On the Participation of Phosphoribulokinase in the Light Regulation of CO₂ Fixation

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ABSTRACT

CO₂ fixation by a suspension of isolated spinach chloroplasts was terminated by turning off the light, and changes of metabolite levels in the chloroplast stroma and the surrounding medium were assayed. Whereas CO₂ fixation comes to a total stop within 15 seconds, a conversion of triose phosphates to hexose, hexose, and pentose monophosphates is found to occur for 1 to 2 minutes afterwards. It seems from these data that an inactivation of fructose and sedoheptulose bisphosphatases proceeds with a lag period. In contrast, the conversion of pentose monophosphates to ribulose 1,5-bisphosphate is inhibited immediately after the stop of illumination. As the stromal level of freely available ATP was not depleted under this condition, these data demonstrate that ribulose 5-phosphate kinase was very rapidly inactivated after darkening of the chloroplasts. Essentially, the same effect is also observed when CO₂ fixation is partially inhibited by addition of moderate concentrations of m-chlorocarbonyl phenylhydrazone, partially uncoupling photophosphorylation. It appears from these results, that the activity of ribulose 5-phosphate kinase is not only regulated by light through the mediation of reduced carriers like thioredoxin but also by alternative parameters, e.g. stromal metabolite levels.

There is ample evidence that light influences the activity of several enzymes of the reductive pentose-P cycle, including fructose bisphosphatase, sedoheptulose bisphosphatase, phosphoribulokinase, ribulose bisphosphate carboxylase, and glyceraldehyde-3-P dehydrogenase (see ref. 2). This effect of light seems to be mediated by several parameters, e.g. reducing equivalents probably in the form of thiolredoxin and the stromal H⁺ and Mg²⁺ concentration (2). Such information has been obtained mainly with extracted enzymes. In order to verify that a given enzyme has a regulatory function, it is necessary to demonstrate that this enzyme is able to limit the flow of carbon under physiological conditions. Such studies require the measurement of stromal metabolites in defined metabolic states. Using isolated chloroplasts as a model where a light-dark transient was partially simulated by changes of the stromal H⁺ and Mg²⁺ concentrations, a very effective regulation of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase has been clearly demonstrated (4, 9, 14, 15). There is no evidence presently available that RuBP carbox-

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2 Abbreviations: RuBP, ribulose 1,5-bisphosphate; Ru5P, ribulose 5-phosphate; CCCP, m-chlorocarbonyl phenylhydrazone; PGA, 3-phosphoglycerate; HMP, sum of hexose and heptose monophosphates; FBP, fructose 1,6-bisphosphate; SBP, sedoheptulose 1,7-bisphosphate; PMP, pentose monophosphate.

MATERIALS AND METHODS

Spinach (Spinacia oleracea, variety United States Hybrid 424 from Ferry Morse Seed Co., Mountain View, CA) was grown in water culture according to Lilley and Walker (12). For the preparation of chloroplasts see reference 14.

The incubation of the chloroplasts (0.1 mg Chl/ml) was carried out in a medium containing 0.33 M sorbitol, 50 mM Hepes adjusted to pH 7.6 with KOH, 1 mM MgCl₂, 1 mM MnCl₂, 2 mM EDTA, 0.04 mg/ml catalase from beef liver (Boehringer, Mannheim), 1 mM Pi, and 10 mM NaHCO₃. In the experiment of Figure 1, carrier-free [³²P]phosphate was added yielding a specific activity of 40 Ci/mol. For experiments where CCCP was added, the pH was lowered to 7.3 and phosphate raised to 1.5 mM. Temperature 20°C. The pH in the stroma and the thylakoid space were determined in parallel samples (8) where ³²P-labeled phosphate was omitted. The samples kept in a 400-μl polypropylene tube were illuminated with a tungsten-halogen light source provided with an RG 630 cutoff filter (Schott, Mainz, West Germany) at a light intensity of 80 w/m². For details of centrifugation and preparation of extracts see reference 8.

The chromatographic assay of ³²P-labeled metabolites was carried out according to Lilley et al. (13).

The determination of ADP and ATP in the stromal extracts was carried out with the luciferase assay (Biotumat, LB 9500, Berthold, Wildbad, West Germany) employing a luciferin-luciferase prep-
chloroplast suspension. RuBP was measured enzymically by $^{14}$CO$_2$ incorporation as in reference 17 and this method was extended to provide a new enzymic assay for the combined pentose-P. In the assay for RuBP, extract (3–5 μg Chl) is incubated for 30 min in a final volume of 150 μl with 100 mM Tris (pH 8.1), 20 mM MgCl$_2$, 2 mM NaHCO$_3$, 4 μCi $[^{14}$C]NaHCO$_3$, 7.5 units activated RuBP carboxylase, and 0.5 mM dichlorophenol indophenol (included to inhibit any traces of Ru5P kinase in the carboxylase preparation). After 30 min, the samples were acidified with 500 μl 1 N HCl, evaporated, and the acid-stable $^{14}$C radioactivity measured. In the pentose-P assay, first an identical incubation was carried out, except that the $[^{14}$C] NaHCO$_3$ was omitted, in order to convert the RuBP in the extract into unradioactive PGA. After 30 min, 3 units phosphoriboseisomerase (Sigma), 0.3 unit Ru5P kinase (Sigma), 0.5 mM ATP, 1 mM DTT, and 4 μCi $[^{14}$C]NaHCO$_3$ were added. The DTT rapidly reduced the dichlorophenol indophenol and activated the Ru5P kinase, and the pentose-P were converted into $[^{14}$C]PGA. After 30 min, the samples were acidified, evaporated, and the acid-stable $^{14}$C counted. This technique gave linear results between at least 0.025 to 1.5 nmol Ru5P. Negligible conversion of a mixture of trioses, pentoses, and fructose-6-P, and fructose-6-P occurred at levels greatly exceeding those found in physiological fractions. All measurements were carried out in triplicates, and internal standards with 0.025 to 1.5 nmol ribose-5-P were also included to ensure that the extract did not interfere with the assay procedures.

The rapid cessation of CO$_2$ fixation by chloroplasts was measured by illuminating chloroplasts with unlabeled NaHCO$_3$ for 11.5 min, before adding $[^{14}$C]NaHCO$_3$ (final specific radioactivity 1 Ci/mol). Five, 15, 25, and 35 s later, aliquots were acidified. At 35 s, the chloroplasts were darkened and further aliquots withdrawn at 5-s intervals. The added $[^{14}$C]NaHCO$_3$ equilibrated with the unlabeled NaHCO$_3$ within 5 s. Since comparatively little $^{14}$CO$_2$ had been fixed before darkening, the continued small increase in the dark could be accurately evaluated. For enzymic measurement of stromal RuBP and pentose-P levels shortly after darkening, aliquots of chloroplast suspension were pipetted into 400-μl microtubes containing silicone oil over 10% (w/v) HClO$_4$, which were transferred into illuminated Beckman microtubes shortly before the end of the illumination, so that the incubation was terminated by centrifugation (13) either in the light or shortly after darkening. Samples for ATP were prepared by pipetting aliquots directly into 10% HClO$_4$, Ru5P kinase was assayed as in reference 10 with 2 mM ATP, 0.5 mM Ru5P, 10 mM MgCl$_2$, 100 mM Tris (pH 7.8), 0.1% Triton X-100. Pentose-P, RuBP, ATP, Ru5P kinase, and CO$_2$ fixation were measured with the same chloroplast preparation.

RESULTS AND DISCUSSION

To study the events occurring in the chloroplasts during a light-dark transition, they first were illuminated for 12 min in the presence of Pi and NaHCO$_3$. This long illumination time was chosen so that the products of CO$_2$ fixation, mainly triose-P and PGA in the medium, would accumulate. The light was then switched off (Fig. 1) and CO$_2$ fixation came rapidly to a stop. In agreement with earlier results (6), darkening leads to a decrease of the stromal pH and an increase of the pH in the thylakoid space, resulting in a considerable decrease of the pH gradient across the thylakoid membrane (Fig. 1A). The metabolite levels in the stroma and the external medium were assayed in parallel samples of the chloroplast suspension. For this, the chloroplasts were preincubated with $^{32}$P-labeled Pi in the light to label uniformly all the phosphorylated intermediates and products of CO$_2$ fixation. The incubation of the chloroplasts was terminated by a rapid separation of the chloroplasts from the surrounding medium through silicone oil into a layer of HClO$_4$. Since the separation is achieved in less than 2 s, and illumination (or darkness) was continued during this process, an effective quenching of the metabolism in the chloroplast stroma is achieved. The separated fractions were then analyzed by ion exchange chromatography. The data of Figure 1 show typical results which have been reproduced several times. Figure 1B shows the balance for the major metabolites in

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**Fig. 1.** Changes of the pH in the stromal and thylakoid space (A) and of metabolite levels, as determined by $^{32}$P ion exchange chromatography, in the external medium (C) and the stromal compartment (D–F) of spinach chloroplasts during the transition from 12-min illumination to darkness. In Figure 1B, the total amount of carbon contained in the metabolites in the stroma and the medium has been calculated from the data of C to F. All data are from the same experiment, for details see “Materials and Methods” and text.
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the unfractionated chloroplast suspension during these experiments. During photosynthesis, some of the triose-P, and to a lesser extent PGA, are exported to the medium, as shown from the continued accumulation of these compounds (Fig. 1C). The remainder are reconverted via hexose and heptose monophosphates into pentose-P, from which RuBP is synthesized, regenerating a CO₂ acceptor and so allowing continued CO₂ fixation. After darkening, the CO₂ fixation stops almost immediately, but initially there is a continued conversion of triose-P into hexose and heptose monophosphates (HMP, these are not separated by our chromatographic techniques) and into pentose-P (see also Kaiser and Bassham [9]). This conversion ceases about 1 to 2 min later indicating that FBPase and SBPase, the limiting enzymes of this conversion, are totally inactivated by then. The lag phase of this inactivation is obvious. This is also confirmed by the corresponding stromal levels of FBP and SBP. One min after darkening, when CO₂ fixation is inhibited totally, the level of SBP has not changed, and that of FBP has even decreased (Fig. 1E). The rise of the SBP level occurring afterwards concurs with the subsequent inhibition of triose-P conversion. This finding clearly indicates that the inactivation of SBPase plays a role in the termination of the conversion of triose-P to sugar monophosphates. The slight fall of total fixed carbon after stop of illumination may be due to prolonged synthesis of carbohydrate polymers and/or pentose-P cycle activity.

The triose-P converted to sugar monophosphates during the initial phase after the termination of CO₂ fixation derives mainly from the external medium. Apparently, there is a switch from export of triose-P in the light, to import in the dark. A preferential export of triose-P, compared to PGA, occurs in the light, probably caused by a light-dependent cation gradient across the envelope (7). Correspondingly, the triose-P/PGA ratio in the external medium is much higher than in the chloroplast stroma. Upon darkening, this difference in the triose-P/PGA ratios dissapears and there is a fall in the stromal PGA level, mainly due to export into the medium (see also Kaiser and Bassham [9]). At the same time, triose-P is transported from the medium into the stroma where it is converted to sugar monophosphates. Due to the specificity of the phosphate translocator (3), the hexose and heptose monophosphates formed are confined to the stroma, whereas pentose-P is being in part released to the medium.

The most prominent and fastest change of stromal metabolite levels is observed with pentose-P. Immediately after turning off the light (Fig. 1F), there is a rapid decrease of RuBP accompanied by an accumulation of pentose-P. The subsequent accumulation of hexose and heptose monophosphates may be visualized in part as a consequence of pentose-P accumulation due to the equilibria between these substrates catalyzed by aldolase and transketolase. These findings clearly show that the formation of RuBP from Ru5P has been blocked under these conditions. A trivial explanation for this blockage would be that in darkness the substrate ATP is not available any more for Ru5P kinase. In the dark, however, ATP could be generated by conversion of triose-P to PGA, and preliminary experiments (not shown here) indeed indicated that there were considerable chloroplast ATP levels also in the dark (see also ref. 5).

To verify these observations, a systematic study was made of the levels of ATP and ADP, when CO₂ fixation was not inhibited by darkening or restricted by the addition of uncoupler. The resulting data shown in Figures 2 and 3 were all obtained in one experiment with the same chloroplast suspension. For comparison with the experiments of Figure 1, stromal RuBP and the sum of stromal pentose-P were determined. In order to verify the results of Figure 1 obtained by ion exchange chromatography, the measurements of RuBP and of the sum of pentose-P in Figures 2 and 3 were carried out by enzymic analysis. In both experiments, the inhibition of CO₂ fixation is accompanied by a marked rise of the

pentose-P, and a decrease of the chloroplast ATP level is found. Inasmuch as chloroplasts are known to contain adenine nucleotides bound to the thylakoid membranes (see 16), the question arises, whether the decreased ATP levels reflect a disappearance of all the ATP which is available for phosphorylating reactions, or if there are still significant levels of ATP available as a substrate for Ru5P kinase. This question can be answered in the positive for both treatments. In the case of CCCP, ample ATP must still be available since CO₂ fixation and PGA reduction continued at 34% and 65%, respectively, of the control rates (CO₂ fixation: 72 μmol/mg Chl-h, PGA reduction: 71 μmol/mg Chl-h). For chloroplasts in the dark, the ATP level falls somewhat further than with CCCP, but ample ATP is still available for phosphorylating reactions, since the chloroplast ATP level assayed 1 min after the termination of illumination could be further decreased by more than 40% when PGA was added to these chloroplasts. These data yield as a minimum about 5 nmol available ATP/mg Chl in the dark. With a stromal space of 25 μl/mg Chl, the ATP concentration 1 min after darkening is estimated to be about 0.2 mM. This concentration is far above the Km of Ru5P kinase for ATP (30 μM) as assayed with the enzyme freshly extracted from illuminated chloroplasts (10) or from darkened chloroplasts (M. Stitt, unpublished work). Assuming the very likely case that the equilibrium between ribose, xylulose, and ribulose monophosphates is not changed markedly, a rise of the pentose-P level in the presence of an ATP level more than 6 times higher than the Km for ATP demonstrates that Ru5P kinase was inhibited very effectively shortly after illumination was stopped. These results establish that Ru5P kinase can indeed limit carbon flow through the Calvin cycle. The question arises as to how this enzyme may be controlled. On the one hand, an interconversion of the enzyme by reducing equivalents, e.g. thioredoxin (see

Fig. 2. Changes in ATP and ADP (A) and RuBP and pentose-P (B) after darkening chloroplasts which had been illuminated for 12 min. For A, the influence of PGA on the levels of ATP and ADP was investigated by adding 5 mM PGA to half of the chloroplast preparation 15 s after darkening. Data obtained by enzymic assay.
introduction) is known. A light-dependent activation and inactivation of this enzyme, probably occurring in this way, has been clearly demonstrated. By rapid extraction of this enzyme from intact chloroplasts in defined metabolic states, an 8-fold activation occurring after the onset of illumination with a half-time of 30 s and a reversal of this activation after turning off the light with a half-time of 90 s has been found (10). This apparent interconversion of the enzyme after darkening, however, does not fully explain our observations made with intact chloroplasts. In experiments with short term resolution, we compared the inhibition of CO₂ fixation after darkening chloroplasts with the rate at which Ru5P kinase was converted back into the less active form typical of chloroplasts in the dark (Table I). After 10 and 30 s darkness, respectively, CO₂ fixation was already inhibited more than 85% and 98%, but the extracted Ru5P kinase activity had decreased only 6% and 21% from the level in the light. At the same time, stromal RuBP fell and pentose-P rose, showing that Ru5P kinase was already rate-limiting. Moreover, although stromal ATP decreased, there was still ample free ATP available to support a high Ru5P kinase activity. When 5 mM PGA was added 10 s before darkening the chloroplasts, the ATP level was decreased reproducibly during the period after darkening. After 10-s and 30-s darkness, the ATP was 1.37 ± 0.05 and 1.43 ± 0.34 nmol/mg Chl lower in the presence of PGA than in untreated chloroplasts. With a stromal volume of 25 μl/mg Chl, this gives a minimal value for free ATP of about 60 μM, which is 2 to 3 times higher than the Kₐ value determined for freshly extracted Ru5P kinase (see above). These data allow the conclusion that factors other than enzyme interconversion are primarily involved in inhibiting Ru5P kinase in these conditions, inasmuch as the flux through the enzyme is nearly totally prevented in the presence of ample levels of substrates, before more than a small portion of the enzyme has been converted from the active form found in the light into the less active form in the dark.

Another clear indication that a change of the reductive state of a substance like thioredoxin is not involved primarily in the inactivation of Ru5P kinase also arises from the experiment of Figure 3. Here adding of uncoupler, which is not supposed to alter the reductive state of electron transport carriers in intact chloroplasts, has principally the same effect as turning off the light. We found that addition of CCCP to chloroplasts produced only small alterations in the extractable activity of Ru5P kinase, even at levels which lead to as much as a 90 to 100% inhibition of photosynthesis (Laeing, Stitt, unpublished work).

We concluded from these model experiments with intact chloroplasts that Ru5P kinase can be regulated efficiently and rapidly in its activity by other parameters beside reducing equivalents. Such parameters may be stromal metabolites and ions. Lavergne et al. (11) reported earlier on a regulatory effect of adenine nucleotide levels on the activity of Ru5P kinase, whereas Anderson (1) did not find such an effect. Furthermore, inhibition of Ru5P kinase activity by 6-P-glucose and PGA has been reported (1). The results of the metabolite measurements shown here stimulated further studies in our laboratory, where Ru5P kinase was rapidly extracted from chloroplasts in a defined metabolic state and the activation state was investigated by a new assay procedure. Our studies so far have shown that the catalytic activity of the light-activated enzyme is strongly influenced by physiological concentrations of stromal metabolites including ADP (10) and PGA (Gardemann, Stitt, Heldt, in preparation). The enzyme appears less sensitive to pH and Mg²⁺, although a 4-fold change in the activity could be produced by changes of stromal Mg²⁺ concentrations and pH occurring during a light-dark transient (see ref. 10). Without questioning the significance of a regulation by light through the mediation of reduced carriers like thioredoxin, a rapid control of the activity by stromal metabolites and ion concentrations may be of equal importance. The precise role of these alternative mechanisms in the control of Ru5P kinase and the Calvin cycle requires further study.

To establish the regulatory role of an enzyme in controlling the flow of carbon through the reductive pentose-P cycle, it is not only necessary to study the influence of different parameters on a particular enzyme in vitro, but also to prove that this enzyme has the capability to limit the metabolism of the cycle in vivo. As a simple model for such in vivo conditions, isolated chloroplasts are a suitable object. In earlier studies of metabolite levels with intact chloroplasts during dark-light transients or during changes of
stromal Mg\(^{2+}\) and H\(^+\) concentrations, fructose and sedoheptulose bisphosphatases have been identified as potent regulatory steps of the CO\(_2\) fixation cycle (4, 9, 14, 15). Our present studies obtained under different experimental conditions, add Ru5P kinase to this list. In the case of the first two enzymes, alternative parameters such as reducing equivalents, stromal metabolite levels and H\(^+\) and Mg\(^{2+}\) concentration contribute to a very rigid metabolic control of enzymic activities. This involves the light-dependent interconversion of enzymes proceeding with a certain time course (and which in the case of FBPase and SBPase is influenced by stromal metabolite levels) as well as the immediate response of the catalytic activity of the activated or inactivated form of these enzymes to stromal metabolites and ions. Although the stromal factors regulating Ru5P kinase activity have not yet been fully elucidated, it seems to follow such a multiple pattern as well. These multiple parameters may enable the enzymes not only to be regulated under extreme situations like illumination and darkness, but also to adjust their activity by fine control to the metabolic conditions during CO\(_2\) fixation.

LITERATURE CITED
1. ANDERSON, LE 1973 Regulation of pea leaf ribulose 5 phosphate kinase. Biochim Biophys Acta 321: 484–488