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Carbonic Anhydrase and Ribulose 1,5-Bisphosphate Regulate Ribulose 1,5-Bisphosphate Carboxylase/ Oxygenase Affinity for Carbon Dioxide

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The carhoxylase activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) was assayed in cowpea (*Vigna unguiculata* [L.] Walp. var. Bambey 21), a C_3 plant, with or without carbonic anhydrase (CA) and ribulose 1,5-bisphosphate (RuBP) added to the reaction mixtures. The apparent K_m (CO_2) and V_{max} were determined using the slatistical formula of Wilkinson and by the direct linear plot method of Cornish-Bowden and Eisenthal. During the experiments, V_{max} remained constant whereas K_m changed according to the assay conditions. It was found that K_m (CO_2) of Rubisco decreased below the commonly accepted value in the absence of added CA and with high concentrations of RuBP. The variations of K_m were discussed on the basis of the relative implication of RuBP and CA in the carboxylase activity. The pracf ical applications of these experiments were also highlighted.

Ribulose 1,5-bisphophate carboxylase/oxygenase (Rubisco, EC 4.1.13.9) is present in all photosynthetic organisms. It constitutes 50% of the soluble proteins in leaves (Lawlor, 1987), 15% of chloroplast proteins and is considered as the most abundant protein in tht biosphere (Hennen, 1998). Ruhisco is a bifunctionnl enzyme ca talyzing both yhotosynthetic CO_2 fixation and the competing photorespiration. In higher plants, this enzyme has a hexadecameric structure, with eight large suhunits (56 kDa) and eight small subunits (14 kDa) (Lawlor, 1987).

The decarbamylation of Rubisco occurs at low pH (-7.5) (Zhu and Jensen, 1991) at low light nnd even in darkness (Sage et al , 1993). The enzyme is also inactivated by the binding of sugar phosphates (i.e., xylulose 1,5-bisphosphate and 2-carboxy-D-arabinitol-1-phosphate) which tightly bind to the activated enzyme CO_2-Mg form (Lawlor, 1987; Portis, 1993).

The kinetic properties of the carboxylase activity of the enzyme are easily determined by spectrophotometric (Lilley and Walker, 1975; Besford, 1984) nnd by radioisotopical assays (Salvucci et al., 1986; Ward and Keys, 1989). The spectrophotometric assav has an advantage over radioisotopical assays in that the use of radioisotopically labeled substrates may change the specific activity during storage (Ward and Keys, 1989). Comparison of the K_m (CO_2) values among grass species suggests that the affinitv for CO_2

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Abbreviations :
CA, carbonic anhydrase; DTT, dithiothritol; HEPES, N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid; PGA, phosphoglyceric acid; PMSF, phenylmethyl-sulfonyl fluoride; PVP, polyvinlpyrrolidone; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate.

could help to discriminate between the photosynthetic C_3 and C_4 plants (Yeoh et al., 1980).

Carbonic anhydrase (CA, E.C. 4.2.1.1) is the other enzyme involved in CO, availability for photosynthesis. Thïs is a zinc containing protein of 180 kDa (Lawlor, 1987). It is an ubiquitous enzyme among living organisms and catalyses the reversible interconversion of bicarbonate (HCO_3^-) and CO_2 at physiological pH (Sultemeyer et al., 1993). The rate of conversion of HCO_3^- to CO_2 is normally slow in alkaline conditions. However, CA increases grea tly the equilibration rate and HCO_3^- is used as well as it is converted to CO_2 (Lawlor, 1987). In C_3 plants, in addition to facilitating CO, diffusion across the plasma membrane and chloroplast envelope, CA is assumed to participate in a special association with Rubisco. This association would allow CO, to be elevatd at the active site of Rubisco (Badger and Price, 1994).

Many workers have investigated Rubisco carboxylase activity by using spectrophotometric assay (Lilley and Walker, 1975; Besford 1984; Ward and Keys, 1989). Besford (1984) considered that Rubisco carboxylase activity in reaction mixture containing enzyme extract starts by addition of RuBP, even when the homogenate was centrifuged at low speed for a short time. Other workers added RuBP to the reaction medium prior to the addition of enzyme extract (Salvucci et al., 1986) without further explanation.

In spite of its presumed functions, CA is not always taken into account during routine spectrophotometric or radioisotopical assays of Rubisco activity. This enzyme lacks in almost all reaction mixtures including KHCO, or $NaHCO_3$ (radioactive or not) as inorganic carbon substrate (Machler et al., 1980; Lan and Mott, 1991; Reddy et al., 1998; Velitchkova and Fedina, 1998). The present payer analyses the effect of addition of CA and RuBP to reaction mixtures during routine spectrophotometric studies of carboxylation kinetic properties of Rubisco, and the importance of RuBP and CA in the determination of Rubisco affinity for CO,, through the specific function of both compounds in the carboxylation process.

MATERIALS AND METHODS

Rubisco was extracted from cowpea (*Vigna unguiculata* [L.] Wlap. var. Bambey 21) obtained from CERAAS. Plants were grown in a greenhouse under the following conditions : 22.7 ± 0.5°C, 35.0 ± 3.5% relative humidity, irradiance of 505 ± 10 µmol $m^{-2}s^{-1}$ and 14/10 day/night photoperiod, on a sand-compost-vermiculite mixtue (7.4% - 19% - 5.6%). Light was provided by SON-T 400 W lamps (Philips, Eindhoven, The Netherlands).

One gram of the third leaf (from the top of plants) was frozen in liquid nitrogen and finely ground on ice using a mortar and a pestle. The powder was suspended in 3 mL 50 mM HEPES-KOH (pH 8.0) containing 20 mM $MgCl_2$, 25 mM KHCO,, 0.2 mM Na,-EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2% insoluble PVP-40. The homogenate was centrifuged at 35000 x g for 30 min in a refrigerated centrifuge (4°C). Fractions of 10 µL of the supernatant were used for protein estimation by the Bio-Rad dye binding protein assay (Spector, 1987) (Bio-Rad Laboratories, Hercules, CA, IJSA). The remaining supernatant was used for Rubisco assay. Total chlorophyll was also extracted in 80% acetone and estimated according to Lichtenthaler and Wellbrun (1983). All the chemicals used, except the dye concentrate, were supplied by Sigma (St. Louis, MO, USA).

Rubisco activity was assayed spectrophotometrically by the continuous measurement of O.D. (Besford 1984). Reaction mixtures was 50 mM HEPES-KOH (pH 8.0) with a total volume of 2 mL in cuvettes of 1 cm path length and included : 20 mM $MgCl_2$, 0.2 mM Na,-EDTA, 5 mM DTT, 3.5 mM ATP, 3.5 mM ATP< 3.5 mM phosphocreatine, 0.35 mM NADH, 0 µmol or 0.66 µmol RuBP, 0 unit or 100 units CA, 10 units 3-PGA-phosphokinase, 6 units glyceraldehyde-3-phosphate-dehydro-genase, 16 units creatine phosphokinase, and KHCO, the concentrations of which ranged between 0.5 and 3 mM.

Extraction buffer and reaction medium were prepared CO_2 and O_2 free from N_2 bubbled wa-

ter for about 15 min before use (Besford, **1984;** Lan and Mott, 1991). Prior to the assay, the enzyme fractions were activated in 50 mM HEPES-KOH, pH 8, containing 20 mM $MgCl_2$ and 25 mM $KHCO_3$ for 35 min at 30°C. The effect of CA and RuBP addition to the reaction mixtures on Rubisco kinetic characteristics was assessed under four experimental conditions : (1) reaction medium with 100 units CA added and no RuBP added (2) reaction medium with 100 units CA and 66 µmol RuBP added, (3) reaction medium without CA and RuBP added, and (4) reaction medium without CA but with 0.66 µmol RuBP added. Enzyme assay was carried out for 4 min at 25°C. The RuBP quantity added (0.66 µmol) was chosen considering its instability in solution.

Rubisco carboxylase activity was assessed on the assumption that oxidation of 1 nmol of NADH reduces optical density by 0.00622, with a stoichiometry of 2:1 between NADH oxidation and RuBP carboxylation (Lilley and Walker, 1975).

The apparent $K_m(CO_2)$ and V_{max} values of the enzyme were calculated by the statistical method of Wilkinson (1961) and by a graphical method using the direct linear plot method (1 /V vs S, V) of Cornish-Bowden and Eisenthal (1977). These two methods were chosen for their more accurate detemination of K_m and V_{max} than the other linear plots which were shown to be statistically objectionable (Markus et al., 1976). Among these linear plots, the double-reciprocal plot was found to be the least accurate method (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1997). The CO_2 concentrations in the solution were calculated, using pKa values corrected for the effects of ionic strength (Umbreit et al., 1972), by the Henderson-Hasselbach equation (Lawlor, 1987).

RESULTS AND DISCUSSION

Rubisco carboxylase activity determined according to Lilley and Walker (1975) is presented as a function of CO_2 concentrations in the reaction mixtures (Fig. 1). This graph indicates the dependence of enzyme activity upon CO_2 concentrations.

Figure 2 represents the graphical determination of K_m and V_{max} for one of the four experimental conditions (100 units CA and without RuBP addcd). The same method was uscd for the other three conditions, and all results are summarized in Table I. When assays were carried out withour RuBP added to the reaction mixtures with 100 units CA added, K_m value was 24 µM (statistical method) and 20 µM (graphical method). When 0.66 µmol RuBP and 100 units CA werc added, this estimate was 14 µ M (statistical method) and 12 µM (graphical me thod).

On the contrary, when CA and RuBP were not added to the reaction mixtures, K_m reached 27 µM (statistical method) **and 20** µM (graphical method).

When CA was not added to the reaction medium, adding 0.66 µmol RuBP led to decrease in K_m value (4 µM by the statisticnl method and 2.8 µM by the graphical method) compared **with the** theoretical value of 12 µM (Lawlor, 1987).

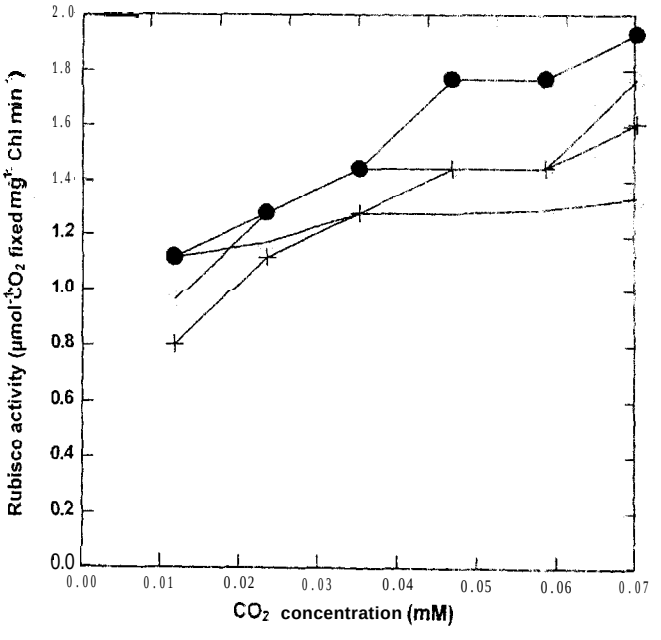
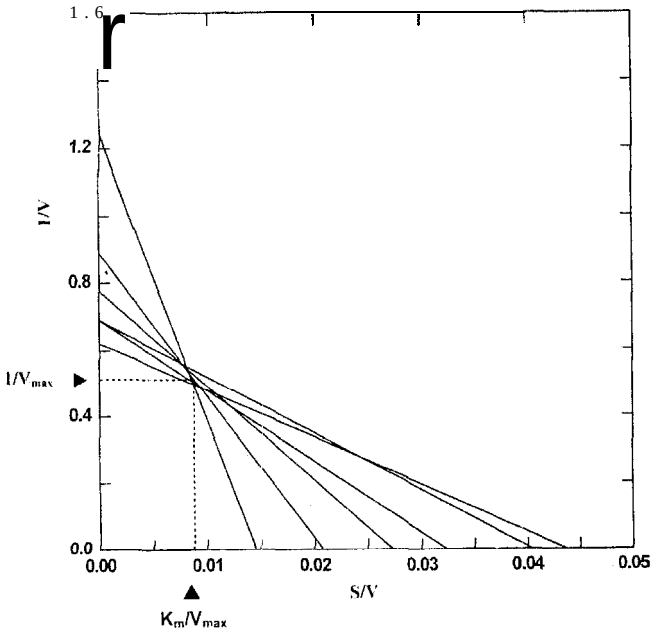


Figure 1. Rubisco activity as a function of CO_2 concentration in the reaction mixtures +CA-RuBP (+), +CA+RuBP (●) -CA-RuBP (□), -CA+RuBP (*). The enzyme activity was determined assuming that oxidation of 1 nmol of NADH reduces optical density by 0.00622, and' a stoichiometry of 2:1 between NADH oxidation and RuBP carboxylation. Data are means of results from three independent experiments with independent protein and chlorophyll extracts.



Figue 2. Direct linear plot of 1/V against S/V under an experimental condition with 100 units carbonic anhydrase and without ribulose 1,5-bisphosphate added. Each observation was plotted as straight line which intercepts another at the point (K_m/V_{max}, $1/V_{max}$). Each intersection point provided an estimate of K_m/V_{max} and an estimate of $1/V_{max}$. The best fit values K_M/V_{max} (0.0086) and $1/V_{max}$ (0.5068) were taken as the medians of the two series, respectively.

Statistical analysis of K_M and V_{max} (x^2 test) revealed no significant difference in V_{max} values ($P>0.30$) irrespective of the method of determination and the experimental conditions. On the contrary, K_m values were significantly different ($P\leq0.038$) according to the experimental condition. The K_m values obtained with both methods were not statistically different.

The different apparent K_m values obtained based upon the assay conditions, suggest that the affinity of the enzyme for CO_2 depends on RuBP and CA concentrations in the reaction mixtures. It is established that under alkaline conditions (i.e. pH 8), the conversion rate of HCO_3^- to CO, increases in the presence of CA, resulting in speeding up the euqilibrium between these two components (Okabe et al., 1980; Sultmeyer et al., 1993). So, when CA was added, the affinity of Rubisco for CO_2 depended on RuBP concentrations. The K_m value obtained when RuBP was not aded (Fig. 2 and Table I) expressed the affinity of the enzyme determined by the intracellular "normal" RuBP concentration of the extract. It is indeed known that leaf extracts to be testcd usually contain RuBP, among other compounds (Keys et al., 1995). This could explain why this K_m value is within the range of experimental K_m (CO_2) values (12 - 24 µM) reported elsewhere for the C_3 plants (Yeoh et al., 1980; 1981). Further, addition of RuBP to the reaction mixtures with 100 units CA added resulted in a significant increase in the affinity of Rubisco for CO, (Table 1) compared with its level when RuBP was not added. Undcr this condition, the RuBP added would have compensated the loss of this substrate due to its high instability in solution. This compensation would have en-

Table 1. *Kinetic parameters of Rubisco carboxylase function in the cowpea variety Bambey 21 under four experimen tal conditions.* Apparent K_m and V_{max} are expressed in µM CO_2 and µmol CO, fixed mg^{-1} Chl min-', respectively. Numbers with asterisk were determined by the direct linear plot method, and the others by the statistical method.

	100 units carbonic anhydrase		**0** unit carbonic anhydrase	
	K_m	V_{max}	K_m	V_{max}
0 µmol RuBP	24	1.52	27	1.98
	20	1.97"	20'	2.36'
0.66 µmol RuBP	14	1.77	4	1.37
	12'	1.93"	2.8"	1.39'

The K_m and V_{max} values are means of data from three independent cxperiments with independent protein and chlorophyll extracts.

hanced carbamylation and carboxylation, as CA made CO_2 available for these processes and hence, enhanced CO_2 fixation (Okabe et al., 1980). On the contrary, when CA and RuBP were not added (Table I), CO_2 concentration as well as RuBP concentration were low. Thus, the enzyme could utilize only RuBP present in the extracts. In addition, as the conversion rate of HCO_3^- to CO_2 is assumed to be very low under alkaline pH conditions without carbonic anhydrase (Okabe et al., 1980; Lawlor, 1987), the affinity of the enzyme would have been limited by the low concentrations of RuBP and free CO_2. This condition would have thereby limited carbamylation and carboxylation. This result suggests that *in vivo* CA concentration would have been strongly reduced in leaf extracts during extraction, even though this enzyme is presumed to be present in substantial excess in C_4 and C_3 leaves (Hatch and Burnell, 1990). As previously recorded, when CA was not added to the reaction mixture, low CO_2 concentrations were noted. So, the addition of RuBP would have slowed decarbamylation of Rubisco by displacing the equilibrium between the activated form (ECM) and the free enzyme (E) by mass action towards formation of ECMRuBP in accordance with the hyperbolic kinetics of catalysis with RuBP (Portis, 1995). This situation would have offered more opportunities for the carboxylation reaction to be performed easily and rapidly with the CO_2 available. This conditon resulted in an important increase in Rubisco activase which promotes maximum carbamylation at low CO_2, but saturation RuBP, by allowing RuBP binding to shift the carbamylation equilibrium towards the formation of ECM-RuBP (Portis, 1995).

These results may confirm that CA and RuBP are important for Rubisco affinity even in solutions. However, this activity seems to be more sensitive to low amounts of RuBP than to low amounts of CA. These could be explained by the implication of inhibitors of the enzyme (Rubisco) in its regulatory process. Indeed, it is established that RuBP may be rapidly converted to ribulose-5-phosphate. The latter compound may be in equilibrium with xylulose-5-phosphate. Xylulose-5-phospha te can by phosphoryla ted to xylulose-1,5-bisphosphate, an inhibitor of Rubisco (Lawlor, 2987; Gutteridge and Ga tenby, 1995). In addition, ribulose-5-phospha te can stimulate the synthesis of RuBP, but consumes ATP. Increased ADP then inhibi ts phosphoglycera te kinase and consequently, decreases CO_2 assi milation (J .awlor, 1987). The low affinity of Rubisco for CO_2, observed when RuBP was not added to the reaction mixtures may be due to such an inhibitory process. High concentrations of RuBP obtained when this substrate was added would therefore tend to supress any inhibi tory effect of the probable inhibi tors (Woodrow, and Berry, 1988; Portis, 1995). The fact that V_{max} remained statistically the same irrespective of experimental conditions applied suggests that inhibitors affected only a part of the catalytic sites (Keys et al., 1995). The uninhibited Rubisco si tes remained in the activated form (Woodrow and Berry, 1988) and may have been able to realize CO_2 fixation until they reached the maximum velocity level of Rubisco carboxylase activity.

From the results of this experiment, i t is suggested that an investigation of the origin of carbon assimilation limitations through Rubisco activity must consider the kinetic properties of the enzyme under these four conditions. This would be important to determine whether the eventual catalytic deficiency of this enzyme is causcd (1) by the lack of one of the substrates involved or (2) by the lack or alteration of its accompanving enzyme, carbonic anhydrase, or even (3) by its own alteration. It is further suggested that the classification of C_3, C_4 plants according to their Rubisco affinity for CO_2 be based on the determination of the apparent K_m (CO_2) values under these four conditions rather than using only one "optimal" condition. This would be useful for the understanding of photosynthetic CO_2 assimilation by these plants

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