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Regulation of Photosynthetic Carbon Reduction Cycle by Ribulose Bisphosphate and Phosphoglyceric Acid[1]

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ABSTRACT

The activation states of a number of chloroplastic enzymes of the photosynthetic carbon reduction cycle and levels of related metabolites were measured in leaves of sugar beet (*Beta vulgaris* L., Klein E-type multigerm) under slowly changing irradiance during a day. The activation states of both phosphoribulokinase and NADP$^+$-glyceraldehyde-3-phosphate dehydrogenase increased early in the light period and remained constant during the middle of the day. Initial ribulose 1,5-bisphosphate carboxylase activity was already about one third of the midday level, did not change for the first 2 hours, but then increased in parallel with the rate of carbon fixation. Because the activation states increased by turns, first phosphoribulokinase and NADP$^+$-glyceraldehyde-3-phosphate dehydrogenase and later ribulose 1,5-bisphosphate carboxylase, the ratios of the activation states changed remarkably. Levels of ribulose bisphosphate and phosphoglycerate, which were high enough to affect enzyme reaction rates and changed in concert with activation state, indicate that these metabolites are involved in feedback/feedforward regulation of enzymes of carbon assimilation. This regulatory sequence is able to explain how the reaction rates for the enzymes of carbon assimilation are adjusted to maintain their activities in balance with each other and with the flux of carbon fixation.

Induction of photosynthesis in nature usually takes place under gradually increasing irradiance. Important to this process is the orderly light-mediated activation of five enzymes of the PCR[2] cycle (19, 28) which establishes a system for regulating photosynthesis. Even with this gradually increasing irradiance, leaves maintain a high level of apparent photosynthetic efficiency throughout most of the day (8). Fundamental to achieving this high efficiency is the ability to maintain a balance between the flux of absorbed photons and that of carbon assimilation. Reaction rates must be adjusted so that the rate of carbon flux at each point in the PCR cycle matches the rate of ATP and NADPH production by electron transport. More needs to be known of the mechanisms that enable plants to use the available light energy for assimilating carbon efficiently under the range of irradiances that occur during a natural daylight period.

In vivo reaction rates are dependent upon several levels of regulation. Gene expression determines potential maximal enzymatic activity in response to general conditions such as irradiance levels or N nutrition (7) which prevail throughout a number of days. Light-driven increases in activation state maintain a general correspondence between enzyme activity and diurnal changes in light-driven electron transport. Finally, precise adjustments in reaction rates are made by altering the level of substrates or effector molecules to control current enzyme activities.

Light activation mechanisms, which prevent futile cycling at night, play an important role in regulating enzyme activity during the diurnal light period. The maximal enzymatic capacity, which depends upon the total amount of enzyme present, can be attenuated via changes in activation state. For PRK, FBPase, Gal3PDH, and MDH, activation involves reduction of sulfhydryl groups via the ferredoxin/thioredoxin system (2). Rubisco activation involves carbamylation of a lysine residue (20), mediated via Rubisco activase (25, 26). Regulation of the sulfhydryl-activated enzymes is complex because the levels of substrates or effector molecules also influences changes in the activation state of these enzymes (28). The mechanisms by which light regulates Rubisco are not completely clear (3). The activation state of some of the enzymes of the PCR cycle appear to change separately and at different rates in response to light (14, 16, 28). For example, the thiol-regulated enzymes, particularly PRK, are activated at much lower light levels than Rubisco (25). Unequal levels of activation of these enzymes imply that additional regulation is needed to maintain balance of flux at various points in the PCR cycle.

Clearly, there is a requirement for precise regulation of flux through the enzymes PRK, Rubisco, PGK, and Gal3PDH. Regulation of the activity of these enzymes adjusts the flux of carbon through the unbranched portion of the PCR cycle, thereby preventing large excursions or oscillations in the levels of intermediate products. During photosynthesis, flux through this pathway is on the order of 12 μmol CO_2 m^{-2} s^{-1}, whereas the concentration of RuBP in the chloroplast during much of the day is near limiting levels, about 35 μmol m^{-2}. If used without being replenished, RuBP would be depleted to half

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[2] Abbreviations: PCR, photosynthetic carbon reduction; PRK, phosphoribulokinase (EC 2.7.1.19); FBP, fructose bisphosphate; FBPase, fructose bisphosphatase (EC 3.1.3.11); Gal3PDH, NADP$^+$-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.13); MDH, NADP$^+$-dependent malate dehydrogenase (EC 1.1.1.40); RuBP, ribulose 1,5-bisphosphate; PGA, 3-phosphoglyceric acid; NCE, net carbon exchange; DHAP, dihydroxyacetone phosphate; PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); TPI, triose phosphate isomerase (EC 5.3.1.1).

its concentration in a few seconds (11). The moderate, orderly changes in the pool sizes of RuBP and PGA observed throughout a diurnal light period (29) indicate that fine adjustment occurs.

The point is well made that regulation of photosynthesis at one or very few steps would be extremely inefficient and, as expected, flux is controlled at a number of points in the PCR cycle (19). Important questions remain to be answered, specifically, how does communication occur between the enzymes involved in these reaction steps and how is this regulation coordinated. In this study, we measured, simultaneously and in the same plant, the activation states of several PCR cycle enzymes and levels of certain key metabolic intermediate products throughout a period of slowly increasing irradiance at the start of a day. Analysis of these parameters revealed how changes in activation state and metabolite levels interact to regulate the unbranched portion of the PCR cycle and allow carbon assimilation to keep pace with the flux of ATP and NADPH produced by light-driven electron transport.

MATERIALS AND METHODS

Plant Material

Sugar beet (*Beta vulgaris* L. cv Klein E-type multigerm) plants were grown indoors under artificial lighting as described previously (8).

NCE Rates

Gas exchange was measured as described previously (13), and flux of CO_2 into the atmosphere of the leaf cuvette was measured by a flow controller (model FC 260, Tylan, Carson, CA). CO_2 concentration was maintained at 350 ± 10 μL L^{-1} (mean ± range), RH was 70%, and air temperature was 24 ± 2°C. A 14-h, sinusoidal light period was generated as described previously (12, 13).

Leaf Sampling and Metabolite Measurement

At various times during the 14-h light period, a 5-cm^2 leaf disc was removed from each of two leaves by freeze clamping the leaf section in a metal-clamp pliers which was previously chilled in liquid N_2 (31). Care was taken to prevent shading the leaf before clamping. The two leaf discs were broken into smaller pieces in a dish of liquid N_2, separated into two equal portions, and stored in liquid N_2 before analysis. One portion was extracted with perchloric acid and used for metabolite analysis as described previously (31). The other portion was used for enzyme measurement. Measurement of $NADP^+$ and NADPH was as described by Maciejewska and Kacperska (21).

Enzyme Extraction and Measurement

Leaf material was homogenized in an ice-cold mortar with 1 mL of extraction buffer, containing 50 mM Tris-HCl (pH 7.4, at 25°C), 10 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The extract was clarified by centrifugation for 30 s at 10,000*g*. Appropriate dilutions of the extract were then made and activities measured immediately. For convenience, all assay mixtures contained 50 mM Tris-HCl (pH 8.0 at 25°C), 0.5 mM EDTA, and 14 mM 2-mercaptoethanol. In addition, the assay mixture for measuring FBP-dependent FBPase activity contained 5 mM $MgCl_2$, 0.3 mM $NADP^+$, 3 units phosphoglucoisomerase, 1 unit glucose-6-phosphate dehydrogenase, and 0.1 mM FBP (4); the oxaloacetate-dependent MDH assay mixture contained 1 mM oxaloacetate and 0.2 mM NADPH (27); the 1,3-bisphosphoglycerate-dependent Gal3PDH assay mixture contained 5 mM $MgCl_2$, 0.2 mM NADPH, 0.6 units phosphoglycerokinase, and 2 mM PGA (14); the ribulose-5-phosphate-dependent PRK assay mixture contained 1 mM ribose-5-phosphate, 2 mM phospho*enol*pyruvate, 5 mM $MgCl_2$, 1 mM ATP, 20 mM KCl, 0.4 mM NADH, 75 units phosphoriboisomerase, 8 units pyruvate kinase, and 4 units lactate dehydrogenase (14). Changes in A_{340} were measured with a Lambda 3B spectrophotometer (Perkin-Elmer, Norwalk, CT). Initial and total Rubisco activities were measured according to the method of Perchorowicz *et al.* (22). Extraction and assay buffers were gassed with N_2 before use to remove dissolved O_2. Assays were initiated by addition of extract.

Enzyme activities were assayed with levels of substrate that were not saturating, and therefore, rates shown do not reflect maximal activities. Substrate levels were chosen to be in the range that allowed changes in activation state to be seen clearly. All enzyme activities, except FBPase, were stable for at least 1 h. FBPase activity was reduced to about one half the initial activity following storage on ice for 1 h. For this reason, FBPase activity was measured immediately after extraction. Enzyme activities were not affected by storage in liquid N_2 even for a period of up to 6 months.

RESULTS

Photosynthesis and Activation State

Five experiments were conducted on different days with separate plants and showed similar results. Data shown for the various parameters measured are from a single representative experiment. NCE rate (Fig. 1A) basically followed the pattern of irradiance as was observed previously (8, 13, 29). At the start of the light period, initial Rubisco activity, which is thought to be a measure of activation state (22), was already about one third of the midday level and remained relatively steady for the first 2 h (Fig. 1B). Thereafter, the activity increased in parallel with irradiance and the rate of carbon fixation until the last 1 h of the day. In contrast, the activation state of PRK increased rapidly during the first 2 h of the light period, remained fairly steady during the middle of the day, and declined rapidly at the end of the day (Fig. 1C). The activation state of Gal3PDH followed a pattern similar to that of PRK, increasing rapidly early in the day and remaining high during the middle of the day (Fig. 1D). By contrast, FBPase and MDH activation states clearly followed irradiance levels throughout most of the day. Total Rubisco activity was two thirds of the midday level at the start of the light period, increased during the first 4 h of the light period, and remained steady for the remainder of the day. These data are indicative of the presence of the endogenous Rubisco inhibitor, carboxyarabinitol-1-phosphate, in leaves of sugar beet (17).

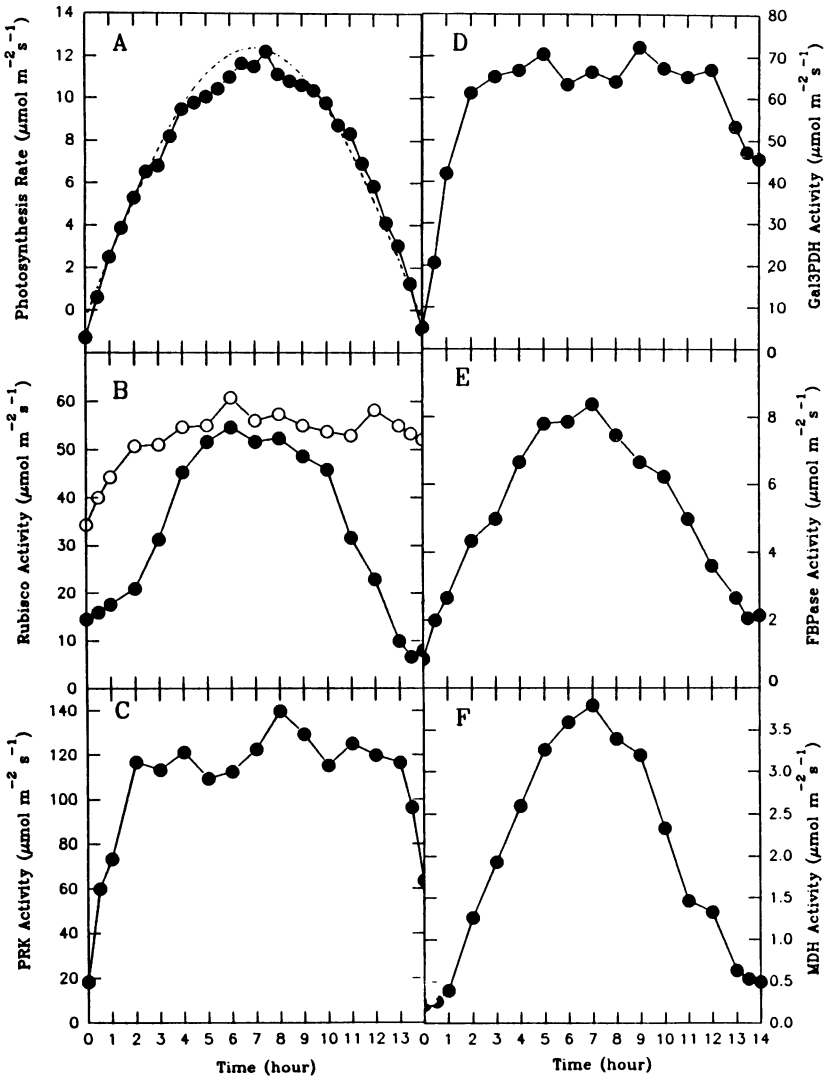

Figure 1. Time course of irradiance, NCE rate, and enzyme activation state during a simulated natural day period. A, Photosynthesis rate (●) and irradiance time course (---). Maximal irradiance level was 800 μmol m^{-2} s^{-1}. B, Initial rubisco activity (●) and total Rubisco activity (○).

Metabolite Levels

Orderly and reproducible changes occurred in the pool sizes of RuBP and PGA (Fig. 2A) throughout the light period in leaves of sugar beet (13, 29). The RuBP level increased rapidly during the first 2 h of the light period, declined to a lower level, and maintained this level during the middle of the day. Under a light period at this intermediate level of irradiance, the PGA level changed in a contrasting manner, in that when RuBP was low PGA was high and *vice versa*. The ATP level (Fig. 2B) decreased rapidly during the first 2 h of the light period, remained relatively steady during the middle of the day, and then increased to the dark level. The levels of ADP and NADPH (Fig. 2C) remained relatively unchanged for the entire day. The levels of $NADP^+$ increased for the first 3 h and then remained relatively steady until near the end of the day.

DISCUSSION

Time Course of Activation State and Photosynthesis

Light activation of the enzymes of the unbranched, carbon assimilation portion of the PCR cycle, namely, PRK, Rubisco, and Gal3PDH, appeared to occur in two phases. During the first phase (0–2 h), the activation states of PRK and Gal3PDH increased from low levels in the dark to maximal levels of activation for the conditions of the experiment (Fig. 1). The second phase of activation (2–6 h) was characterized by an increase in the activation state of Rubisco, but the activation state of the other two enzymes remained constant. Specific metabolites have been shown to be involved in the activation process of these enzymes (28). The lag observed in the activation of Rubisco (Fig. 1B) may be attributed to the requirement for RuBP to initiate the action of Rubisco activase (32). Comparison of the time courses of RuBP and initial Rubisco activities shows that Rubisco only began to activate when RuBP had approached its maximal level. Although no metabolites have been implicated in the activation of PRK, activation of Gal3PDH involves the substrate, 1,3-bisphosphoglycerate (28). This metabolite is thought to be readily available for activation of Gal3PDH at all times because of the high activity of PGA kinase (14) and the high levels of PGA and ATP.

Changes in the activation state of the enzymes of the

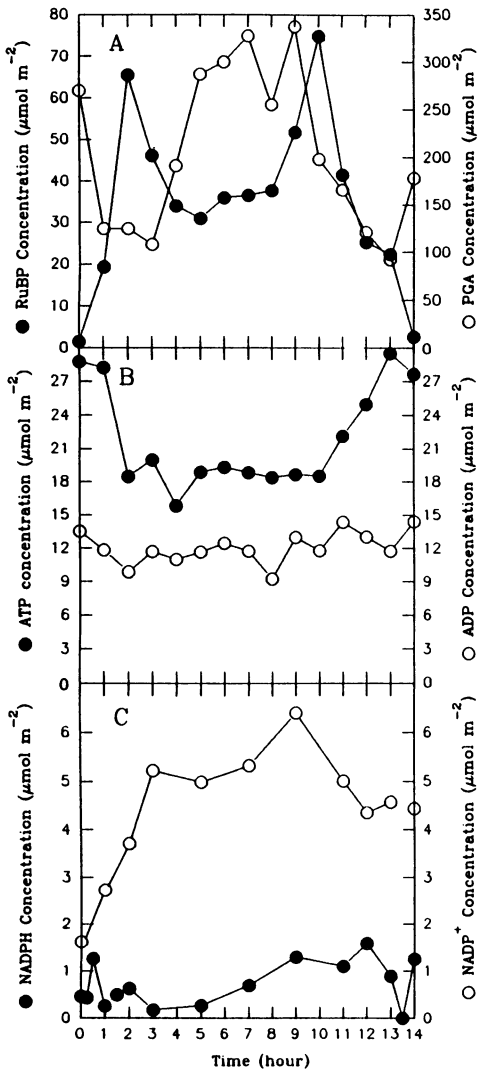

Figure 2. Time course of metabolite levels during a simulated natural day.

unbranched pathway did not correlate well with changes in carbon assimilation rate during the day, and the extractable activities of these enzymes as measured here were quite different from one another (Fig. 1). A striking feature of the time courses of the extractable activities of PRK, Gal3PDH, and Rubisco is that they increased by turns, first PRK and Gal3PDH and then Rubisco. Unlike the time courses of FBPase and MDH, which were similar to that of irradiance in these experiments, the activation states of PRK, Rubisco, and Gal3PDH were sometimes unchanging, whereas flux through the pathway was continuously changing with irradiance. The disparity between the time course of activation state and NCE rate could be reconciled if there are mechanisms in addition to those that regulate activation state, which attenuate activities of individual enzymes to enable them to match their reaction rate with that of photosynthesis. Clearly, the requirement that the reaction rates of each step must match the rate of carbon flux is not met by changes in activation state alone. To do this practically demands that enzymes involved in the synthesis and use of a particular metabolite communicate by a feedback/feedforward system centered on this common metabolite.

By comparing the time courses of enzyme activation state and metabolite levels, one should be able to identify which metabolites are most likely involved in enzyme regulation. Likely, these metabolites will show changes that are related to, but not necessarily identical with, the time course of the activation state that they modify. The time course of the ratio of the extractable activity of PRK to that of Rubisco shows that the activation state of PRK increased to a point where it substantially exceeded that of Rubisco during the first half of the morning and then decreased to a minimum by midday, and this pattern was repeated later in the day (Fig. 3A). The time course of the ratio of the extractable activity of Rubisco to that of Gal3PDH shows that the activation state of Gal3PDH substantially exceeded that of Rubisco very early in the day, but the opposite was true by midday (Fig. 3B). A striking feature of the plot of PRK/Rubisco activation ratio is that it closely resembled the time course of RuBP. Likewise, the PGA time course closely resembled the ratio of Rubisco/Gal3PDH, *i.e.* PGA was high when Rubisco activation state was considerably higher than that of Gal3PDH. The dynamic interaction between activation state and metabolite level implies that the levels of RuBP and PGA that result from differences in time courses of the activation states also are involved in modulating the flux of carbon through the pathway. The particular shape of the time courses of RuBP and

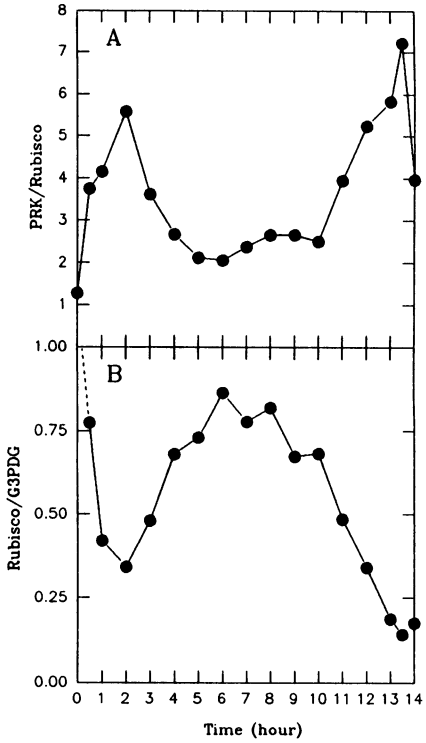

Figure 3. Ratio of activation state of PRK/Rubisco (A) and Rubisco/Gal3PDH (B) during a simulated natural day period.

PGA, bicuspid under these conditions, reflects the action needed to restore balance between enzymes. Under other conditions, the shape of the time courses for these metabolites might be quite different depending upon the requirements for regulation.

A Feedback/Feedforward System Regulates the Activities of PRK, Rubisco, and Gal3PDH

Components of the System

It is helpful to consider the unbranched portion of the PCR cycle as an operational unit (Fig. 4). This sequence of five enzymes utilizes the energy potential of the light reactions for the phosphorylation, carboxylation, and reduction reactions of the PCR cycle. The ensemble of these enzymes catalyzes a sequence of reactions together having a large negative change in the Gibbs free energy (24). Lacking branching, this pathway has a fixed stoichiometry among the carbon entering, moving through, and exiting each enzyme reaction. By contrast, the branched part of the PCR cycle, which involves the enzymes aldolase, transketolase, FBPase, and sedoheptulose bisphosphatase, allocates carbon for RuBP regeneration or for other synthetic pathways outside of the PCR cycle. For instance, FBPase, in addition to providing carbon for regeneration of RuBP, also provides carbon for starch synthesis.

Necessity for Regulating Flux

For the unbranched pathway, the flux of carbon is high but, as mentioned above, individual pool sizes of substrates and products are small. For example, although the net carbon fixation rate gradually increased to 5 μmol m^{-2} s^{-1} during the first 2 h (Fig. 1A), the RuBP pool size accumulated at a rate of only 12 nmol m^{-2} s^{-1} (Fig. 2A). Clearly, the flux of carbon through the RuBP pool is several hundred times larger than the rate of increase of the RuBP pool. The activity of PRK needed to produce the increased RuBP levels need only exceed the rate of Rubisco activity by a practically negligible amount. Hence, the activities of the two enzymes at any time are essentially equal. Regulation of RuBP synthesis and utilization must be very precise to maintain the size of a small pool size and maintain a large flux. Furthermore, because this pathway is unbranched, the flux of carbon at any given carbon assimilation rate clearly must be the same at all points. This requirement is yet another reason why the activities of these enzymes need to be regulated closely.

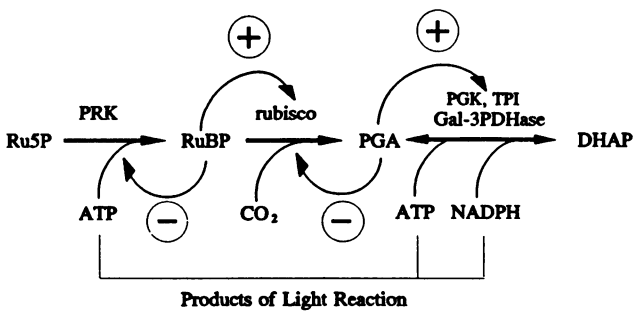

Figure 4. Diagram of the unbranched portion of the PCR cycle showing the interaction of metabolites on enzyme activity.

Mechanism of Regulation

So that the reaction rates of enzymes of the unbranched PCR pathway will match the rate of carbon fixation, common metabolites can communicate between enzymes and regulate their rates. RuBP can be considered to be such a control metabolite if it inhibits PRK and promotes the activity of Rubisco. RuBP has been shown to be a potent competitive inhibitor of PRK activity (K_i = 0.7 mM) (10), and RuBP levels change in relation to the activation states of PRK and Rubisco. The dynamic changes observed in the levels of RuBP contrast with those of ADP (Fig. 2B). The later is also an inhibitor of PRK activity (10), but its level does not change during the diurnal light regimen. Furthermore, the RuBP level changes from essentially zero to several times higher than that of ADP.

The K_m(RuBP) of Rubisco is usually found to be quite low, about 40 μM (24). However, it has been shown that Rubisco activity continues to increase with increasing RuBP levels, demonstrating that it does not follow strict Michaelis-Menten kinetics (18, 30). Recent evidence (J. C. Servaites, A. R. Portis, and D. R. Geiger, unpublished data) demonstrates that Rubisco exhibits negative cooperativity with respect to RuBP, *i.e.* the binding of each RuBP molecule decreases the intrinsic affinities of the vacant sites on Rubisco. Such a response gives physiological meaning to the wide dynamic concentration range of RuBP found in leaves, which ranges from 0 to 25 mM (Fig. 2A).

Likewise, PGA can be considered to be a control metabolite if it inhibits Rubisco activity and promotes its own conversion to DHAP (Fig. 4). Although PGA is a weak inhibitor of Rubisco (K_i = 0.85 mM) (1), it is present *in vivo* in a concentration range from 20 mM at 2 h to 70 mM at midday, assuming that only one half of the PGA is present in the chloroplast (Fig. 2A). PGA present at this high concentration can effectively inhibit carboxylation with respect to RuBP (9). Although Foyer *et al.* (9) found that physiological concentrations of DHAP and FBP only marginally altered the K_m(RuBP) for Rubisco, 20 mM PGA increased the apparent K_m by 10-fold. When irradiance was decreased rapidly, the PGA level was found to increase to 15-fold that of RuBP (23). We observed that at very low light (~0 h), the PGA/RuBP ratio was >100, decreased to <1 at 2 h, and then increased and was maintained at about 6 during the middle of the day.

In the light, the three reactions involved in the readily reversible conversion of PGA and DHAP are catalyzed by the enzymes PGK, Gal3PDH, and TPI. The extractable activities of PGK and TPI are known to be very high in light and dark (5, 14), whereas the Gal3PDH activation state increases from near zero in the dark to full activity at very low light (14) (Fig. 1). Hence, at most light intensities, enzyme activities are high and ensure that the reaction is not far from thermodynamic equilibrium (5, 14). The equilibrium is dependent upon the ratios of [ATP]/[ADP][Pi], [NADPH][H^+]/[$NADP^+$], and [PGA]/[DHAP]. This integration of many metabolic parameters generates metabolic flexibility (14). Balanced flux is thus maintained by adjustment of these ratios in response to

conditions. Depending on the light intensity, which influences activation state and flux, each of these individual ratios will have a certain value. These ratios may vary when conductance is altered in response to changes in activation state or levels of control metabolites. The time courses of some of these parameters indicate that [PGA], [Pi], and [H^+] likely are significant in regulating this portion of the PCR cycle.

Operation

Examining the changes in the levels of the two metabolites, RuBP and PGA, in conjunction with the changes in the activation state of PRK, Rubisco, and Gal3PDH shows how these metabolites restore balance between flux of carbon fixation and reaction rates of individual enzymes during slowly increasing irradiance (Fig. 4). At the beginning of the light period (0–2 h), the activation state of PRK is low and increasing, and the activity of PRK is restrained by RuBP to keep pace with the rate of carbon assimilation. In contrast, the activation state of Rubisco is constant, and its activity is gradually increased by complementary changes in the levels of RuBP and PGA. The activation state of Gal3PDH is low initially and begins to increase with increasing light. Because the flux of carbon through this reaction exceeds that of Rubisco, the PGA level declines thus restraining the rate of PGA conversion to DHAP and increasing the activity of Rubisco.

At increasing irradiance during the middle of the day (2–5 h), the needed changes in Rubisco activity appear to occur by a light-mediated increase in its activation state. During this period, levels of PGA and RuBP may restrain Rubisco if it exceeds that required to follow the rate of carbon fixation. A gradual lowering of RuBP increases PRK activity, and increasing PGA level increases the activity of Gal3PDH to the maximum needed to support the rate of carbon fixation.

Implications of This Mode of Regulation

The experiments described here were conducted with leaves of sugar beet plants growing under an intermediate level of light, a midday maximum of 800 μmol m^{-2} s^{-1}, and atmospheric levels of CO_2. The particular pattern of time courses for activation of PRK and Rubisco and the levels of RuBP and PGA are characteristic of sugar beet leaves under these conditions. Different light levels, CO_2 concentrations, growth conditions, or other species may show different patterns of RuBP and PGA, depending upon the requirements for attenuating the activities of PRK, Rubisco, and Gal3PDH. However, the involvement of RuBP and PGA in the feedback/feedforward regulation of these enzymes is flexible enough to deal with these differences.

The current levels of PGA and RuBP are the consequence of their present rates of formation and utilization. The resulting levels both communicate the state of balance among reaction rates and act to restore balance. In a regulatory system having both feedforward and feedback communication, the level of a metabolite influences both the rate of its synthesis and utilization. Although changes in the rate of light energy input are communicated through changes in the rate of synthesis of ATP and NADPH, the levels of these metabolites do not follow the time course of photosynthesis (Fig. 2, B and C). This lack of correlation prompted Heber *et al.* (14) to suggest that the levels of these metabolites do not have a major role in the regulation of photosynthesis rate. Although total leaf ATP was measured here, Dietz and Heber (6) sampled leaves at different irradiances and used nonaqueous fractionation techniques to determine the ratio of ATP/ADP in chloroplasts of spinach leaves but also found little change. Changes in the flux of carbon fixation may be more effectively communicated and regulated through changes in RuBP and PGA level. Carbon assimilation rate can follow the time course of irradiance even though ATP level does not follow carbon assimilation rate, because the activation state of Rubisco as modified by RuBP and PGA changes in response to the time course of irradiance.

In view of the above considerations, it appears inappropriate to attribute the moment to moment control of photosynthesis rate to constraints in the activity of a single enzyme or factor, for example, the activity of Rubisco or the ability to regenerate RuBP. The appropriate response to the question as to which factor is controlling photosynthesis rate is that all of them, as a regulatory unit, share control over assimilation rate. A value can be assigned for the amount of change in the rate of carbon assimilation produced by changing a given parameter by a certain amount (15), but it also seems important to understand that these parameters all interact and that a change in any one will result in a change in the activity of the others. When the activity or level of any one of the components is reduced, that member temporarily assumes an increased importance until equilibrium is restored. A small change in any component will quickly result in a swing of the system back to steady state where no one of these system elements itself controls the rate but all regulate jointly.

CONCLUSION

Efficient utilization of light energy under changing light levels is dependent upon mechanisms that maintain the stoichiometric balance between the reaction rates of the enzymes of the PCR cycle and the rate of carbon assimilation. The basic pattern of diurnal regulation of the unbranched portion of the PCR carbon assimilation pathway involves an interaction between the activation state of key enzymes and levels of key metabolites with small pool size and high flux, which both control and are controlled by the enzymes that flank them. The enzymes PRK, Rubisco, and Gal3PDH, the metabolites RuBP and PGA, along with the flux of ATP and NADPH, interacting as a system, share control over assimilation rate. No one of these system elements controls the rate but all regulate jointly. The impressive aspect of the system is not its ability to make rapid responses to transients in light level but that it exhibits a gradual, well-regulated response to the gradually changing irradiance of the diurnal light regimen.

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