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Regulation of Ribulose-1,5-Bisphosphate Carboxylase Activity in Response to Changing Partial Pressure of O_2 and Light in *Phaseolus vulgaris*[1]

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ABSTRACT

The regulation of ribulose-1,5-bisphosphate (RuBP) carboxylase (rubisco) activity in *Phaseolus vulgaris* was studied under moderate CO_2 and high light, conditions in which photosynthesis in C_3 plants can be insensitive to changes in O_2 partial pressure. Steady state RuBP concentrations were higher, the calculated rate of RuBP use was lower and the activation state of rubisco was lower in low O_2 relative to values observed in normal O_2. It is suggested that the reduced activity of rubisco observed here is related to feedback effects which occur when the rate of net CO_2 assimilation approaches the maximum capacity for starch and sucrose synthesis (triose phosphate utilization). The activation state of rubisco was independent of O_2 partial pressure when light or CO_2 was limiting for photosynthesis. Reduced activity of rubisco was also observed at limiting light. However, in this species light dependent changes in the concentration of an inhibitor of rubisco controlled the apparent V_{max} of rubisco in low light while changes in the CO_2-Mg^{2+} dependent activation of rubisco controlled the apparent V_{max} in high light.

Efforts to understand the biochemical factors which determine the rate of photosynthetic CO_2 assimilation by intact leaves have focused on the reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39). When photosynthesis is light saturated and CO_2 is in rate limiting supply, the rate of catalysis is linearly related to the V_{max} of rubisco[2] (5, 13, 19). When photosynthesis is light limited, the potential rate of RuBP regeneration determines the rate of photosynthesis (23). Models of photosynthesis assumed that when light limited photosynthesis, rubisco activity would be regulated by substrate (*i.e.* RuBP) availability (5). However, measurements of steady state RuBP levels in leaves indicated that enzyme activation rather than substrate availability can often regulate rubisco activity (1, 16, 17, 27). Experiments by Mott *et al.* (16) showed that RuBP levels measured immediately after a rapid decrease in light were linearly related to the rate of photosynthesis and only after some time in low light did the RuBP levels recover to their original level. Mott *et al.* suggested that this increase was a consequence of rubisco deactivation. The rate of photosynthesis did not change during the time when RuBP levels were increasing and rubisco was presumably deactivating. We interpret these results to imply that the reduction of rubisco activity does not limit photosynthesis in low light.

While most studies of rubisco activation and RuBP levels are interpreted in terms of either light or CO_2 limitations, Walker and Herold (30) pointed out that TPU could also limit the rate of photosynthesis. Recent studies (22–26) have related the phenomenon of O_2 insensitive photosynthesis in C_3 plants to a TPU limitation of photosynthesis. This can be observed consistently under conditions of high light intensity and high partial pressure of CO_2 (500 μbar internal). When the partial pressure of O_2 is lowered from 180 to 20 mbar there is often little or no change in net CO_2 uptake even though there is a substantial reduction in photorespiration (12, 24). It has been suggested that the expected increase in CO_2 assimilation does not occur because the capacity to use triose-P in starch and sucrose synthesis is lower than the potential rate of triose-P production. This causes a feedback inhibition of the photosynthetic carbon reduction cycle. The RuBP level is high under conditions which cause O_2 insensitivity (25), indicating that regulation of rubisco activity may also be important in TPU limited photosynthesis.

We have investigated the regulation of rubisco activity in *Phaseolus vulgaris* under conditions which caused O_2 insensitivity and under conditions of limiting light. We found that rubisco activation state varied with changes in partial pressure of O_2 sufficient to account for O_2 insensitivity.

We have also considered the mechanism of control of activation in *Phaseolus*. There are two known mechanisms of regulation of rubisco activity: CO_2-Mg^{2+} activation (15) and a light modulated inhibitor of rubisco (20, 21) which is apparently found in many species (29), including *P. vulgaris*. When the catalytic site of rubisco does not have both CO_2 and Mg^{2+} bound to it is catalytically incompetent (14); therefore activation state regulation affects the apparent V_{max} of rubisco. Sites which have the inhibitor bound to them are catalytically incompetent; therefore, the inhibitor also affects the apparent V_{max} of rubisco. The regulation of rubisco activity in high light was accomplished by the CO_2-Mg^{2+} activation mechanism while in low light the inhibitor of rubisco regulated rubisco activity.

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[2] Abbreviations: A, rate of photosynthetic CO_2 assimilation; C_i, partial pressure of CO_2 in the intercellular spaces of the leaf; R, calculated rate of RuBP use; rubisco, ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39).; RuBP, ribulose 1,5-bisphosphate; TPU, triose phosphate utilization; CABP, carboxyarabinitol bisphosphate; PGA, P-glycerate.

MATERIALS AND METHODS

Plant Material. *Phaseolus vulgaris.* L. var Tendergreen (seeds from Northrup King) plants were grown in 4-L plastic pots in compost:sand:perlite mixture (2:1:1, v:v:v) in a greenhouse. The temperature was controlled at 27°C day, 15°C night; RH was controlled at 60%.

Gas Exchange. Air was mixed from N_2, O_2, and 3% v/v CO_2 in air using mass flow controllers (FC 260, Tylan, Carson, CA). Some of this synthetic air passed through an aluminum leaf chamber. The air flow through the chamber was controlled by a mass flow controller. Some of the synthetic air and air from the leaf chamber was compared for water content and CO_2 content with a Binos IR gas analyzer (Leybold-Hereaus, Köln W. Germany). Cross sensitivity of the CO_2 measuring section to water was eliminated by condensing the water out of the air as the air passed from the water measuring tube to the CO_2 measuring tube.

Leaf temperature was measured with a copper-constantan thermocouple probe (SCPSS-020G-6; Omega Engineering Inc., Stamford, CT).

Calculations of evaporation, conductance to gas exchange, photosynthesis, and intercellular CO_2 partial pressure were made according to von Caemmerer and Farquhar (28). The flux of RuBP was calculated from the measured rate of net CO_2 fixation and the intercellular partial pressures of O_2 and CO_2 as described in (5).

"Light" is used to describe photosynthetic photon flux (areal) density. Light was measured with a LiCor quantum sensor (190 SB and LI 188B). Light was provided by a quartz halogen lamp through light guides. Lenses at the end of the light guides were used to adjust the intensity of the beams so that the light quality did not change when the intensity was changed. The unit of pressure used for gas partial pressures is bars because this has the same relative magnitude as mole fraction. One bar is equal to 10^5 Pa.

Stopping Metabolism. The metabolites involved in photosynthesis turn over extremely fast. Theoretical calculations suggest that RuBP pool turnover times of 0.5 s may occur. The fastest turnover time measured so far has been 1 s (TD Sharkey, JR Seemann, unpublished data). (Pool turnover rate = pool size [μmol m^{-2}]/assimilation rate [μmol $m^{-2}s^{-1}$] = s. This is actually a slight under estimate of pool turnover rate because photorespiration has not been taken into account.) To stop metabolism faster than the pool turnover time a device was made in which lead screws turned by an electric motor coupled through a magnetic clutch caused circular copper heads cooled with liquid N_2 to be driven through the Saran windows of a gas exchange chamber. Between the time that the light beams were interrupted and the time when the leaf material was below 0°C, 0.25 s elapsed. Freezing of the leaf material could be judged by a transient increase in temperature that occurred when the leaf was −5°C, about 0.3 s after light interruption.

Analysis of Rubisco Activity and RuBP Pool Sizes. The leaf piece produced by the freeze clamp machine was usually divided into two 3 cm^2 pieces and stored under liquid N_2 until analysis. One piece was rapidly extracted in ice-cold 100 mM Bicine, pH 7.8, 5 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, and 1.5% PVPP which had been prepared CO_2 free. After centrifugation in a microfuge (Eppendorf model 5414) for 10 s an aliquot of this extract was assayed for 30 s at 25°C for rubisco activity. This procedure required approximately 2 min from extraction to assay. This activity is called the 'initial' activity and is believed to reflect the *in vivo* activity of rubisco. Another aliquot of this extract was made up to 10 mM $NaHCO_3$ and 20 mM $MgCl_2$ by addition of a small amount of concentrated stock. After incubation at 23°C for 10 min rubisco activity was again assayed. This 'total' activity (representing the maximum activable activity of rubisco) was divided into the initial activity to arrive at the percent activation which is reported in this paper.

Rubisco activity was assayed as described in Seemann *et al.* (18) and described briefly here. RuBP was generated 15 min prior to the assay in the assay buffer (100 mM Bicine, pH 8.2, 20 mM $MgCl_2$, 1 mM EDTA) using phosphoriboisomerase (6 units/ml; Sigma, from yeast), phosphoribulokinase (free of rubisco activity, 2 units/ml), 2 mM ATP (Sigma), and 1.5 mM ribose 5-P (Sigma). $NaH^{14}CO_3$ (0.8 Ci/mol) (Amersham) was 15 mM in the assay. Assays (final volume = 0.5 ml) were started by the addition of extract and stopped with 0.3 ml of 2 N HCl. Acid stable ^{14}C was determined by liquid scintillation counting.

The concentration of rubisco was determined by radiolabeling each catalytic site of the enzyme with [^{14}C]CABP and precipitation of the enzyme-CABP complex with antibodies, as described by Collatz *et al.* (2) and Evans and Seemann (4). The total activity was expressed as apparent kcat. Reductions from the maximum kcat are presumed to result from the inhibitor. The maximum kcat observed during these experiments was 24.2 s^{-1}. To calculate the inhibitor free sites relative to the total binding sites the apparent kcat of each sample was divided by 24.2 s^{-1}. This gives a number which is conceptually similar to the activation state which is often calculated.

RuBP pool size was measured using the other 3 cm^2 portion of leaf. This was ground to a fine powder in liquid N_2 and extracted in 1.3 ml of 3% $HClO_4$. This extract was kept on ice for at least 15 min, and then centrifuged for 3 min in a microfuge. Ten μl of saturated KCl were then added to the supernatant and the pH of this mixture was adjusted to pH 7 with 5 N KOH containing 400 mM Hepes. After centrifugation, the supernatant was frozen, thawed and recentrifuged to remove additional potassium perchlorate. Aliquots of this supernatant were then freeze-dried in vials in which the RuBP assay would be carried out. RuBP concentration was determined as ^{14}C incorporation into acid stable counts using purified spinach rubisco in an assay buffer similar to that described above. RuBP concentration is expressed relative to the concentration of rubisco active sites (binding sites for CABP) determined on the other half of the sample.

RESULTS

Regulation of Rubisco During O_2 Insensitive Photosynthesis. The rate of CO_2 assimilation by leaves of *P. vulgaris* in high light and C_i about 500 μbar was the same in either 180 mbar O_2 (the normal partial pressure of O_2 in Reno, 1300 m elevation) or 30 mbar O_2 (Table I). Because the CO_2 assimilation rate stayed constant and oxygenation of RuBP was eliminated, the calculated rate of RuBP use declined by about 20% when O_2 was removed. Under these conditions the activation state of carboxylase was also 20% less in leaves freeze-killed in 30 mbar O_2 than in leaves killed in 180 mbar O_2.

When C_i was controlled at 190 μbar in 30 mbar O_2, photosynthesis was slightly lower than when C_i was 500 μbar (Table I) and the activation state of rubisco was nearly the same as in 180

Table I. *Assimilation Rate (A) Intercellular CO_2, C_i, and CO_2-Mg^{2+} Activation State of Rubisco at Normal and Low O_2 Partial Pressure*

R is the calculated rate of use of RuBP. Leaf temperature was 25°C and the light intensity was 1500 μmol quanta m^{-2} s^{-1}. The leaf to air water vapor pressure difference was 10 mbar.

O_2	A	C_i	R(calc)	Activation
mbar	*μmol m^{-2} s^{-1}*	*μbar*	*μmol m^{-2} s^{-1}*	%
180	18 ± 2	510 ± 3	24 ± 2	76 ± 1
30	18 ± 2	508 ± 3	19 ± 2	60 ± 1
30	15 ± 1	190 ± 4	18 ± 1	73 ± 1

mbar O_2 and 500 μbar C_i.

The effect of light on O_2 sensitivity of activation is shown in Table II. At 200 μmol quanta m^{-2} s^{-1}, CO_2 assimilation exhibited the O_2 sensitivity expected under these conditions (22). The activation of rubisco was the same at both partial pressures of O_2. About 30% of the rubisco sites were apparently blocked by inhibitor but this was not affected by O_2 partial pressure. At 600 μmol quanta m^{-2} s^{-1} the ratio of A in normal O_2 to that in low O_2 was 86 ± 1% for all leaves used. Some deactivation of rubisco occurred in low O_2 and the level of RuBP was greater in low O_2. This result may indicate that triose-P use limited photosynthesis to some degree, especially in low O_2, even though substantial O_2 sensitivity was observed. At 1200 μmol quanta m^{-2} s^{-1}, saturating for photosynthesis in these plants, CO_2 assimilation was lower in 18 mbar O_2 than in 180 mbar O_2. Each leaf used was tested and any leaf which did not have this behavior was not used. The RuBP level was higher and the activation state of rubisco was lower in low O_2. The change in RuBP level had no effect on the rate of CO_2 assimilation but may have influenced the Pi metabolism of the leaf. The proportion of inhibitor free sites was the same in both partial pressures of O_2.

Insensitivity to O_2 can be induced by feeding mannose to leaves (8). 2-Deoxyglucose causes similar effects but is less toxic, so we tested the effects of deoxyglucose on the activation state of rubisco. At C_i = 340 μbar, CO_2 assimilation was marginally O_2 sensitive. The ratio of A in normal O_2 to that in low O_2 was 96 ± 3% for the leaves used in this experiment. In the control leaves there was some deactivation of rubisco at low O_2. After feeding 5 mM deoxyglucose, CO_2 assimilation was inhibited by low O_2, and the activation state of rubisco was very low in low O_2. The level of RuBP was less than binding site concentration but did not vary with O_2 after feeding deoxyglucose (Table III).

Regulation of Rubisco in Response to Light. We tested the role of activation state of rubisco by measuring samples freeze clamped after 20 min in darkness, 20 min in 100 μmol photons m^{-2} s^{-1} or 20 min in 1000 μmol m^{-2} s^{-1}. Leaves in darkness had greater than 100% activation but very low activity (Table IV), a condition which occurs when the inhibitor of rubisco is present (20). In 100 μmol m^{-2} s^{-1} the activation state was only slightly lower than in 1000 μmol m^{-2} s^{-1}. The effect of light on the proportion of inhibitor free sites was greater than its effect on activation state.

DISCUSSION

The CO_2-Mg^{2+} activation of rubisco varied sufficiently to account for a constant rate of photosynthetic CO_2 assimilation with changing O_2 under TPU limited conditions. These results confirm the prediction made by Sharkey (23) that rubisco deactivation is the mechanism by which net photosynthetic CO_2 assimilation in C_3 plants becomes O_2 insensitive. The light-dependent inhibitor recently described by Seemann *et al.* (20) and Servaites (21) accounted for the bulk of the regulation of rubisco in response to light in *Phaseolus* but appeared to play no role in the regulation of rubisco under conditions of TPU limitation.

Speculation on the Limitation which Causes O_2 Insensitivity. Feeding deoxyglucose caused both the RuBP pool size and rubisco activation state to be lower than in control leaves. Deoxyglucose inhibits photosynthesis by sequestering Pi in the cytosol, making it unavailable for photosynthesis (11). Since feeding deoxyglucose also leads to O_2 insensitivity (8), low Pi availability has been postulated to be the cause of O_2 insensitivity (25). Chloroplasts photosynthesizing in a medium containing low Pi exhibit higher RuBP levels and lower rubisco activation states than control chloroplasts (9) just as observed for leaves in low O_2 in the present study. While the level of Pi inside chloroplasts from leaves exhibiting O_2 insensitivity has not been measured, low ATP/ADP ratios in such leaves have been measured (26). It has been hypothesized that Pi levels fall when triose-P are not used as fast as produced (24) and so Pi is not released during sucrose synthesis as fast as required. This form of feedback inhibition of photosynthesis was predicted by Herold (10). The results presented here indicate that the mechanism of this feedback is deactivation of rubisco rather than substrate limitation of the rubisco reaction.

The steps leading from TPU limitation to rubisco deactivation may be as follows. Lack of availability of Pi leads to a low ATP level which causes the PGA pool to increase because of inhibited PGA reduction. Since PGA is an acid, for every PGA produced, one H^+ is also produced (assuming a pH near 8 inside the chloroplast). This H^+ is normally consumed during the reduction of PGA to triose-P but without sufficient ATP, the PGA and the H^+ will build up. This buildup can be as great as 200 nmol mg^{-1} Chl inside the chloroplast (26). The lowered stromal pH would then affect key regulatory enzymes of the photosynthetic carbon reduction cycle. Rubisco is deactivated by low pH (15) and Ru5P kinase is strongly inhibited by PGA^{2-} making PGA a potent inhibitor at low pH (7). In addition both stromal bisphosphatases are directly inhibited by low pH (3, 6). While any other regulatory mechanisms are important, especially in the regulation of the bisphosphatases, this single point of regulation by pH would affect all parts of the cycle in a coordinated manner.

Under low light it is believed that the total level of rubisco does not limit the rate of photosynthesis but that the ATP supply is the primary limiting factor (23). Under O_2-insensitive conditions ATP supply is also suspected to limit photosynthesis. We propose that when photosynthesis is limited by ATP supply, the apparent V_{max} of rubisco is reduced and so the RuBP pool may not decline. Therefore, the reduction of rubisco activity is a symptom of limited capacity for ATP generation caused by either

Table II. *Effect of Light on O_2 Insensitivity and Rubisco Activation*

Measurement conditions given in legend for Table I. Experiments were done so that values at each light intensity are comparable but between light intensities the values are not strictly comparable. RuBP levels are expressed as mol RuBP/mol of CABP binding sites of rubisco, assuming 8 mol sites/mol rubisco and a mol wt of 550,000.

Light	O_2	A	C_i	RuBP	Activation	Inhibitor Free Sites
μmol m^{-2} s^{-1}	*mbar*	*μmol m^{-2} s^{-1}*	*μbar*	*mol mol^{-1}*	*%*	*%*
1200	180	20 ± 3	403 ± 30	3.9 ± 0.4	95 ± 5	100 ± 5
1200	18	16 ± 3	431 ± 48	6.4 ± 1.4	77 ± 10	99 ± 3
600	180	16 ± 2	529 ± 6	0.9 ± 0.3	82 ± 2	84 ± 1
600	18	20 ± 2	499 ± 23	1.6 ± 0.5	65 ± 2	88 ± 2
200	180	8 ± 1	510 ± 19	2.1 ± 0.1	88 ± 1	70 ± 0
200	18	11 ± 1	467 ± 20	3.2 ± 0.6	86 ± 4	70 ± 4

Table III. *Effect of 2-Deoxyglucose (2-DOG) on Assimilation Rate, RuBP Pool Size and CO_2-Mg^{2+} Activation at Normal and Low O_2 Pressure*

Other measurement conditions are given in Table I.

	O_2	A	C_i	RuBP	Activation
	mbar	*μmol m⁻² s⁻¹*	*μbar*	*mol mol⁻¹*	*%*
Control	180	21 ± 5	333 ± 12	0.7 ± 0.2	68 ± 0
	18	24 ± 2	355 ± 3	1.4 ± 0.3	57 ± 3
5 mM 2-DOG	180	15 ± 1	355 ± 13	0.8 ± 0.3	60 ± 3
	18	11 ± 1	364 ± 2	0.7 ± 0.2	42 ± 7

Table IV. *Effect of Light on Activity of Rubisco in Air*

Measurement conditions given in legend of Table I.

Light	A	C_i	RuBP	Activation	Inhibitor Free Sites
μmol m⁻² s⁻¹	*μmol m⁻² s⁻¹*	*μbar*	*mol mol⁻¹*	*%*	*%*
Dark	−2	298	0.3	141	10
100	3	274	1.0	92	66
1000	9	274	3.0	98	83

low light or limited capacity for starch and sucrose synthesis, rather than an inherent limitation or colimitation of photosynthesis. An increase in the activity of rubisco would only transiently increase the rate of photosynthesis until the underlying limitation (*e.g.* TPU or low light) reduced the rate of photosynthesis by some other mechanism. We speculate that regulation of rubisco may serve to minimize negative feedback interactions and so enhance rather than limit photosynthesis.

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