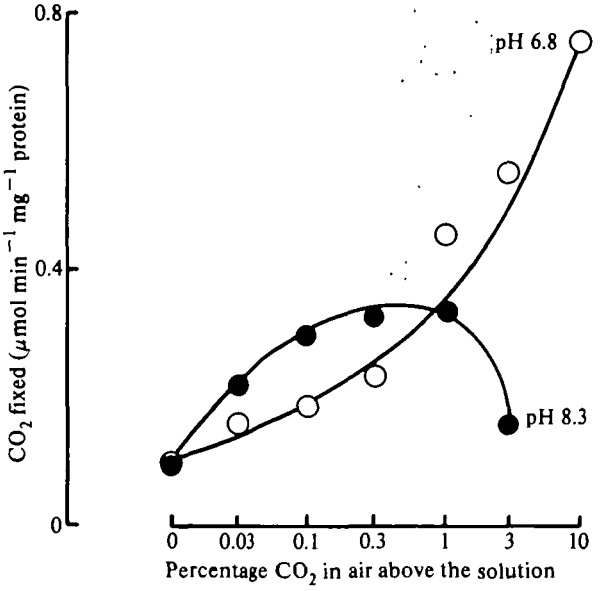

FIG. 6. Influence of CO_2 concentration and pH (○:pH 6·8, ●:pH 8·3) on the activity of RuBPCase in crude extracts from wheat leaves. The CO_2 concentrations in the extracts were in equilibrium with the indicated CO_2 concentrations in the air above the extracts. The enzyme activity was tested at 25 °C. For details see Materials and Methods.

to the equilibrium with an increased concentration of HCO_3^-. The high activity of enzyme extracted from leaves which had been incubated in 10% CO_2 (Fig. 5) is probably due to a low concentration of HCO_3^- in the stroma, since the pH of the stroma must be decreased by high concentrations of carbonic acid.

A comparison of the activity of rapidly extracted enzyme with purified enzyme activated by CO_2 and Mg^{2+}

V_{max} of enzyme rapidly extracted from illuminated leaves was 0·12 μmol CO_2 min^{-1} mg^{-1} protein at 10 °C. Assuming activity to be as similarly dependent on temperature as purified enzyme (Mächler *et al.*, 1980) and assuming 50% RuBPCase present in soluble protein, a specific activity of 1·8 μmol CO_2 min^{-1} mg^{-1} protein at 25 °C was calculated. This value is high compared with the estimates of activated purified enzyme. Purified wheat RuBPCase activated by CO_2 and Mg^{2+} attained a specific activity of 0·75 μmol CO_2 min^{-1} mg^{-1} protein at 25 °C (Mächler *et al.*, 1980). Purified spinach extracts showed a specific activity of 1·8 μmol CO_2 min^{-1} mg^{-1} protein at 30 °C (Lorimer *et al.*, 1977). However it is suggested that purified enzyme fully activated by CO_2 and Mg^{2+}, but not inhibited by HCO_3^-, would attain a much higher specific activity.

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