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LIGHT-DARK REGULATION OF STARCH METABOLISM IN CHLOROPLASTS

I. LEVELS OF METABOLITES IN CHLOROPLASTS AND MEDIUM DURING LIGHT-DARK-TRANSITION
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Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Chemical Name</th>
</tr>
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<tbody>
<tr>
<td>PGA</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>DHAP</td>
<td>(OH)$_2$-acetone phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>glucose-6-phosphate</td>
</tr>
<tr>
<td>G1P</td>
<td>glucose-1-phosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>fructose-6-phosphate</td>
</tr>
<tr>
<td>R5P</td>
<td>ribose-5-phosphate</td>
</tr>
<tr>
<td>Ru5P</td>
<td>ribulose-5-phosphate</td>
</tr>
</tbody>
</table>

1 This work was supported in part by the Biomedical and Environmental Division of the U.S. Department of Energy.

2 Recipient of a post-doctoral fellowship of the Deutsche Forschungsgemeinschaft (DFG).
ABSTRACT

The kinetics of CO₂ fixation, of starch formation and of changes in the levels of metabolites in chloroplasts and the surrounding medium have been investigated during light-dark and dark-light transitions with isolated intact chloroplasts.

The internal level of Pi stays constant throughout a light-dark-light cycle. The concentration of 3-phosphoglycerate in the chloroplasts decreases (from about 4 mM in the light to 1.6 mM in the dark within 3 min), whereas the level of the hexosemonophosphates increases at the same time (from 2.2 mM in the light to about 6 mM in the dark). In the subsequent light period both compounds reach their original levels within 3 min. The chloroplastic concentrations of dihydroxyacetone phosphate, of the pentose monophosphates and of the hexose- and heptose-bisphosphates remain constant at about 0.4 mM 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 trans-envelope concentration gradients of 3-phosphoglycerate, dihydroxyacetone phosphate and Pi which exist in the light, is completely abolished after 2-3 min 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.
INTRODUCTION

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 and hexose monophosphates (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 seconds upon light-dark transition (10).

Recently it was demonstrated by different authors (6,8,16) 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 phosphates in the chloroplast due to the reactions catalyzed by the phosphate translocator (4). These results (6,8,16) 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 CO₂ fixation and of starch formation under the same conditions.
MATERIALS AND METHODS

**Plant Material.** Spinach was grown in vermiculite under artificial light (3000 ft-c) with an 8 h light period and a 16 h 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 (7). Chlorophyll was measured by the procedure of Arnon (1).

**Incorporation of $^{14}$CO$_3$.** 50 µl of chloroplast suspension (50 µg chlorophyll) was added to solution C (7), 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 x m$^{-2}$ x sec$^{-1}$. At given intervals 50 µl were removed with micro-syringes and injected into 450 µl 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 glacial acetic acid, dried by flushing with nitrogen 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 chlorophyll) were incubated in a total volume of 3 ml solution C, labeled with 150 µCi $^{32}$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 (Spinco). 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 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 (7). Aliquot samples (150 μl) of the chloroplast-methanol mixture were spotted on Whatman #1 paper and developed in two dimensions, first for 60 h with a phenol-water-acetic acid solvent and then for 48 h in the second direction with butanol-water-propionic acid (13). The insoluble fraction (obtained from chromatogram origins) was hydrolyzed with trifluoroacetic acid for 1 h at 100°C and an aliquot was chromatographed as above, but only for 24 h 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 starch.

RESULTS

Time course of CO₂ fixation and starch formation during light-dark transition. In the first light-phase, CO₂ was fixed by whole chloroplasts with a linear rate of about 90 μmol/mg chlorophyll x h (Fig. 1). After the light was switched off, CO₂ fixation ceased within 30-60 sec of darkness. Under our conditions, there was no detectable decrease of the fixed CO₂ during the 4 min dark period (e.g. by oxidative pentose phosphate cycle). In the second light phase, CO₂ 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 CO₂ fixation. During the dark period, there was usually little or no post-illumination incorporation of CO₂ into starch and there was no or only little starch degraded in the dark period.
It is known that the overall percentage of carbon incorporated into the starch depends largely on the Pi concentration in the medium (6,8,16). 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 CO₂ fixation in agreement with other published results (6,16).

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 ³²P-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 chlorophyll as a reference. This is well in the range found by other authors (5) as well as by our own measurements of chloroplast volumes by coulter-counter distribution (not shown here). However, it should be kept in mind that 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.

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 3-4 mM and then remains nearly constant (9,12). Our first samples were taken after 7 min in the light. Therefore, 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 2-3 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 hexose monophosphates (F6P + G6P + G1P) in the chloroplasts was about 50 nmol/mg chlorophyll corresponding to a concentration in the chloroplast of 2 mM (Fig. 2). Upon light-dark transition, the hexose monophosphate concentration in the chloroplasts increased dramatically up to about 6 mM within 2-3 min and decreased once more to about 2.5 mM in the second light period. Thus, the concentration change of the hexose monophosphates and of PGA during light-dark-light transitions were opposite, the increase in the hexose monophosphate concentration being somewhat greater than the decrease in [PGA] (see Discussion).

The concentration of pentose monophosphates (R5P, Ru5P) in the chloroplasts was about 0.4 mM and the fructose- and sedoheptulose-bisphosphates 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 Fig. 2.

Metabolites in the Medium. The changes of metabolite levels in the medium 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 about 30-60 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 McLilley 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 2-3 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 monophosphates, hexose monophosphates and of the bisphosphates 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 ref. 2,12).

Changes in the $^{14}$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, $^{14}$CO$_3$ was added during photosynthesis instead of $^{32}$P so that possible changes in labelled 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 2-4 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 (see discussion). It was therefore of interest to find that 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 monophosphate level in the dark was completely abolished by adding PGA, and addition of Pi even induced a considerable decrease in total hexose monophosphates (Fig. 4). In this experiment we found a small amount of degradation of starch in the dark. This starch degradation, however, was too small to account for the observed increase in hexose monophosphate. In the presence of high [PGA], this starch degradation was inhibited, thus confirming similar observations of other authors (6,16).

DISCUSSION

Analysis of the Changes in Metabolite Concentrations. Since the increase in the hexose monophosphates 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-bisphosphatase into hexose monophosphate (as long as the latter enzyme is active in the dark). One molecule of Pi would be released (from fructose-bisphosphate) for two DHAP molecules consumed. This released Pi could serve as a counter-ion for only half the imported DHAP so that, in addition, another counter-ion has to be exported. Since the internal level of Pi remains constant, this counter-ion has to be PGA. Infact, 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 monophosphates, 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 monophosphates 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 monophosphate.
The total amount of PGA formed and of DHAP consumed in the dark phase is usually higher than the change in hexose monophosphate 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 post-illumination CO$_2$ fixation.

It is known that PGA or Pi compete with DHAP for translocation by the phosphate translocator (4). We have demonstrated that the increase in concentration of hexose monophosphates 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 monophosphates in the dark is due to a re-import of DHAP from the medium, as it should be expected that this reimport is inhibited by high [Pi] or [PGA] in the medium.

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 (Fig. 2,3). In other words, in the light, a large difference in the trans-envelope concentration gradients of PGA, Pi, and DHAP is maintained (Table I). After three min in the dark, all concentration gradients of the compounds transported by the phosphate translocator have become equal (Table I). Therefore, it seems reasonable to conclude that the driving force for the observed light to dark changes in metabolite concentrations is an imbalance of the trans-envelope 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 two negative charges, whereas Pi and DHAP have only one. 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 decreases the external [Pi] and increases the internal [Pi]) would lead to an even higher trans-envelope gradient of Pi, thus increasing the imbalance between the concentration gradients of DHAP and Pi.

Significance of the Observed Changes in Metabolite Levels for the 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 overall 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 monophosphates 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. It should be kept in mind, however, that PGA is more active in regulating the enzyme than hexose monophosphates so that [PGA] could still modulate the enzyme activity to a great extent (3,14,15).

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). Therefore, 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$^{++}$ concentrations. All 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 the following paper.
ACKNOWLEDGEMENTS

The skilled technical assistance of Ms. Sherry Gee is gratefully acknowledged.
LITERATURE CITED


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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A Concentration in Chloroplast (mM)</th>
<th>B Concentration in Medium (mM)</th>
<th>A/B Trans-envelope gradient</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Light</td>
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</tr>
<tr>
<td>PGA</td>
<td>4</td>
<td>0.05</td>
<td>80</td>
</tr>
<tr>
<td>DHAP</td>
<td>0.4</td>
<td>0.05</td>
<td>8</td>
</tr>
<tr>
<td>Pi</td>
<td>4</td>
<td>0.25</td>
<td>16</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGA</td>
<td>1.6</td>
<td>0.1</td>
<td>16</td>
</tr>
<tr>
<td>DHAP</td>
<td>0.4</td>
<td>0.025</td>
<td>16</td>
</tr>
<tr>
<td>Pi</td>
<td>4</td>
<td>0.25</td>
<td>16</td>
</tr>
</tbody>
</table>
LEGENDS

Fig. 1: Time course of CO₂ fixation and starch formation with intact chloroplasts during light-dark-light transition. Reaction was started by adding 50 µl of chloroplasts (50 µg chlorophyll) to 450 µl reaction medium in the light. Numbers next to the curves give the rate of carbon incorporation (µmol/mg chlorophyll x h). For further details see Materials and Methods.

Fig. 2: Changes in the levels of ³²P-labeled metabolites in the chloroplasts during light-dark-light transition. Concentrations (right scale) were calculated for an average chloroplast volume of 25 µl/mg chlorophyll (see text). 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). For further details see Materials and Methods.

Fig. 3: Changes in the levels of ³²P-labeled metabolites in the medium (same experiment as in Fig. 2). Note that the scales for each section of the figure are different. Abbreviations as in Fig. 2.
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 Fig. 2. For further details see Materials and Methods.
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Fig. 1
Fig. 2

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Fig. 3

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Fig. 4

Time (min)

---

 PGA

DHAP

HMP

STARCH

XBL 782 - 3818

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Fig. 4
This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.