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Communication

Regulation of Ribulose-1,5-Bisphosphate Carboxylase Activity by the Activase System in Lysed Spinach Chloroplasts

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MARTIN A. J. PARRY*, ALFRED J. KEYS, CHRISTINE H. FOYER, ROBERT T. FURBANK[1], AND DAVID A. WALKER
AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Hertfordshire, United Kingdom, AL5 2JQ (M.A.J.P., A.J.K.); *and Research Institute for Photosynthesis, University of Sheffield, Sheffield, United Kingdom, S10 2TN* (C.H.F., R.T.F., D.A.W.)

ABSTRACT

Ribulose-1,5-bisphosphate (RuBP) carboxylase in lysed spinach (*Spinacia oleracea* L. cv virtuosa) chloroplasts that had been partly inactivated at low CO_2 and Mg^{2+} by incubating in darkness with 4 millimolar partially purified RuBP was reactivated by light. If purified RuBP was used to inhibit dark activation of the enzyme, reactivation by light was not observed unless fructose-1,6-bisphosphate, ATP, or ADP plus inorganic phosphate were also added. Presumably, ADP plus inorganic phosphate acted as an ATP-generating system with a requirement for the generation of ΔpH across the thylakoid membrane. When the RuBP obtained from Sigma Chemical Co. was used, light did not reactivate the enzyme. There was no direct correlation between ΔpH and activation. Therefore, thylakoids are required in the ribulose-1,5-bisphosphate carboxylase activase system largely to synthesize ATP. Inactivation of RuBP carboxylase in isolated chloroplasts or in the lysed chloroplast system was not promoted simply by a transition from light to dark conditions but was caused by low CO_2 and Mg^{2+}.

To attain catalytic competence, RuBP[2] carboxylase must first be activated with CO_2 and Mg^{2+} (14). The concentration of CO_2 required to produce maximal activity *in vitro* is an order of magnitude greater than that which occurs in chloroplasts of C_3 plants *in vivo*. However, Pi and several phosphate esters that are present in the chloroplasts cause the enzyme to reach full activity *in vitro* at ambient concentrations of CO_2 and it can be assumed that these effectors also influence the activity of the enzyme *in vivo* (2, 8, 12, 15). The effectors are generally competitive inhibitors of RuBP binding and so have a dual effect on activity. 2-Carboxyarabinitol 1-P inhibits carboxylase in the dark in certain plant species but is lost in the light when the activity of RuBP carboxylase is restored (3, 7, 22–24, 28).

The recent discovery of a protein, which has been called RuBP carboxylase activase, that can activate RuBP carboxylase at low CO_2 concentrations in the presence of RuBP (17, 19, 25) may indicate the existence of a further means of regulating RuBP carboxylase activity. Alternatively, the activase could be involved in the removal of 2-carboxyarabinitol 1-P from the active site. Seemann *et al.* (22) demonstrated that a phosphatase relieved the inhibition of RuBP carboxylase by 2-carboxyarabinitol 1-P.

The requirements for RuBP carboxylase activation in broken chloroplasts were investigated to confirm the published observation and to seek any relationship to regulation by 2-carboxyarabinitol 1-P. Initial difficulties in reproducing the light activation of RuBP carboxylase in this system were overcome by use of RuBP produced in the laboratory rather than a use of commercial sample (AR Portis, private communication).

MATERIALS AND METHODS

Plant Material and Intact Chloroplast Preparation. *Spinacia oleracea* L. cv virtuosa was grown in hydroponic culture in a glasshouse (5) and intact spinach chloroplasts were prepared as described by Walker (26).

Assay System for Broken Chloroplasts. Intact chloroplasts (100 μg Chl) were lysed in 10 mM $MgCl_2$, and the other components of the reaction medium were added to the final concentrations of 0.35 M sorbitol, 1 mM EDTA, 50 mM Hepes/KOH buffer (pH 7.6), 3 μM 9-aminoacridine, 6000 units catalase, 1500 units carbonic anhydrase, and 1 mM methyl viologen with either low (2 mM) or high (10 mM) $NaHCO_3$. The final volume of the reaction mixture was 2 ml. Incubations and O_2-uptake measurements were performed at 20°C in a Clark-type oxygen electrode (Hansatech Ltd., U.K.) illuminated with a quartz halogen projector lamp (150 W) with a calflex heat filter (Balzers, F.R.G.) and a red filter giving an irradiance of 1800 $\mu E \cdot m^{-2} \cdot s^{-1}$. 9-Aminoacridine fluorescence was measured as described previously (10). RuBP (4 mM) was added in the dark prior to dark incubation. Samples (50 μl) were taken at the times indicated and assayed immediately for RuBP carboxylase activity.

Preparations of RuBP. RuBP was prepared from AMP using the method of Wong *et al.* (27). Samples were retained at three stages of purification. Proteins were removed by precipitation with acid and nucleotides by absorption on charcoal. Crude RuBP (RuBP 1) was precipitated from the filtrate by precipitation with $BaCl_2$. The bulk of this crude fraction was purified by absorption on Dowex 1 Cl^- and elution with 40 mM HCl to remove ribose 5-P and then with 150 mM HCl containing 0.1 M NaCl to elute the RuBP. The product was precipitated with $BaCl_2$ to yield RuBP 2. This RuBP was further purified by chromatography on a Dowex 1 column equilibrated with 3 mM HCl using a linear LiCl gradient (0.0–0.5 M) to yield RuBP 3 as the barium salt. Barium salts were converted to sodium salts before the samples were tested in the lysed chloroplast system.

[1] Present address: CSIRO, Division of Plant Industry, P. O. Box 1600, Canberra 2601 ACT, Australia.

[2] Abbreviations: RuBP, ribulose-1,5-bisphosphate; FBP, fructose 1,6-bisphosphate.

RuBP Carboxylase Activity. RuBP carboxylase activity was measured at 25°C in 0.5 ml reaction mixtures containing 0.1 M Bicine/HCl buffer (pH 8.2), 20 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (1 Ci mol^{-1}), and 0.6 mM RuBP (Sigma Chemical Co.). The reaction was started by the addition of 50 μl of sample from the primary reaction mixture. Reactions were stopped when 200 μl samples of the mixture were taken after 30 s and mixed rapidly with 400 μl of 10 N formic acid. Carboxylase activity was calculated from ^{14}C incorporation measured after the acidified samples were dried in an air stream and resuspended in Hydro Luma scintillation fluid (Lumac/3M, The Netherlands).

Other Assays. Protein was estimated by the dye-binding protein assay (Bio-Rad Laboratories, München, FRG). Chl was estimated by the method of Arnon (1). RuBP was measured by the acid-stable ^{14}C formed when test samples were incubated with excess purified RuBP carboxylase in the presence of saturating concentrations of $NaH\ ^{14}CO_3$ (13).

RESULTS

RuBP carboxylase activity was measured in intact chloroplasts incubated with low (2 mM) $NaHCO_3$ or high (10 mM) $NaHCO_3$. With intact chloroplasts, RuBP carboxylase activity was high and constant during a dark to light transition, and light-dependent activation of the enzyme was not observed. With broken chloroplasts, RuBP carboxylase activity was highly dependent on the $NaCHO_3$ concentration of the medium, being high at 10 mM $NaHCO_3$ and much lower with 2 mM $NaHCO_3$ (Fig. 1). In the absence of RuBP and with 2 mM $NaHCO_3$, the RuBP carboxylase activity of broken chloroplasts was not significantly increased by illumination after a dark preincubation (Fig. 1). The addition of commercial RuBP failed to promote light-dependent activation of RuBP carboxylase under any of the experimental conditions described here. RuBP synthesized from AMP was used in the experiments reported here. RuBP samples at different stages of purification ("Materials and Methods") were incubated with the broken chloroplast mixture in the dark. Illumination then caused light activation with two of the RuBP samples tested (RuBP 1 and RuBP 2) (Fig. 1). RuBP 1, the least pure of the RuBP samples, produced a twofold increase in RuBP carboxylase activity following illumination but depressed the level of maximum activity when compared to the high $NaHCO_3$ control. No significant depletion of the RuBP was detected in any of the experiments. However, analysis of the contents of RuBP 1 revealed that it was contaminated with 3.1 mol of Pi per mol of RuBP, which would be inhibitory to catalysis and depress the maximum level of activity. Light-dependent activation of RuBP carboxylase was pronounced in the broken chloroplasts incubated with RuBP 2, which had only 1.33 mol Pi per mol of RuBP. In addition, light activation in the presence of RuBP 2 resulted in a final level of activity that approached that of the fully activated enzyme (measured in the presence of 10 mM $NaHCO_3$). RuBP 3, the most pure RuBP sample that had been freed from contaminating Pi by the ion-exchange chromatography, did not promote light activation of RuBP carboxylase. This latter result suggested that a necessary component of the activating system had been removed from the RuBP during the purification of RuBP 2 to RuBP 3. Analysis of the components present in RuBP 1 and RuBP 2 by thin layer chromatography suggested that these samples contained little or no adenylates but some free sugar and sugar monophosphate. In the absence of RuBP 3 the presence of FBP or ADP plus Pi had no effect on the activation of RuBP carboxylase. However, when broken chloroplasts were incubated in the presence of RuBP 3 with FBP or ADP plus Pi, light activation of RuBP carboxylase was restored (Fig. 2).

Activation of RuBP carboxylase in a reconstituted chloroplast system has previously been correlated with the extent of transthylakoid ΔpH (18). This parameter was monitored during the time course of the experiments by 9-aminoacridine fluorescence (with simultaneous measurements of RuBP carboxylase activity). A comparison of the ΔpH measured after 6 min illumination and the accompanying RuBP carboxylase activity attained in the presence of RuBP 3 showed no positive correlation between the two parameters (Fig. 3). Indeed, the lower ΔpH measured in the presence of ADP and Pi resulted in a higher RuBP carboxylase activity.

DISCUSSION

The evidence for the existence of an enzyme, RuBP carboxylase activase, involved in the activation of RuBP carboxylase, comes from studies of the *rca* mutant of *Arabidopsis thaliana* (20). The initial activity of carboxylase in extracts of leaves of this mutant declines rather than increases when the leaves are illuminated (20). Two polypeptides were absent from leaves of the mutant that were present in the wild-type plant (19). Purified proteins from spinach chloroplasts containing polypeptides with behavior similar, upon gel electrophoresis, to those missing from the *rca* mutant catalyzed activation of RuBP carboxylase that had been inactivated by excess RuBP at low CO_2 and Mg^{2+}

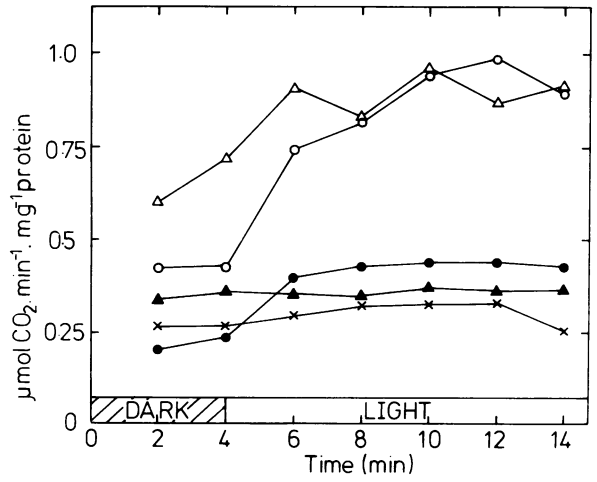

FIG. 1. Effects of 4 mM RuBP, at different stages of purification, in the presence of 2 mM $NaHCO_3$ on the activity of RuBP carboxylase in broken chloroplasts (RuBP 1 [least pure] [●]; RuBP 2, [○]; RuBP 3 [purest sample] [×]). The controls without RuBP contained 2 (▲) or 10 mM (△) $NaHCO_3$.

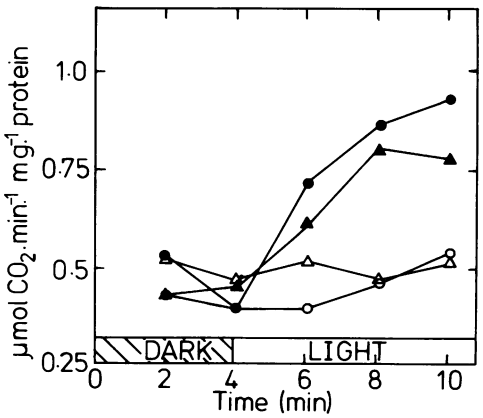

FIG. 2. Effect of 1 mM ADP and 5 mM Pi (●, ○) and 1 mM FBP (▲, △) on the activity of RuBP carboxylase in lysed chloroplasts in the presence of 2 mM $NaHCO_3$ but in the absence (open symbols) or presence (closed symbols) of RuBP 3.

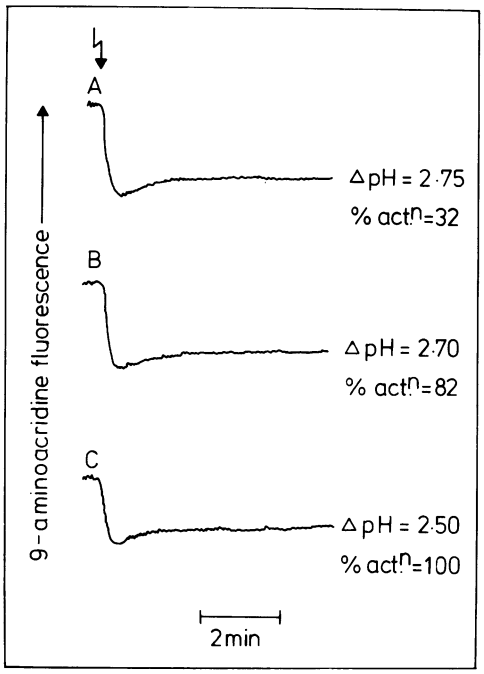

FIG. 3. Measurements of 9-aminoacridine fluorescence following a dark to light transition in broken chloroplasts in the presence of 2 mM $NaHCO_3^-$ and supplied with 4 mM RuBP 3 alone (A), with RuBP 3 and 1 mM FBP (B), or with RuBP 3, 1 mM ADP, and 5 mM Pi (C). The percentage activation of RuBP carboxylase (relative to the fully active enzyme) after 6 min illumination under each condition is given (% act^n).

concentrations. The assay system required to demonstrate activase activity was complex, and the presence of thylakoid membranes and light were reported to be essential (8, 20, 21). It is of major concern that activation can only be successfully achieved if the RuBP used is made in the laboratory since commercial RuBP did not allow reactivation. It is not clear whether the commercial RuBP lacks an additional component essential for the activase system or contains an inhibitor of it. In our analysis by TLC, the commercial RuBP appeared to be pure and therefore the absence of some additional component essential for the activase system appears most likely. Adenine nucleotides have been reported to be an impurity in samples of RuBP and have been implicated in the activation process (17). We confirm a role for adenine nucleotides, but our results also indicate that they may be replaced by FBP. Partially purified samples of the RuBP that we made allowed reactivation of RuBP carboxylase in the light but the most pure RuBP fraction alone would not facilitate activation. When the partially purified RuBP was purified further, the activation could only be demonstrated if either ADP and Pi or FBP were also added to the system of thylakoids and chloroplast extract. With commercial RuBP, activation could not be demonstrated even when FBP or ADP plus Pi were added.

The hypothesis that activation depended on the ΔpH existing in the thylakoids (18) is clearly at variance with the measurements we now report. This hypothesis has presumably also been negated by the observed involvement of ATP (17) in activation catalyzed by the activase. However, it remains impossible to decide what components of the test system might be directly involved in displacing RuBP from noncarbamylated sites on the enzymes.

The observed changes in the activation state of RuBP carboxylase in leaves are not consistent with the involvement of inactivation by the binding of RuBP to noncarbamylated sites even in those species where there is no evidence for the involvement of 2-carboxyarabinitol 1-P. Thus, RuBP carboxylase in extracts of wheat leaves exposed to low light (6) or to photoinhibitory treatments (4) is mostly reactivated by a short incubation with CO_2 and Mg^{2+}, whereas experiments with purified RuBP carboxylase suggest that RuBP removal from noncarbamylated sites would have a $t_{1/2}$ of at least 60 min at 24°C (11). We can assume that activase must be present in the crude extracts and presumably in other substances that might be required for removal of RuBP from noncarbamylated sites. Many effectors present in the chloroplast stroma *in vivo* inhibit decarbamylation (12) and permit high activities in the presence of limiting CO_2 and Mg^{2+} (16). There are strong indications that in the intact chloroplast Pi and phosphate esters affect the activation status of RuBP carboxylase in a reversible manner (9). The activase enzyme might therefore be involved in reactions changing the concentrations of particular phosphate esters in the chloroplast stroma. The requirement for impurities in the RuBP causes speculations that these include a precursor of an activator. FBP could be converted into many different phosphate esters in reconstituted chloroplast systems. Hence, more information is needed about the binding and release of RuBP in the presence of effectors.

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