Plant Physiol. (1979) 63, 105–108
0032-0089/79/63/0105/04/$00.50/0

Light-Dark Regulation of Starch Metabolism in Chloroplasts

I. LEVELS OF METABOLITES IN CHLOROPLASTS AND MEDIUM DURING LIGHT-DARK TRANSITION[1]

Received for publication April 10, 1978 and in revised form June 20, 1978

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ABSTRACT

In *Spinacia oleracea* the kinetics of CO_2 fixation, of starch formation, and of changes in the levels of metabolites in chloroplasts and the surrounding medium has been investigated during light-dark and dark-light transitions with isolated intact chloroplasts.

The internal level of orthophosphate stays constant throughout a light-dark-light cycle. The concentration of 3-phosphoglycerate in the chloroplasts is about 4 millimolar in the light and decreases in the dark within 3 minutes to about 1.6 millimolar. The level of the hexose monophosphates shows a reverse trend, increasing from about 2.2 millimolar in the light to 6 millimolar in darkness. In the subsequent light period both compounds reach their original levels within 2 minutes. The chloroplastic concentrations of dihydroxyacetone phosphate, of the pentose monophosphates, and of the hexose- and heptose bisphosphates remain constant at about 0.4 millimolar throughout the light-dark-light cycle.

In the medium, the concentration of 3-phosphoglycerate increases and dihydroxyacetone phosphate decreases in the dark phase: this is due to an exchange of internal 3-phosphoglycerate for external dihydroxyacetone phosphate. Part of the reimported dihydroxyacetone phosphate is converted into hexose monophosphates via aldolase and fructose bisphosphatase during the first minutes of darkness. Due to the observed exchange transport reactions, the large difference between the transenvelope concentration gradients of 3-phosphoglycerate, dihydroxyacetone phosphate, and orthophosphate which exist in the light, is completely abolished after 2 to 3 minutes in the dark.

The kinetics and the magnitudes of the changes of metabolite concentrations during the light-dark-light cycle are compared to the kinetics of starch formation, and their relevance for a possible light-dark regulation of starch synthesis is discussed.

Starch synthesis in plants seems to be mainly regulated via modulation of the activity of ADP-glucose pyrophosphorylase (14). This enzyme is strongly inhibited by Pi and stimulated by several glycolytic intermediates, particularly PGA[3] and hexose-monoP (3, 15). Based on these findings, it was proposed (3, 14, 15) that a possible light-dark regulation of starch metabolism in chloroplasts might occur via changes of the chloroplastic levels of these intermediates. Evidence for a fast light-dark regulation of ADP-glucose pyrophosphorylase *in vivo* came from the finding that the level of ADP-glucose in *Chlorella pyrenoidosa* dramatically changed within sec upon light-dark transition (10).

Recently it was demonstrated by different authors (4, 7, 16) that the relative amount of carbon incorporated into starch in isolated intact chloroplasts depends on the Pi concentration in the medium, which, in turn, affects the level of Pi, PGA, and triose-P in the chloroplast due to the reactions catalyzed by the phosphate translocator (5). These results (4, 7, 16) present direct evidence that starch synthesis in chloroplasts might be regulated via the ratio of [Pi]/[PGA].

There have been no reports on the extent and the kinetics of changes in metabolite levels in isolated intact chloroplasts during the light-dark transitions, nor of the effects of those changes on rate of ADP-glucose formation. In the following studies we describe the levels of photosynthetic metabolites in isolated intact chloroplasts and in the surrounding medium during light-dark transition and compare them with the kinetics of CO_2 fixation and of starch formation under the same conditions.

MATERIALS AND METHODS

Plant Material. Spinach (*Spinacia oleracea*) was grown in Vermiculite under artificial light (3,000 ft-c) with an 8-hr light period and a 16-hr dark period at a temperature of 13 C. Plants were fertilized with a modified Hoagland solution. Chloroplasts were isolated from young leaves according to the method of Jensen and Bassham (8). Chl was measured by the procedure of Arnon (1).

Incorporation of $H^{14}CO_3$. Fifty μl of chloroplast suspension (50 μg of Chl) was added to solution C (8), containing 7 mM $NaH^{14}CO_3$ (50 μCi) in a total volume of 500 μl. The suspension was assayed in serum-stoppered flasks in a water bath illuminated from below with fluorescent tubes, providing a light intensity of 450 μE m^{-2} sec^{-1}. At given intervals 50 μl were removed with microsyringes and injected into 450 μl of methanol (final methanol concentration 90%). Total CO_2 fixation was measured by removing from the chloroplast-methanol mixture aliquots which were acidified with 200 μl of glacial acetic acid, dried by flushing with N, and counted by liquid scintillation.

Separation of Pellet and Supernatant in ^{32}P Experiments. The chloroplasts were separated from the medium as described by Bassham *et al.* (2) but with some modifications. Chloroplasts (containing a total of 150 μg of Chl) were incubated in a total volume of 3 ml of solution C, labeled with 150 μCi of ^{32}P (0.5 mM), under the conditions mentioned above. At given intervals, 200 μl of the suspension was quickly transferred into ice-cold microfuge tubes and then centrifuged for about 12 sec in a microfuge (Beckman). Aliquot samples (150 μl) of the supernatant were removed and injected into 300 μl of methanol. The rest of the supernatant was discarded. The pellet was then inactivated by

[1] This work was supported in part by the Biomedical and Environmental Division of the United States Department of Energy.

[2] Recipient of the postdoctoral fellowship of the Deutsche Forschungsgemeinschaft (DFG).

[3] Abbreviations: PGA: 3-phosphoglycerate; DHAP: dihydroxyacetone phosphate; G6P: glucose 6-phosphate; G1P: glucose 1-phosphate; F6P: fructose 6-phosphate; R5P: ribose 5-phosphate; Ru5P: ribulose 5-phosphate.

injecting 200 μl of methanol into the microfuge tubes. Samples removed in the light or dark were centrifuged and treated in the light or dark, respectively. The average time from sampling to deactivation was about 40 sec.

Analysis of Products. Products of chloroplast photosynthesis were separated by descending paper chromatography and radioautography (8). Aliquot samples (150 μl) of the chloroplast-methanol mixture were spotted on Whatman No. 1 paper and developed in two dimensions, first for 60 hr with a phenol-water-acetic acid solvent and then for 48 hr in the second direction with butanol-water-propionic acid (13). The insoluble fraction (obtained from chromatogram origins) was hydrolyzed with trifluoroacetic acid for 1 hr at 100 C and an aliquot was chromatographed as above, but only for 24 hr in each direction. After that treatment, more than 90% of the radioactivity was found in glucose, indicating that the insoluble material at the origin consisted nearly exclusively of a polyglucan, presumably starch or amylopectin.

RESULTS

Time Course of CO_2 Fixation and Starch Formation during Light-Dark Transition. In the first light phase, CO_2 was fixed by whole chloroplasts with a linear rate of about 90 μmol/mg Chl·hr (Fig. 1). After the light was switched off, CO_2 fixation ceased within 30 to 60 sec of darkness. Under our conditions, there was no detectable decrease of the fixed CO_2 during the 4-min dark period (*e.g.* by oxidative pentose-P cycle). In the second light phase, CO_2 fixation started without a significant lag phase, but sometimes with a slightly diminished rate. The time course of starch formation closely followed the kinetics of total CO_2 fixation. During the dark period, there was usually little or no postillumination incorporation of CO_2 into starch and there was no or only little starch degraded in the dark period.

The overall percentage of carbon incorporated into the starch depends largely on the Pi concentration in the medium (4, 7, 16). Under our conditions (*i.e.* at a Pi concentration in the medium of 0.5 mM at the beginning of the experiment), starch formation in both light periods was approximately 10% of the rate of total CO_2 fixation in agreement with other published results (4, 16).

Levels of Metabolites in the Chloroplast during Light-Dark-Light Transition. The concentrations of metabolites found with our method of separating chloroplasts from medium and determined with ^{32}P labeling agree well with reported data obtained by silicon oil centrifugation (12). For the calculation of metabolite concentrations we used a chloroplast volume of 25 μl/mg Chl as a reference. This is well in the range found by other authors (6) as well as by our own measurements of chloroplast volumes by Coulter Counter distribution (not shown here). A considerable part of the chloroplast volume might be occupied by the thylakoid space, which might be impermeable to the metabolites. In this case, all concentrations would be somewhat underestimated.

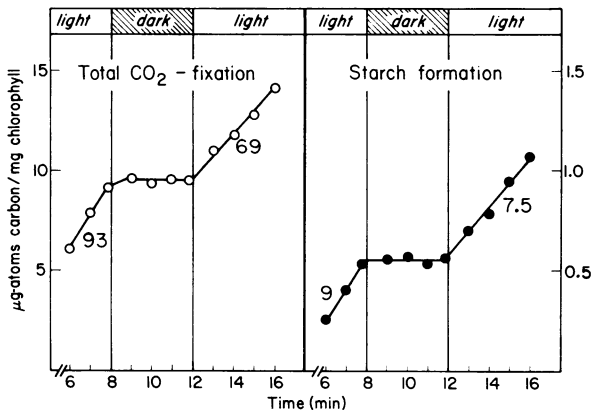

FIG. 1. Time course of CO_2 fixation and starch formation with intact chloroplasts during light-dark-light transition. Reaction was started by adding 50 μl of chloroplasts (50 μg of Chl to 450 μl of reaction medium in the light. Numbers next to curves give rate of carbon incorporation (μmol/mg Chl·hr).

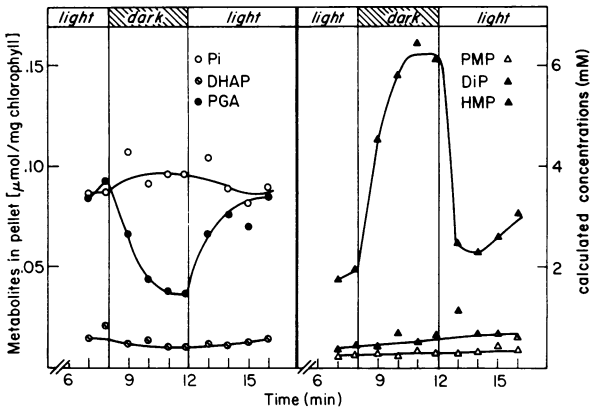

FIG. 2. Changes in levels of ^{32}P-labeled metabolites in chloroplasts during light-dark-light transition. Concentrations (right scale) were calculated for an average chloroplast volume of 25 μl/mg Chl. Abbreviations: PGA: 3-phosphoglycerate; DHAP: dihydroxyacetone phosphate; HMP: hexose monophosphates (fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate); DIP: bisphosphates (fructose 1,6-bisphosphate, sedoheptulose 1,7-bisphosphate, ribulose 1,5-bisphosphate); PMP: pentose monophosphates (ribose 5-phosphate, ribulose 5-phosphate).

The internal Pi level of isolated chloroplasts is known to be approximately 12 mM, if measured directly after isolation in the dark (9, 12). Upon illumination this level decreases to 3 to 4 mM and then remains nearly constant (9, 12). Our first samples were taken after 7 min in the light. At the end of the first light period, the internal Pi level had already reached its low steady-state value of about 4 mM (Fig. 2). Under our conditions this level stayed constant throughout the light-dark-light cycle.

The level of PGA decreased from about 4 mM in the light to 1.6 mM in the dark within 2 to 3 min, and it returned to the level of about 4 mM in the second light period. In the light, the level of DHAP was only 10% of the PGA level, and this low concentration of DHAP did not change in the dark. A very low ratio of [DHAP]/[PGA] in the stroma during illumination was also found by other authors (2, 12).

The steady-state concentration of F6P + G6P + G1P in the chloroplasts was about 50 nmol/mg Chl corresponding to a concentration in the chloroplast of 2 mM (Fig. 2). Upon light-dark transition, the hexose-monoP concentration in the chloroplasts increased dramatically up to about 6 mM within 2 to 3 min and decreased once more to about 2.5 mM in the second light period. The concentration changes of the hexose-monoP and of PGA during light-dark-light transitions were opposite, the increase in the hexose-monoP concentration being somewhat greater than the decrease in [PGA] (see under "Discussion").

The concentration of R5P and Ru5P in the chloroplasts was about 0.4 mM and the fructose- and sedoheptulose-BisP reached about the same level. Both pool sizes did not change significantly during light-dark-light transition (Fig. 2). Also, the level of ribulose 1,5-bisphosphate (RuDP) was usually very low in our experiments. Therefore, all bisphosphates are plotted together in Figure 2.

Metabolites in Medium. The changes of metabolite levels in the medium (Fig. 3) were very different from those in the chloroplasts (Fig. 2). The level of Pi decreased constantly during the first light phase, and the amount of Pi disappearing from the medium approximately equaled the sum of PGA and DHAP which were exported (Fig. 3). In the dark, the decrease of [Pi] stopped after

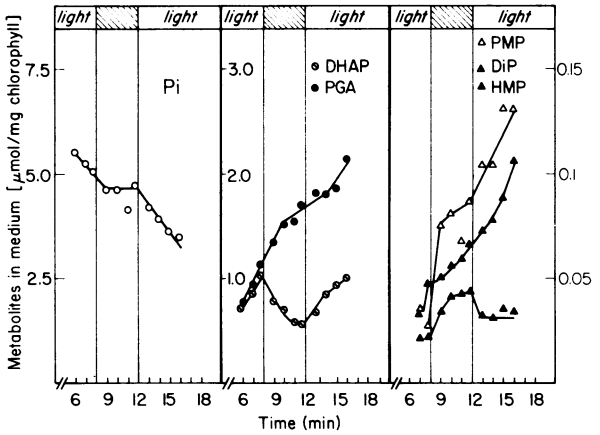

FIG. 3. Changes in levels of ^{32}P-labeled metabolites in medium (same experiment as in Fig. 2). Note that the scales for each section of the figure are different. Abbreviations as in Figure 2.

about 30 to 60 sec.

The levels of PGA and DHAP were about the same at the end of the first light period, reaching a concentration of 0.05 mM in the medium, a value which is again close to the findings of Lilley *et al.* (12), confirming the existence of a very high concentration gradient of PGA across the chloroplast envelope. In the dark, there was a dramatic decrease of DHAP in the medium, which stopped after 2 to 3 min. At the same time, the level of PGA increased by about the same amount. In the second light phase, the levels of both compounds again increased.

The levels of the other metabolites, pentose-monoP, hexose-monoP, and of the -BisP were two orders of magnitude lower than those of PGA and DHAP, indicating that these compounds are to a high degree retained inside the chloroplasts (compare refs. 2 and 12).

Changes in ^{14}C Labeling of Metabolites in Chloroplasts plus Medium during Light-Dark Transition. Some experiments were carried out without separating the chloroplast pellet from the medium in order to eliminate possible inaccuracies in the total metabolite pools which might have resulted from differential rates of conversion in pellet and supernatant fractions during separation and killing. In these experiments, $H^{14}CO_3$ was added during photosynthesis instead of ^{32}P so that possible changes in labeled starch could be observed.

The total amount of PGA continued to increase in the dark nearly as rapidly as in the light for a period of 2 to 4 min, whereas the total amount of DHAP decreased to about the same extent (Fig. 4). Several reactions could contribute to the observed changes of metabolite levels, *e.g.* an oxidation of DHAP to PGA or a reimport of external DHAP. Both the increase in PGA and the decrease in DHAP could be significantly inhibited if either PGA or Pi were added in high concentrations (5 mM) to the chloroplast suspension immediately after the light was switched off (Fig. 4). Even more striking, the increase of the hexose-monoP level in the dark was completely abolished by adding PGA, and addition of Pi even induced a considerable decrease in total hexose-monoP (Fig. 4). In this experiment we found a small amount of degradation of starch in the dark. This starch degradation was too small to account for the observed increase in hexose-monoP. In the presence of high [PGA], this starch degradation was inhibited, thus confirming similar observations of other authors (4, 16).

DISCUSSION

Analysis of Changes in Metabolite Concentrations. Since the increase in the hexose-monoP in the dark could not be accounted for by starch degradation, another source was required. We assume that in the dark DHAP is reimported from the medium, being partly converted via aldolase and fructose-bisP into hexose-monoP (as long as the latter enzyme is active in the dark). One molecule of Pi would be released (from fructose-bisP) for 2 DHAP molecules consumed. This released Pi could serve as a counterion for only half the imported DHAP so that, in addition, another counterion has to be exported. Since the internal level of Pi remains constant, this counterion has to be PGA. In fact, an exchange of internal PGA against external DHAP can account for both the decrease of internal [PGA] and partly also for the increase of [PGA] in the medium. In this case it should be expected that the decrease in [PGA] would just balance the increase in hexose-monoP, and the total amount of Pi + sugar phosphates in the chloroplast would then stay constant as postulated (12). But in most of our experiments, the increase in hexose-monoP was somewhat higher than the decrease in PGA. This discrepancy might be due to a certain contamination of the pellet with medium, which contained a much higher amount of PGA than of hexose-monoP.

The total amount of PGA formed and of DHAP consumed in the dark phase is usually higher than the change in hexose-monoP concentration (Fig. 4). Therefore we conclude that part of the DHAP is also oxidized to PGA in the dark, either inside the chloroplast or perhaps in the medium by contaminating cytoplasmic enzymes. Some additional PGA might be formed during the observed postillumination CO_2 fixation.

It is known that PGA or Pi competes with DHAP for translocation by the phosphate translocator (5). We have demonstrated that the increase in concentration of hexose-monoP in the dark is inhibited by adding high concentrations of PGA or Pi to the medium immediately after switching the light off (Fig. 4). This finding strongly supports the idea that the formation of hexose-monoP in the dark is due to a reimportation of DHAP from the medium, which would be inhibited by high [Pi] or [PGA] in the medium.

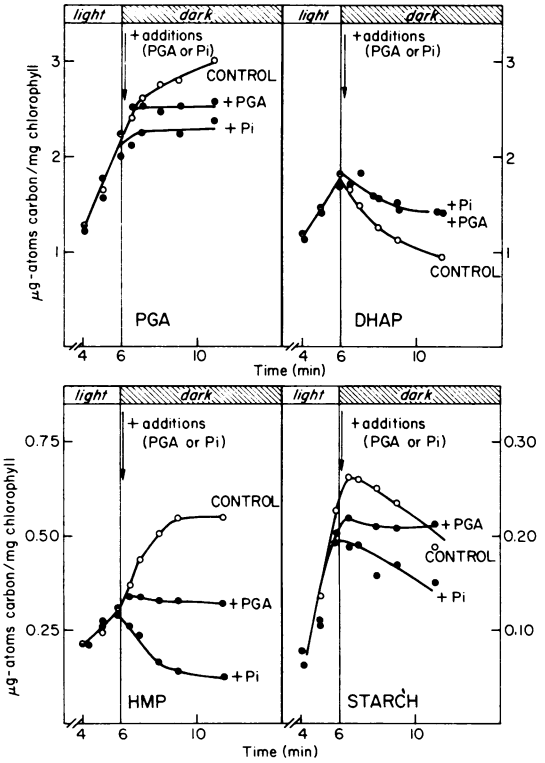

FIG. 4. Levels of ^{14}C-labeled compounds during light-dark transition (without separation of chloroplasts and medium) showing effect of high concentrations of PGA or Pi (added in the dark). Where indicated, 10 μl of PGA or Pi were added to the reaction mixture to give a final concentration of 5 mM. Abbreviations as in Figure 2.

What is the driving force for these exchange transport reactions and the coupled changes in metabolite concentrations? Consistent with the findings of other authors (2, 12), we observed that in the light the internal concentration of PGA is much higher than that of DHAP, whereas in the medium, both compounds have about the same concentration (Figs 2 and 3). In other words, in the light, a large difference in the transenvelope concentration gradients of PGA, Pi, and DHAP is maintained (Table I). After 3 min in the dark, all concentration gradients of the compounds transported by the phosphate translocator have become equal (Table I). It seems reasonable to conclude that the driving force for the observed light-to-dark changes in metabolite concentrations is an imbalance of the transenvelope gradients of Pi, PGA, and DHAP maintained in the light, but not in the dark.

Explanations for retention of PGA in the chloroplast during illumination have been recently discussed (12). It was suggested that PGA might be retained in the light in the chloroplast, because at the more alkaline pH of the stroma in the light PGA would carry three negative charges, whereas Pi and DHAP have only two. A decrease of the stromal pH in the dark would then necessarily lead to the observed release of PGA into the medium. One might ask why the released PGA is obviously exchanged only for DHAP and not for Pi, despite the fact that at the end of the first light period, the Pi concentration in the medium is still higher than that of DHAP. But a consideration of the concentration gradients listed in Table I shows that an import of Pi (which dercreases the external [Pi] and increases the internal [Pi]) would lead to an even higher transenvelope gradient of Pi, thus increasing the imbalance between the concentration gradients of DHAP and Pi.

Significance of Observed Changes in Metabolite Levels for Light-Dark Regulation of Starch Formation. According to Preiss *et al.* (3, 14, 15), the main candidates for a possible light-dark regulation of ADP-glucose pyrophosphorylase are PGA, Pi, and F6P. But the level of Pi in the chloroplasts did not change significantly during light-dark-light transition. Therefore, it could not contribute to the observed light-dark regulation of starch formation in our experiments. Nevertheless, the level of Pi can play an important role for the over-all amount of carbon flowing into starch during photosynthesis, as internal chloroplastic [Pi] will change in response to changes of the external (cytoplasmic) Pi level (12).

The levels of PGA and of hexose-monoP change dramatically during light-dark transition. Both are known to stimulate ADP-glucose pyrophosphorylase (3, 14, 15). As the changes in the concentrations of these compounds are opposite, the resulting change in the activity of ADP-glucose pyrophosphorylase might be questioned. Only F6P of the hexose-monoP is an activator, and only one-third of the hexose-monoP is present as F6P. Also, PGA is a more effective activator of ADP-glucose pyrophosphorylase than F6P (14, 15).

One might further assume that the high concentration of PGA in the chloroplasts in the dark as well as in the light might be far beyond the saturation level for activation ($A_{0.5}$ of PGA = 20 μM, compare refs. 14 and 15). However, it has been shown that the important aspect for regulation is the ratio of activator (PGA) *versus* inhibitor (Pi) rather than the absolute concentrations (14, 15). As shown in our experiments, the Pi concentration is in the same range as the concentration of PGA. Therefore the observed changes of [PGA] during light-dark-light transition could still modulate the activity of ADP-glucose pyrophosphorylase to a great extent.

When compared with the kinetics of starch formation during light-dark-light transition (Fig. 1), the changes in metabolite levels (mainly of PGA) seem to be too slow to account for the fast light-dark regulation of starch formation observed in our experiments and also in investigations *in vivo* (10). Additional factors must be involved in light-dark control of starch formation, such as changes in ATP/ADP levels, in pH, or in the stromal Mg^{2+} concentration. All of these factors are known to change during light-dark transitions (11) and they also influence the activity of ADP-glucose pyrophosphorylase (3, 15). These problems are investigated in a following paper.

Table I. Concentrations of PGA, DHAP and Pi in chloroplasts and medium in light and dark

The numbers are taken from Fig. 2 and 3, at the end of the first light period (light) or at the end of the dark period (dark). For further details see text.

Compound	A Conc. in Chloroplast (mM)	B Conc. in Medium (mM)	A/B Trans-envelope gradient
	Light		
PGA	4	0.05	80
DHAP	0.4	0.05	8
Pi	4	0.25	16
	Dark		
PGA	1.6	0.1	16
DHAP	0.4	0.025	16
Pi	4	0.25	16

Acknowledgments—The skilled technical assistance of S. Gee is gratefully acknowledged. We thank S. Krohne, J. Paul and K. Hammel for helpful discussions and a critical reading of the manuscript.

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