Influence of Leaf Age on Photosynthesis, Enzyme Activity, and Metabolite Levels in Wheat

Received for publication February 10, 1987 and in revised form April 15, 1987

SHIGETOSHI SUZUKI 2, HITOSHI NAKAMOTO 3, MAURICE S. B. KU, AND GERALD E. EDWARDS 4

Botany Department, Washington State University, Pullman, Washington 99164-4230

ABSTRACT

The rate of photosynthesis under high light (1000 micromole quanta per square meter per second) and at 25°C was measured during development of the third leaf on wheat plants and compared with the activity of several photosynthetic enzymes and the levels of metabolites. The rate of photosynthesis reached a maximum the 7th day after leaf emergence and declined thereafter. There was a high and significant correlation between the rate of photosynthesis per leaf area and the activities of the enzymes ribulose 5-phosphate kinase ($r = 0.91$), ribulose 1,5-bisphosphate (RuBP) carboxylase ($r = 0.94$), 3-phosphoglycerate (PGA) kinase ($r = 0.82$), and fructose 1,6-bisphosphatase ($r = 0.80$) per leaf area. There was not a significant correlation of photosynthesis rate with chlorophyll content. The rate of photosynthesis was strongly correlated with the level of PGA ($r = 0.85$) and inversely correlated with the level of triose phosphate (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate) ($r = 0.92$). RuBP levels did not change much during leaf development; therefore photosynthesis rate was not correlated with the level of RuBP. The rate of photosynthesis was at a maximum when the ratio of PGA/triose phosphate was high, and when the ratio of RuBP/PGA was low. Although several enzymes change in parallel with leaf development, the metabolite changes suggest the greatest degree of control may be through RuBP carboxylase. The sucrose content of the leaf was highest under high rates of photosynthesis. There was no evidence that later in leaf development, photosynthesis (measured under high light and at 25°C) was limited by utilization of photosynthate.

There is considerable evidence that in C3 plants there is a high degree of correlation between the maximum extractable activity of Rubisco and the rate of photosynthesis measured under high light intensity (3, 4). It is less clear to what extent a change in the capacity of photosynthesis correlates with a change in the activity of other photosynthetic enzymes or in the levels of associated metabolites. Recently, the pool size of certain metabolites and the activity of certain photosynthetic enzymes have been evaluated relative to the rate of photosynthesis in C3 plants under different light intensities, leaf temperatures, and CO2 and O2 concentrations (1, 4, 9, 15, 17, 18, 22). Considerable evidence has been obtained on the relative limitation by photochemistry, reactions of carbon assimilation, and by the capacity for utilization of photosynthate from these measurements.

It is known that, typically, during leaf development the rate of photosynthesis increases rapidly, reaches a maximum before full expansion, and subsequently declines. Also, there is an increase in Rubisco activity and a decline upon reaching maturity; and some researchers have suggested Rubisco may become a limiting factor for photosynthesis (21). There have been few studies on the activity of photosynthetic enzymes other than Rubisco during development, particularly relative to the rate of photosynthesis. In Perilla frutescens, Batt and Woolhouse (2) found two patterns of changing photosynthetic enzyme activity, one group reaching a maximum activity prior to full leaf expansion, and the other group reaching maximum activity later in leaf development. In order to understand the basis for developmental changes in the capacity of photosynthesis it is necessary to examine a large number of factors. Some of these are considered in the present study with the C3 plant wheat, in which we evaluated the rate of photosynthesis during leaf development relative to the activity of several photosynthetic enzymes and the levels of certain related metabolites.

MATERIALS AND METHODS

Plant Material. Wheat seeds (Triticum aestivum L. var Argee) were germinated in the dark at 25°C. After 24 h, germinated seeds were selected for uniformity and planted in pots (14 × 14 × 15 cm, three plants per pot) filled with premixed soil (55% peat moss, 30% pumice, and 15% sand). Plants were grown in the growth chamber under a 15 h photoperiod and a day/night temperature regime of 25°C/18°C. Photon flux density (400–700 nm) at the top of the plants was about 300 μmol m−2 s−1. Plants were watered with diluted commercial nutrient solution (Hypex) twice a week.

The third leaf from the bottom of each seedling was chosen for investigation of the effect of leaf age on photosynthesis. On the 10th d after germination, the third leaves had emerged above the sheath of the second leaves (emergence) and the third leaves were fully expanded on the 17th d after germination. Leaf age was expressed in days after emergence. A 13 cm long section measured from the tip of the leaf was used for gas exchange measurements and for extraction of enzymes and metabolites. Since the tip is the older part of the monocot leaf, the sections sampled represent an increase in age during leaf development.

Gas Exchange Measurement. CO2 exchange of attached individual leaves was measured using an Anarad AR-600 IRGA in an open system as previously described by Wolf et al. (25) which allows the leaf to be inserted in the cuvette without sealing. The rate of air flow in the leaf cuvette was 0.8 L/min. Temperature and relative humidity were maintained at 25°C and approximately 60%, respectively. The CO2 concentration in the cuvette was maintained at 320 μL/L (±0 μL/L). The photon flux density...
LEAF AGE AND PHOTOSYNTHESIS IN WHEAT

The volume of the tube.

Gas exchange measurements were performed on plants 2 to 5 h into the light period. A steady-state CO₂ exchange rate was usually obtained after 30 min. After the measurement the leaf blade was quickly removed from the cuvette and clamped with two copper plates (13 × 2.5 × 0.9 cm) which were precooled to the temperature of liquid N₂. The killing time was approximately 2 s which is longer than the calculated turnover time for some metabolites in photosynthesis. However, the clamping of the leaf was performed in the growth chamber under the same light intensity and temperature as existed in the leaf cuvette. There were 4 to 5 replications on separate leaves at each leaf age. Leaf samples from different replicates were combined and stored in liquid N₂ until used for enzyme or metabolite assays.

Enzyme Assays. A 100 mg leaf sample from the pooled replicates of each leaf age was rapidly homogenized in a chilled mortar with 4 mg of insoluble polyvinylpolypyrrolidone and 0.25 g of acid-washed sand plus 2 ml of ice-cold medium containing 50 mM Tricine-KOH (pH 8.0), 10 mM MgCl₂, 1 mM EDTA-Na₂, and 10 mM β-mercaptoethanol. After complete homogenization, 1 ml of the same medium was added to the extract. The homogenates were filtered through one layer of Miracloth and centrifuged at 10,000g for 2 min. The supernatant was used as the crude enzyme extract.

All enzyme assays were carried out at 25°C. Rubisco was assayed spectrophotometrically after activation with CO₂ and Mg²⁺ (14). PAG kinase and NADP-triose-P dehydrogenase activities were measured according to Latzko and Gibbs (11). Ru5P kinase was assayed in a medium containing 50 mM Tricine-KOH (pH 8.0), 10 mM MgCl₂, 40 mM KCl, 0.2 mM NAD₂, 1 mM ATP, 2.5 mM DTT, 1 mM PEP, 5 units of lactate dehydrogenase, and 2 units of ribose-5-P isomerase (12). The reaction was initiated by the addition of ribose-5-P to a final concentration of 2 mM. The assay mixture for total FBPase activity (chloroplast + cytosolic) contained 100 mM Tricine-KOH (pH 8.0), 5 mM MgCl₂, 0.4 mM NADP, 1 mM EDTA-Na₂, 4 units of glucose-P isomerase, and 2 units of glucose-6-P dehydrogenase. The reaction was started by addition of FBP to a final concentration of 0.5 mM and followed spectrophotometrically. For extraction and assay of cytosolic FBPase, the procedure of Rufty and Huber (19) was followed. The extraction and assay of Ru5P kinase, NADP-triose-P dehydrogenase, and FBPase were performed to measure the catalytic capacity of the enzymes in the leaf relative to leaf photosynthesis rates (enzymes were not extracted and incubated with a di-thiol reagent whereby further in vitro activation might occur).

Metabolite Assays. Extracts for metabolite assays were prepared by a slight modification of the method of Leegood and Furbank (13). Leaf samples (approximately 100 mg fresh weight) were pulverized to a fine powder using a prechilled pestle and mortar. About 0.5 ml of frozen 0.7 N HClO₄ was added to the powder which was then gently pulverized. The mixture was allowed to thaw slowly and then was transferred to an ice-cold centrifuge tube. The mortar was washed twice with a small amount of ice-cold 0.07N HClO₄. The extracts were combined, left on ice for 30 min, and centrifuged at 10,000g for 2 min. The pellet was washed with 0.25 ml of water and centrifuged at 10,000g for 2 min. The supernatants were combined and neutralized with 10 N KOH. Four mg of charcoal were added as a suspension in 100 μl of water to remove pigments and lipids. The extract was then centrifuged at 10,000g for 2 min and the volume of the supernatant was brought to 2 ml. The supernatant was used for metabolite assays.

Trioise-P (dihydroxyacetone-P plus glyceraldehyde-3-P), PGA, and RuBP were determined spectrophotometrically by sequential assays on the same sample (9). The initial assay medium contained 50 mM Tricine-KOH (pH 8.0), 20 mM MgCl₂, 20 mM NaHCO₃, 10 mM KCl, 1 mM EDTA, 5 mM ATP, 5 mM phosphocreatine, 0.3 mM NADH, and 2 units phosphocreatine kinase.

Metabolites were assayed by the sequential addition of 5 units of triose-P isomerase and 0.4 unit glycerol-P dehydrogenase (for determining triose-P), 3 units PGA kinase, and 1 unit glyceraldehyde-P dehydrogenase (for determining PGA), and 0.5 unit Rubisco (for determining RubBP). The small changes in volume after additions of various coupling enzymes were taken into account for calculation of metabolite concentration. Sucrose was determined enzymatically using the sucrose/glucose/fructose assay kit from Boehrhringer Mannheim Biochemicals. Chl was determined on original extracts prior to centrifugation, using the method of Wintermans and De Mots (24).

The coupling enzymes used in enzyme and metabolite assays, except for Rubisco, were purchased from Sigma Chemical Co. Rubisco was purified from spinach leaves obtained from a local market using a modification of the procedure of Paech and Dybing (16). Fifty g of spinach leaves were rinsed in distilled water, exposed to light for 30 min, frozen in liquid N₂, and ground into a fine powder in a chilled mortar with 0.5 g insoluble PVP and 25 g acid-washed sand. Three hundred ml of ice-cold grinding medium were added and grinding continued until the leaf tissue was completely macerated. The grinding medium contained 50 mM Tricine-KOH (pH 8), 10 mM MgCl₂, 1 mM EDTA-Na₂, and 10 mM β-mercaptoethanol. The following purification procedures were carried out at 4°C except for the 60°C heat treatment. The homogenate was filtered through two layers of Miracloth and the filtrate centrifuged at 10,000g for 10 min. The supernatant was quickly heated to 60°C and maintained at this temperature for 5 min, cooled, and centrifuged at 10,000g for 10 min. The Rubisco in the supernatant was fractionated by (NH₄)₂SO₄ between 40 and 55% saturation. The protein precipitate formed in the last step was collected by centrifugation and resuspended in a small amount of grinding medium. Further purification was achieved by centrifugation of the protein on a 10 to 30% linear sucrose density gradient which included 25 mM Tricine-KOH (pH 8.0), 2.5 mM MgCl₂, 5 mM β-mercaptoethanol, and 0.2 mM EDTA as previously described (10). Fractions that contained high specific activity of Rubisco were pooled, and glycerol was added to a final concentration of 50%. Relatively pure enzyme is obtained with this procedure (16). The preparation contained no Ru5P kinase activity which is important in using the enzyme to specifically determine RubBP levels. The enzyme was stored at 4°C prior to use.

RESULTS

The rate of photosynthesis per unit area in the third leaf of wheat reached a maximum of about 32 μmol m⁻² s⁻¹ on the 7th day after leaf emergence and then declined to ½ of the maximum after 22 d (Fig. 1). The activity of Rubisco was very similar to the rate of photosynthesis throughout leaf development. The other enzymes also tended to have maximum activities when photosynthesis was at its maximum, including total FBPase (chloroplast + cytosolic), cytosolic FBPase and PGA kinase. Chl content was also highest 7 d after emergence, although even after 22 d the Chl level was still high. Figure 2 shows plots of the day on enzyme activity versus photosynthetic rate. The highest correlation between enzyme activity and photosynthesis rate was with Ru5P kinase and Rubisco (r = 0.91 and 0.94, respectively) followed by PGA kinase and FBPase. The lowest degree of correlation was with NADP-triose-P dehydrogenase which had relatively high activity throughout development. The correlation between photosynthesis and Chl content was low and not significant.
During development the synthesis rate was measured from replicates (4–5 replicates) from photosynthesis assays in Figure 1. Therefore, each data point represents a measurement from the pooled leaf samples of the 4 to 5 replications from photosynthesis assays in Figure 1.

In measuring metabolite pools under photosynthetic conditions, the level of PGA was found to closely parallel the photosynthesis rate (Fig. 3). However, the level of Rubisco did not change as much during leaf development. Triose-P increased with leaf age. The sucrose content of the leaf was higher early in leaf development and declined 11 days after leaf emergence. Figure 4 shows that the ratio of PGA/triose-P reached a peak early in leaf development, and declined sharply thereafter. There was a reverse pattern in the ratio of RuBP/PGA, which was low earlier in development and increased as the leaf aged.

There was a very high correlation between PGA content of the leaf and photosynthesis rate and a strong inverse relationship between triose-P level and photosynthesis rate. There was a significant correlation between sucrose content of the leaf and photosynthetic rate. There was no correlation between the rate of photosynthesis during leaf development and the level of RuBP (Fig. 5).

**DISCUSSION**

There has been considerable interest in the level and activity of Rubisco in leaves of C3 plants relative to rates of photosynthesis (1, 4, 8, 9, 15, 17, 18, 20). In most of these studies the rate of photosynthesis has been examined and compared to the activity of the enzyme and the RubBP pool size under different environmental conditions. In the present study with developing leaves of wheat the total activity of Rubisco (initial extractable activity not measured) was strongly correlated with the rate of photosynthesis. However, there were also similar changes in other photosynthetic enzymes, including PGA kinase of the reductive
phase and Ru5P kinase of the regenerative phase of the reductive pentose-P pathway. This suggests that changes in photosynthesis during leaf development are associated with changes in the capacity of the cycle through control of the level of a number of enzymes. The level of the cytosolic FBPase, as well as the total FBPase activity, also had a similar pattern to those of photosynthetic activity. The rate of photosynthesis was not correlated with Chl content, which is consistent with other studies where photosynthesis has been measured under high light (3).

The rate of photosynthesis was strongly correlated with the level of PGA. Most of the PGA is considered to be located in the chloroplast (5, 23). Although there were changes in RuBP content, it did not parallel the changes in photosynthesis rate during leaf development. However, from 6 to 16 d after emergence the level of RuBP was similar (about 40–45 \( \mu \text{mol m}^{-2} \)) (see von Caemmerer and Edmondson [4]), and based on the carboxylase activity at these respective leaf ages (note total activity was measured rather than initial extractable activity) (see “Materials and Methods”), there are about \( 1 \mu \text{mol} \) of binding sites on the enzyme \( \text{m}^{-2} \) at 7, and 5 \( \mu \text{mol} \) of binding sites \( \text{m}^{-2} \) at 16. This results in a ratio of moles of RuBP to moles of binding sites on the enzyme of 3.5 at 7 (similar to the ratio of 4 with radish under normal atmospheric conditions) (4) and 9 at 16. This suggests that RuBP may not be rate limiting when photosynthesis is at its maximum, or particularly in older leaves.

However, not all of the RuBP may be available to bind to the enzyme, either because of chelation of RuBP or competition by other organic phosphates for binding sites on the enzyme (4).

Besides the high levels of RuBP to calculated substrate binding sites on Rubisco, the high RuBP/PGA ratio in older leaves suggests Rubisco is rate limiting for photosynthesis. It is also apparent that the reduction in activity of Rubisco with increasing leaf age is accommodated by a decrease in other enzymes, including PGA kinase of the reductive phase and Ru5P kinase of the regenerative phase. Rubisco activity may be a primary limitation on photosynthesis in older leaves. Alternatively, as the activity of other enzymes in the cycle decline with leaf age, the activity of Rubisco may be controlled in a manner which accommodates the capacity of the cycle and at the same time maintains a high ratio of RuBP to substrate binding sites on the enzyme.

A further consideration is whether the photochemical generation of assimilatory power is limiting photosynthesis in the older leaves. The level of the assimilatory power in the form of NADPH and ATP is correlated with the ratio of dihydroxyacetone phosphate to PGA in chloroplasts (7). The high ratio of triose-P (most of which is DHAP) (8) to PGA in older leaves of wheat suggests that assimilatory power is not limiting, although it should be noted we do not know the levels of these metabolites in the chloroplast. While the photochemical capacity was not measured in the present study, Evans (8) found that the Hill reaction and Ru5P and Rubisco changed in parallel with aging of flag leaves in wheat. It is also of interest that in studies with radish the ratio of RuBP/PGA remains constant with varying light intensity under atmospheric levels of \( \text{CO}_2 \) (4). This suggests that as the supply of energy for conversion of PGA to RuBP is decreased the carboxylase is controlled, such that the PGA/RuBP ratio remains unchanged. In contrast, the ratio of RuBP/PGA increased with leaf development in wheat. The generation of assimilatory power does not appear to be limiting photosynthesis in the older leaves.

It is possible that photosynthesis decreases during leaf development due to an accumulation of carbohydrate and a limitation on utilization of products, rather than because of limitation on the capacity for photosynthesis. However, the results on sucrose content suggest that this was not the case in the present study, since the sucrose content decreased during leaf development. In fact, it is likely that adjustments in the cytosol regulate sucrose synthesis when the capacity for photosynthesis is reduced. The large decrease in cytosolic FBPase activity as photosynthesis decreases may be one means of preventing a drain of carbon from the reductive pentose-P pathway under this condition (coarse control) (23).

Acknowledgments—Discussions with Dr. Christian Paech on Rubisco purification and with Dr. John Kobza on some of the methodology are greatly appreciated.

LITERATURE CITED

1. BADGER MR, TD SHARKEY, S VON CAEMMERER 1984 The relationship between steady-state gas exchange of bean leaves and the levels of carbon reduction cycle intermediates. Planta 160: 305–313

Fig. 5. Plots of photosynthetic rate versus metabolite level during development of the third leaf of wheat. The data are from Figure 3. *, \( P < 0.05 \) (significant at 5% level). **, \( P < 0.01 \) (significant at 1% level).


20. MOTT, KA, RG JENSEN, JW O'LEARY, JA BERRY 1981 Photosynthesis and ribulose 1,5-bisphosphate concentrations in intact leaves of Xanthium strumarium L. Plant Physiol. 76: 968-971


26. SESTAK Z 1985 Photosynthesis During Leaf Development. Dr. W. Junk, Boston, pp 396


