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Communication

Activity Ratios of Ribulose-1,5-Bisphosphate Carboxylase Accurately Reflect Carbamylation Ratios[1]

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ABSTRACT

Activity ratios and carbamylation ratios of ribulose-1,5-bisphosphate carboxylase (RuBPCase) were determined for leaves of *Phaseolus vulgaris* and *Spinacia oleracea* exposed to a variety of partial pressures of CO_2 and O_2 and photon flux densities (PFD). It was found that activity ratios accurately predicted carbamylation ratios except in extracts from leaves held in low PFD. In particular, it was confirmed that the loss of RuBPCase activity in low partial pressure of O_2 and high PFD results from reduced carbamylation. Activity ratios of RuBPCase were lower than carbamylation ratios for *Phaseolus* leaves sampled in low PFD, presumably because of the presence of 2-carboxyarabinitol 1-phosphate. *Spinacia* leaves sampled in darkness also exhibited lower activity ratios than carbamylation ratios indicating that this species may also have an RuBPCase inhibitor even though carboxyarabinitol 1-phosphate has not been detected in this species in the past.

The activity of RuBPCase[2] *in vivo* is modulated to maintain the efficiency of CO_2 fixation under fluctuating environmental conditions which include changes in PFD (13), partial pressure of CO_2, $p(CO_2)$ (14), partial pressure of O_2, $p(O_2)$ (18), and mineral nutrition (3). Investigations focusing on regulation of CO_2 fixation by RuBPCase have shown that it is primarily variations in the activation state of the enzyme rather than fluctuations in the pool size of RuBP which modulate RuBPCase activity (1, 12).

We have been particularly concerned with feedback from triose phosphate use to RuBPCase activity. This feedback effect is studied by changing $p(O_2)$ at high PFD. When the $p(O_2)$ is reduced from 180 to 20 mbar, there is no change in net CO_2 uptake even though there is a substantial reduction in photorespiration (17). It is believed that phosphate availability is reduced by this feedback. In feedback limited *Phaseolus* leaves (18) and phosphate limited spinach chloroplasts (8) RuBPCase activation state was reduced. However, a recent report questions these observations (6).

Activation state determinations were made by taking the ratio of the RuBPCase activity immediately upon extraction to the activity obtained after incubation with CO_2 and Mg^{2+}. From this point, these measurements will be referred to as activity ratios instead of activation states. It has been assumed that under high PFD, changes in activity ratios are caused by changes in the CO_2-Mg^{2+} dependent activation of RuBPCase. Before a site on RuBPCase is catalytically competent, it must contain an activator CO_2 covalently bound to the epsilon amino group of a specific lysine residue believed to reside in the cleft of the site (11). The CO_2 bound to an amino group is called a carbamate. Once the carbamate is formed, Mg^{2+} is rapidly coordinated onto the site which is then capable of carboxylating RuBP. This process has been referred to as CO_2-Mg^{2+} dependent activation, but it may more accurately be termed carbamylation. It is believed that in the plant, carbamylation is catalyzed by the enzyme activase (19). By determining the number of sites which are carbamylated *in vivo* and dividing by the number of carbamylated sites after incubation with CO_2 and Mg^{2+}, the carbamylation ratio is obtained.

We have tested the assumption that activity ratios accurately reflect carbamylation ratios when the $p(O_2)$ is changed by comparing activity ratios with carbamylation ratios in *Phaseolus*. We found that activity ratios and carbamylation ratios were equivalent.

We also tested whether activity ratios directly reflect carbamylation ratios as PFD varied in different types of plants. Carboxylation rates are controlled not only by changes in the carbamylation of RuBPCase (1, 12) but also by changes in the level of CA1P (15, 16), a naturally occurring inhibitor of RuBPCase found in a number of species including *Phaseolus*. We have investigated the relationship between activity ratios and carbamylation ratios in *Phaseolus* and spinach under a variety of conditions which resulted in modulation of the rate of carboxylation by fluctuations both in carbamylation and in the level of CA1P present. We found that activity ratios accurately predict carbamylation ratios, except under low PFD when naturally occurring inhibitors increase carbamylation but not activity.

MATERIALS AND METHODS

Plant Material

Plants used in this study were grown in a controlled environment chamber with 60% RH on a photoperiod of 12 h light/12 h darkness and 24°C/16°C day/night temperatures.

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[2] Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); PFD, photo flux density; CABP, 2-carboxyarabinitol-1,5-bisphosphate; CA1P, 2-carboxyarabinitol 1-phosphate.

Photon flux density was 600 μmol $m^{-2}s^{-1}$. Plants of *Phaseolus vulgaris* L. cv Linden were grown in 4-L plastic pots in a soil:peat:perlite:rice hull mixture (3:3:3:2; v:v:v:v). Plants of *Spinacia oleracea* L. cv Long Standing Gaudry, were grown hydroponically in half-strength Hoagland solution B (9) with aeration. Experiments were carried out using samples from plants of various ages with the age of the plant proving inconsequential to the results obtained.

Treatments and Sampling

Prior to sampling, a variety of PFD, $p(CO_2)$, and $p(O_2)$ conditions were imposed on individual plants in order to obtain samples with various levels of CA1P and different activity ratios of RuBPCase. Plants of *Phaseolus* and spinach were sampled at midday in the growth chamber in order to determine the relationship between activity ratios and carbamylation ratios under conditions of normal PFD and $p(O_2)$ for comparison with the various treatments. Samples of *Phaseolus* exhibiting a lack of O_2-inhibition of photosynthesis were obtained from leaves exposed to 20 mbars O_2 and 800 μbars CO_2 for 20 to 30 min. Samples of *Phaseolus* containing CA1P were obtained from plants sampled after being exposed to PFD of 40 μmol m^{-2} s^{-1} for 4 h at 24°C. Because activity ratios were low in spinach leaves taken from the growth chamber, we incubated some leaves in high PFD (1500 μmol m^{-2} s^{-1}) and 200 μbars CO_2 for 1 h since it is known that low CO_2 and high PFD can increase the activity ratio (20).

Samples of healthy-looking, mature leaves of *Phaseolus* and spinach were taken using a hand-held freeze clamp prechilled in liquid N_2 and were kept in liquid N_2 until assayed. The freeze clamp produced 8.22 cm^2 samples. Individual samples not used the same day they were taken were transferred to −80°C for storage until assayed. All samples were used within 14 d of being taken. Storage at −80°C did not affect the relationship between carbamylation ratio and activity ratio, nor did it cause loss of activity of purified enzyme stored in 20% glycerol. We did not test whether storage at −80°C allowed the degree of carbamylation to change.

Determination of Activity Ratios and Carbamylation Ratios

Using a Ten Broeck tissue homogenizer, extracts were obtained by rapidly grinding 4.11 cm^2 *Phaseolus* leaf tissue or 2.05 cm^2 spinach leaf tissue in 2 ml ice-cold 50 mM Bicine (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 1.5% PVPP which had been prepared CO_2-free. The resulting solution was immediately transferred to a 1.5 mL microfuge tube and centrifuged in 6 s in a Beckman Microfuge E. Aliquots of the supernatant were used as the initial extract for activity and carbamylation determinations. After initial carbamylation and activity determinations were underway, the remaining extract was transferred to another tube and activated by addition of $MgCl_2$ and $NaHCO_3$ to a final concentration of 20 mM $MgCl_2$ and 10 mM $NaHCO_3$. This mixture was maintained at 25°C for 30 min then used for determination of the total carbamylation and total activity.

Activity ratios were obtained by dividing the activity of the initial extract by the total activity obtained after incubation with CO_2 and Mg^{2+}. Carbamylation ratios were obtained by comparing the retention of label by the initial extract with the retention of label by the activated extract.

Activity Measurements

RuBP was generated in 50 mM Bicine (pH 8.2), 10 mM $MgCl_2$, and 1 mM EDTA using phosphoriboisomerase (6 units/mL), phosphoribulokinase (free of RuBPCase activity, 2 units/mL), 2 mM ATP, and 1.5 mM ribose-5-P. After incubation for 20 min at room temperature, this solution was made CO_2-free and $NaH^{14}CO_3$ (American Radiolabelled Chemicals, St. Louis, MO) was added to give a concentration of 15 mM $NaHCO_3$ with a specific activity of 0.72 Ci/mol. Activity assays were started by adding 100 μL of extract to 250 μL of RuBP-containing buffer and stopped after 30 s by addition of 200 μL of 2 N HCl. The resulting solution was dried, and the residue was dissolved in 100 μL of H_2O. Bio-Safe II scintillation cocktail (3 mL) was added, and the acid stable ^{14}C was determined by liquid scintillation counting.

Carbamylation Measurements

Carbamylation assays were initiated by adding 25 μL of extract to 100 μL of 50 mM Bicine (pH 7.8), 5 mM $MgCl_2$, and 1 mM EDTA which had been prepared CO_2-free containing 0.5 μL of 2 mM ^{14}CABP. The mixture was vortexed and left at 35°C for 2 to 20 min; there was a modification when initial extract was used in that the microfuge tubes were kept on ice for this incubation. Five μL of ^{12}CABP was added and left for 1 to 5 min. The enzyme-CABP complexes were then precipitated and washed.

Two different methods were employed for precipitation of the RuBPCase-CABP complexes. One method, immunoprecipitation with rabbit anti-RuBPCase serum (4, 5), was carried out by adding 150 μL of rabbit antiserum to the enzyme-CABP mixture. After 2 to 12 h, the precipitate was collected by vacuum filtration on GA6-S Gelman membrane filters and washed with 0.85% NaCl, 10 mM $MgCl_2$, 35 mM Na_2HPO_4, and 5 mM NaH_2PO_4 (pH 7.6). Each filter was transferred to a scintillation vial; 100 μL of H_2O was added followed by 3 mL of Bio-Safe II scintillation cocktail.

For the second precipitation method, PEG 4000 and $MgCl_2$ were added to the RuBPCase-CABP mixture to give a final concentration of 20% PEG 4000 and 25 mM $MgCl_2$ (10). The resulting solution was vortexed and placed on ice for 30 min for precipitation. After centrifugation at 27,000*g* for 10 to 12 min, the supernatant was discarded and the pellet was washed three times using a total volume of 3 mL of 50 mM Bicine (pH 7.8), 15 mM $MgCl_2$, 1 mM EDTA, and 20% PEG 4000. The pellet remaining after the third wash was resuspended in 100 μL of 50 mM Bicine (pH 7.8), 5 mM $MgCl_2$, and 1 mM EDTA. Each sample was transferred to a scintillation vial, and 3 mL of Bio-Safe II scintillation cocktail was added. The ^{14}C retained by each sample was determined by liquid scintillation counting.

Preparation of CABP

Radiolabeled CABP was prepared by combining 17.2 μmol RuBP (Sigma Chemical Co.) in 50 mM Bicine (pH 7.8), 10

mM $MgCl_2$, and 0.5 mM EDTA with 19.2 μmol $K^{14}CN$ (53 Ci/mol) (Amersham). This mixture was allowed to react for 16 h at which time 3.5 mL of 1% formic acid was added to hydrolyze the nitriles to the carboxylic acids. The products were dried under vacuum on a rotary film evaporator, washed once with H_2O, and brought to dryness a second time. The residue was dissolved in H_2O to give a final concentration of 2mM ^{14}CABP. We did not separate CABP from 2-carboxyribitol 1,5-bisphosphate, and we assumed complete conversion of RuBP to CABP + 2-carboxyribitol 1,5-bisphosphate. The first step in the preparation of ^{12}CABP was generating RuBP in 50 mM Bicine (pH 7.8), 10 mM $MgCl_2$, and 0.5 mM EDTA using phosphoriboisomerase (24 units/mL), phosphoribulokinase (free of RuBPCase activity, 4 units/mL), 0.21 mmol ATP, and 0.22 mmol ribose-5-P. After 45 min, 0.22 mmol NaCN was added. This reaction mixture was left for 16 h at 25°C at which time 3.5 mL 2% formic acid was added for hydrolysis of the nitriles to the carboxylic acids. After freeze drying, the products were dissolved in H_2O to give a final concentration of 200 mM ^{12}CABP.

To test the binding of CABP we isolated RuBPCase from spinach leaves on a sucrose density gradient (2). This enzyme had a specific activity of 1.7 μmol mg^{-1} protein min^{-1} (Bradford dye binding protein assay with BSA as a standard). We found about 6.5 binding sites per mol of enzyme as was found by Hall et al. (7). It is unclear why only 6.5 sites are often found but given the correlation between activity ratios and carbamylation ratios, we suggest that some protein in the RuBPCase preparation may be inactive and also not capable of binding CABP.

RESULTS AND DISCUSSION

Determination of Carbamylation of RuBPCase

A simple description of the use of ^{14}CABP in quantitating the number of carbamylated sites in a RuBPCase-containing extract is shown in Figure 1. As seen in the top half of Figure 1, CABP bound to native sites of RuBPCase is rapidly exchanged. This allows any ^{14}CABP bound to such uncarbamylated sites to be replaced by ^{12}CABP when a 500-fold excess of ^{12}CABP is supplied. Carbamylation of a catalytic site produces some conformational change which results in very tight binding of CABP (as represented by the bottom half of Fig. 1). Radiolabeled CABP is thus bound essentially irreversibly to carbamylated sites and is retained on the carbamylated RuBPCase when challenged by a 500-fold excess of ^{12}CABP. This provides the basis for our measurement of the carbamylation ratio (7).

Carbamylation Ratio is Correlated with Activity Ratio in the Absence of CA1P

Under high PFD and normal atmospheric conditions (210 mbars O_2 and 350 μbars CO_2), activity ratios and carbamylation ratios were the same in *Phaseolus vulgaris* (Table I, line 1). The catalytic constant for RuBPCase, labeled k_{cat}, is the total activity divided by the total number of sites. Under these normal conditions, the k_{cat} was 20 mol CO_2 mol^{-1} enzyme s^{-1} which is similar to that seen previously in this species (18).

It has been suggested that the lack of O_2-inhibition of photosynthesis observed under conditions of high PFD and high $p(CO_2)$ is due to an imbalance between the rate of triose-P production and the capability for the utilization of triose-P in starch and sucrose synthesis. In order to generate this feedback limitation of photosynthesis, leaves of *Phaseolus* were placed in low $p(O_2)$ and high $p(CO_2)$. Under these conditions, the activity ratio was reduced to 54% and the carbamylation ratio was reduced to the same extent (Table I, line 2). The k_{cat} was unaffected by changing O_2 and CO_2 conditions; therefore, the activity ratio measurements reflected the degree of carbamylation of RuBPCase.

Spinach was used because it is thought that it does not produce the inhibitor CA1P (15). The relationship between activity ratio and carbamylation ratio over a wide range of conditions is shown in Figure 2. A linear regression of all the points not taken in darkness had a slope of 1.003 and a y-intercept of −0.4% ($r^2 = 0.996$). The results indicate that the activity ratio is equivalent to the carbamylation ratio when samples taken in darkness are excluded.

Comparison of Carbamylation Ratio to Activity Ratio in the Presence of CA1P

Leaves of *Phaseolus* were sampled in low PFD in order to assess the effect of the presence of CA1P on activity and carbamylation ratio determinations. Under low PFD, CA1P was present as indicated by the reduced k_{cat} of 11 s^{-1} (Table I, line 3). In the presence of CA1P, there is no longer agreement between the activity ratio and the carbamylation ratio (Table

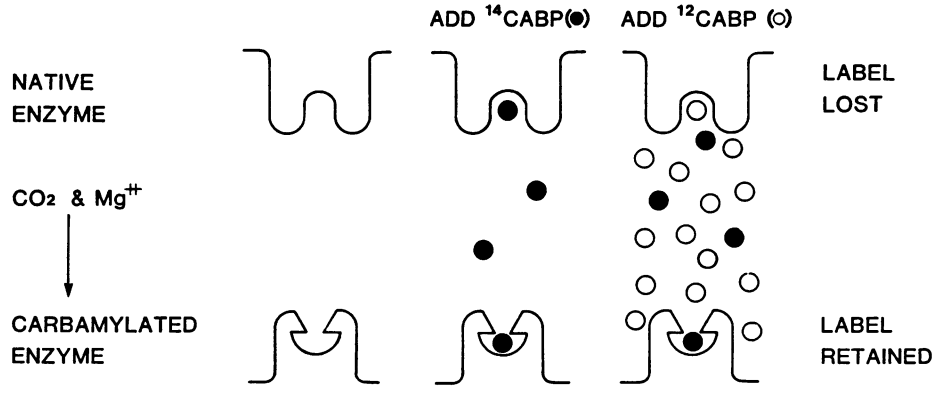

Figure 1. Measurement of RuBPCase using ^{14}CABP and ^{12}CABP.

Table I. *Carbamylation Ratio, Activity Ratio, and Catalytic Constant of RuBPCase from P. vulgaris*

	Carbamylation Ratio	Activity Ratio	k_{cat}
	%		s^{-1}
Normal air	80 ± 5	80 ± 4	20 ± 1
Low O_2 and high CO_2	56 ± 3	54 ± 1	18 ± 1
Low light	69 ± 2	59 ± 6	11 ± 1

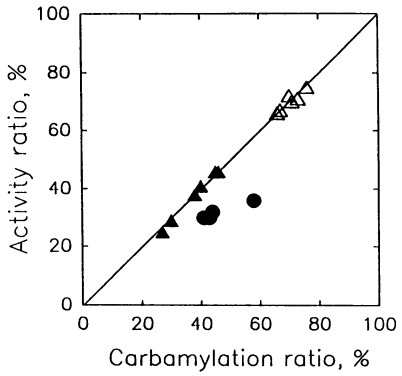

Figure 2. Activity ratio of RuBPCase as a function of carbamylation ratio for leaves of *Spinacia oleracea*. Samples were taken in darkness (circles), in the growth chamber with the lights on (solid triangles), or 1500 μmol photons m^{-2} s^{-1} and 200 μbar CO_2 to increase the activation state (open triangles). The line is a linear regression on all points taken in light and has a slope of 1.003, a *y* intercept of −0.4% (r_2 = 0.996).

I, line 3). A similar effect was observed in samples of spinach taken in darkness. The carbamylation ratio was higher than the activity ratio which indicates the presence of CA1P or a similar compound. However, RuBP bound to inactive enzyme would not account for these results.

The activity ratio is representative only of sites not bound with CA1P. The carbamylation ratio measures all of the sites, whether they are bound with CA1P or not. This is because CABP has a higher affinity for carbamylated sites than does CA1P. A decreased activity ratio relative to carbamylation ratio indicates that sites bound with CA1P are more often carbamylated than sites not bound with CA1P. This is expected considering the higher affinity of CA1P for carbamylated sites relative to uncarbamylated sites. Since the activity ratio tells the amount of carbamylated RuBPCase not bound with CA1P, the activity ratio can be multiplied by the proportion of inhibitor-free sites to determine the RuBPCase activity *in vivo*. For example, in Table I, assuming the ratio of k_{cat} in low light to that in high light to reflect the proportion of inhibitor free sites (18), then

$$0.59 * 11/20 = 0.32.$$

In this case, RuBPCase activity in the dark treated leaves of *P. vulgaris* was 32% of the potential maximum.

CONCLUSIONS

In the absence of CA1P, the activity ratio of RuBPCase is equal to the carbamylation ratio of RuBPCase. These results confirm the conclusion of Sharkey et al. (18), which was based on activity ratio determinations, that it is the carbamylation (CO_2-Mg^{2+} dependent activation) of RuBPCase which varies sufficiently to account for a lack of O_2-inhibition of photosynthesis. When CA1P is present, activity ratios may be used to calculate the activity of uninhibited RuBPCase while carbamylation ratios, when compared to activity ratios, provide information on the carbamylation of the inhibitor-bound sites.

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