Adenine nucleotide status, phosphoglycerate reduction and photosynthetic phosphorylation in a reconstituted chloroplast system

K. A. CARVER,* A. B. HOPE† and D. A. WALKER*

*A.R.C. Research Group on Photosynthesis, Department of Botany, University of Sheffield, Sheffield S10 2TN, U.K., and †School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia 5042, Australia

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The concentrations of ADP and ATP were measured during illumination of a reconstituted chloroplast system to which NADP⁺, 3-phosphoglycerate and ribose 5-phosphate were successively added. Their effects on photosynthetic oxygen evolution and on the concentrations of ATP and ADP are discussed in terms of the regulation of photosynthetic carbon assimilation.

In recent years considerable emphasis has been placed on the regulatory role of P_i in photosynthetic carbon assimilation. In experiments with isolated chloroplasts there is no doubt that high concentrations of P_i inhibit photosynthesis by enforcing export of metabolites via the phosphate translocator (Walker & Crofts, 1970) or that low concentrations of P_i limit photosynthesis (Cockburn *et al.*, 1967) by restricting the synthesis of Gri3P and sugar phosphates. Low [P_i] may make itself felt by lowering the [ATP]/[ADP] ratio [either by slowing the rate (Ryrie & Jagendorf, 1971) or diminishing the extent of photophosphorylation]. Attention has also been drawn to the sensitivity of the 3-phosphoglycerate kinase reaction (EC 2.7.2.3) to inhibition by low [ATP]/[ADP] ratios (Lavergne *et al.*, 1974; Robinson & Walker, 1979) and to the possibility that the effects of [ATP], [ADP] and [Fri3P] on this reaction play a key role in the regulation of photosynthetic carbon assimilation (Walker & Robinson, 1978). Experiments on a reconstituted chloroplast system, reported here, add weight to this proposition.

Experimental

Materials

Spinach (*Spinacia oleracea* var. U.S. Hybrid 424; Ferry Morse Seed Co. Ltd., Mountain View, CA, U.S.A.) was grown in pots or water culture as described previously by Lilley & Walker (1974).

Abbreviations used: Gri3P, 3-phosphoglycerate; Rib5P, ribose 5-phosphate; Gri1,3P₂, glycerate 1,3-bisphosphate; Gri2,3P₂, glycerate 2,3-bisphosphate; Rbl5P, ribulose 5-phosphate; Rbl1,5P₂, ribulose 1,5-bisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Spinach ferredoxin was prepared by a method similar to that of Rao *et al.* (1971).

The reconstituted chloroplast system

Intact chloroplasts were prepared as described previously (Lilley & Walker, 1974). The isolation medium was 0.33 M-sorbitol/10 mM-Na₄P₂O₇/5 mM-MgCl₂, and 2 mM-D-isoascorbate, adjusted to pH 6.5 with HCl. The resuspension medium was 0.33 M-sorbitol/2 mM-EDTA/1 mM-MgCl₂/1 mM-MnCl₂/50 mM-Hepes, adjusted to pH 7.6 with KOH. The reconstituted chloroplast system was prepared by a method similar to that of Lilley & Walker (1974), except that intact chloroplasts [100 µg of chlorophyll, measured by the method of Arnon (1949)], were osmotically shocked by water in the oxygen-electrode vessel. Ferredoxin (200 µg), NADP (0.1 µmol), ADP (0.3 µmol), NaHCO₃ (10 µmol), KH₂PO₄ (1 µmol), MgCl₂ (10 µmol), dithiothreitol (1 µmol), sodium isoascorbate (4 µmol) and catalase (200 units) were added to the shocked chloroplasts. Reaction mixtures were adjusted to 1 ml by the addition of 500 µl of chloroplast-resuspension medium. Rib5P (0.5 mM) and Gri3P (2 mM) were added as indicated.

O₂ evolution

O₂ evolution was measured polarographically (Delieu & Walker, 1972) by using twin-channel Hansatech electrodes (Hansatech Ltd., King's Lynn, Norfolk, U.K.). Illumination was by red light in the wavelength range 590–750 nm at an irradiance of 300 W·m⁻².

Adenine nucleotides

ATP and ADP were determined in 10 µl samples of chloroplast suspension added to equal volumes of

0.1 M-HCl. These mixtures were adjusted to 0.4 ml with 25 mM-Hepes buffer, pH 7.9, and further diluted with Hepes buffer if necessary. Adenine nucleotides were assayed in an LKB Wallac Luminometer 1250 with luciferin-luciferase in the form of 'ATP Monitoring Reagent' (LKB Wallac) by using procedures similar to those described by Kobayashi *et al.* (1979). ATP gave a signal that was calibrated against standard ATP (10 μ M). ADP was converted into ATP by using 5 units of pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate (0.5 mM). This conversion was monitored in the Luminometer and a second calibration was made.

Results

It has been previously established that Gri3P-dependent O₂ evolution in a reconstituted chloroplast system is inhibited by ADP or by a variety of ADP-generating systems (Slabas & Walker, 1976a; Walker *et al.*, 1976; Walker & Robinson, 1978; Robinson & Walker, 1979) and that this is probably a mass-action effect. The reaction catalysed by phosphoglycerate kinase has a $\Delta G'$ value of approx. +16.74 kJ (+4 kcal) in the direction of Gri1,3P₂ formation and therefore requires a high [ATP]/[Gri3P]/[ADP][Gri1,3P₂] in order to proceed. For this reason it is readily reversed by the addition of ADP. This reversal stops Gri3P reduction, NADPH utilization and non-cyclic electron transport. As phosphoribulokinase (EC 2.7.1.19) has a high affinity for ATP (Lavergne *et al.*, 1974), it has been argued that the addition of Rib5P provides an ATP sink (Slabas & Walker, 1976b), since it is converted via Rbl5P into Rbl1,5P₂:

The inhibition of O₂ evolution would be overcome, in time, as cyclic and/or pseudocyclic photophosphorylation restored a favourably high [ATP]/[ADP] ratio. The time course of O₂ evolution in Fig. 1 is in accord with this proposal and with the earlier work by Lilley & Walker (1974) and Slabas & Walker (1976a,b). It will be seen that there was an initial and rapid burst of O₂ evolution, which was in stoichiometric accord with the reduction of the NADP added to the system. Non-cyclic electron transport then ceased, for want of an oxidant, until NADP was regenerated from NADPH by the reduction of added Gri3P (2 mM). Subsequently, Gri3P reduction and its associated O₂ evolution was stopped by the addition of Rib5P (0.5 mM), which functions as an ATP sink. After a short interval, O₂ evolution was resumed at a slightly lower rate. It eventually ceased upon darkening and re-started upon further illumination.

It will be seen that the major changes in

adenine-nucleotide status were also in accord with the earlier conclusions of Robinson & Walker (1979). Some ATP and AMP (the latter not measured in this experiment) was formed in the dark because of the presence of adenylate kinase (myokinase) (EC 2.7.4.3), which catalyses the interconversion:

In the presence of adenylate kinase, interconversion of ATP and AMP will occur, which explains why the changes in ATP levels are larger than those of ADP.

Upon illumination there was a rapid rise in [ATP] and a corresponding fall in [ADP], which slowed as the added NADP was reduced and non-cyclic gave way to cyclic or pseudocyclic electron transport. Eventually, steady-state levels were reached. The addition of Gri3P then brought about a sharp, but transient, decline in [ATP] as an initial consumption of ATP in the phosphoglycerate kinase reaction was followed by even faster ATP formation occasioned by the restoration of non-cyclic electron transport to NADP.

When Rib5P was added, there were similar, but larger, excursions in adenine nucleotide values. This time, however, the initial decrease in the [ATP]/[ADP] ratio stopped non-cyclic electron transport and its associated O₂ evolution so that a favourable ratio could only be restored by cyclic and/or pseudocyclic photophosphorylation. It is noteworthy that, in this particular experiment, the steady-state ratio of [ATP]/[ADP] after Gri3P addition was about 5; O₂ evolution ceased as this ratio approached 1. It was resumed at something less than 3 and reached a new steady-state (for both O₂ evolution and adenine nucleotides) at about 4. This final value was presumably lower than the initial value (of 5) because of the continuing drain on ATP in the presence of Rib5P. The less-favourable final value (of 4) could also account for the correspondingly lower rate of O₂ evolution. By this time, however, the reaction mixture was extremely complex, with new Gri3P being derived from Rib5P and *vice versa*.

On darkening, the [ATP]/[ADP] ratio fell and rose again upon subsequent illumination.

Discussion

The experiment illustrated in Fig. 1, which was typical of a number carried out at this time, reinforces earlier proposals that photosynthetic carbon assimilation can be regulated by the controlling influence of adenine-nucleotide status on the reaction catalysed by phosphoglycerate kinase, and that this in turn may reflect the attenuating effect of low [P_i].

Fig. 1. Time course of O_2 evolution (----) and adenine nucleotide changes (—) in a reconstituted chloroplast system. The reconstituted system contained catalytic concentrations of NADP (0.1 mM) and ADP (0.3 mM). The transition to light and dark conditions is indicated by L and D respectively. Gri3P (2 mM) and Rib5P (0.5 mM) were added as indicated. For further details see the Experimental section. O, ADP; ●, ATP.

The point at issue is not whether adenine-nucleotide status exerts its effect by mass action (Robinson & Walker, 1979) or by some allosteric modulation of phosphoglycerate kinase (Lavergne *et al.*, 1974), since these effects would, in any case, simply reinforce one another. In fact, these experiments were undertaken in order to seek direct, rather than inferential, evidence (cf. Slabas & Walker, 1976b) that the addition of Rib5P would constitute an ATP sink and that the ensuing decrease in the [ATP]/[ADP] ratio would halt Gri3P-dependent O_2 evolution until such time as cyclic or pseudocyclic electron transport could restore the ratio to a value (approx. 2.3 in this case) which would again support Gri3P reduction. This, indeed, proved to be the case.

The transitory nature of the excursions in [ATP] and [ADP] seen upon the addition of Gri3P is noteworthy. Since two molecules of ATP are required for every molecule of O_2 evolved in the photosynthetic reduction of Gri3P, the consumption of ATP at this juncture can readily be quantified from the oxygen-electrode trace and was found to be $132 \mu\text{mol} \cdot \text{h}^{-1} \cdot (\text{mg of chlorophyll})^{-1}$. Nevertheless,

this consumption was rapidly exceeded by ATP formation associated with the renewed onset of non-cyclic electron transport, so that the earlier steady-state [ATP]/[ADP] ratio was quickly restored.

It should be noted that the rate of Gri3P reduction is not only governed by the [ATP]/[ADP] ratio but also by the concentration of Gri3P (high [Gri3P] would offset a low [ATP]/[ADP] ratio). Nevertheless, as P_i limitation of photosynthesis is induced (*in vitro* by withholding P_i from reaction mixtures or *in vivo* by lowered availability of P_i within the cytosol), a point must eventually be reached when the [ATP]/[ADP] ratio is lower than that which would obtain in the presence of optimal P_i . It seems inescapable, therefore, that the decline in O_2 evolution that is observed under these circumstances must reflect a slowing in the conversion of Gri3P into Gri₁3P₂. Similarly, this conversion will modulate the formation of Rb15P just as the concentration of Rb15P will, in turn, govern the reduction of Gri3P. As previously noted (Slabas & Walker, 1976b), it is this mechanism which main-

tains a balance between Gri3P reduction and the regenerative phase of photosynthetic carbon assimilation and, likewise, demands a high concentration of Gri3P and low concentration of Rb15P in steady-state photosynthesis.

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