Vol. 89, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 12, 1979 Pages 300-306

CHLOROPLAST RIBULOSE-5-PHOSPHATE KINASE: LIGHT-MEDIATED ACTIVATION, AND DETECTION OF BOTH SOLUBLE AND MEMBRANE-ASSOCIATED ACTIVITY

Kurt H. Fischer[1] and Erwin Latzko

Botanisches Institut der Universität, Schloßgarten 3,
D-4400 Münster, Germany (BRD)

Received June 4,1979

SUMMARY: Phosphoribulokinase from spinach chloroplasts held in darkness was inactive when measured at pH 6.8 but active when measured at pH 7.8. Following the onset of illumination of the chloroplasts, the enzyme activity measured at either pH increased rapidly. Significant activation by dithiothreitol was observed only at pH 7.8 with enzyme from chloroplasts held in darkness. A portion of the total phosphoribulokinase can be pelleted by centrifugation after chloroplast breakage, irrespective of whether or not the chloroplasts were illuminated immediately prior to breakage. The pelleted activity could be easily solubilised by extraction with buffer.

INTRODUCTION: Although light-mediated activation of phosphoribulokinase is a well established phenomenon (1-4), reported activities of phosphoribulokinase from chloroplasts held in darkness are nevertheless quite considerable (1). This is surprising for two reasons. Firstly, light-mediated activation would not seem to serve any purpose if the activity in darkness approaches or exceeds the rate of CO_2 fixation of intact chloroplasts in the light (5). Secondly, based on thermodynamic considerations (6), phosphoribulokinase *in vivo* appears to be more or less completely inactive in the dark: the concentrations of ribulose-5-phosphate and ATP in the stroma of chloroplasts in darkness are essentially the same as under illumination (7,8), yet accumulation of ribulose-1,5-bisphosphate, which would be expected with active phosphoribulokinase in the absence of CO_2 fixation, does not occur (9). In an attempt to clarify these anomalies, estimates have been made of phosphoribulokinase acti-

[1] This investigation represents partial fulfilment of the requirements of K.H. Fischer for the degree of Dr. rer. nat.

0006-291X/79/130300-07$01.00/0

vity from darkened chloroplasts and assayed under conditions believed similar to those of the chloroplast stroma in darkness. The results confirm that the enzyme is essentially inactive in darkness, but is rapidly activated following even a low intensity of illumination.

During these experiments phosphoribulokinase was found to exist both as a soluble enzyme and as enzyme loosely bound to chloroplast membranes. The latter association may be relevant to the mechanism of light-induced activation mediated by the electron transport chain.

MATERIALS AND METHODS: Spinach (Spinacea oleracea L.) was grown hydroponically. The plants were placed in darkness prior to experiments. Chloroplasts were isolated under weak green light following the procedure of Lilley et al. (10). 8 g leaves were blended for 5 sec in 40 ml of isolation medium (0.33 M sorbitol, 10 mM $Na_4P_2O_7$, 5 mM $MgCl_2$ and 2 mM ascorbate adjusted to pH 6.5 with HCl). The homogenate was filtered through Miracloth, centrifuged at 760 x g for 80 sec and the supernatant discarded. The upper layer of the pellet was suspended in 2 ml of resuspending medium (0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 50 mM HEPES-KOH, pH 7.8) and then discarded. The remaining chloroplasts were suspended in 5 ml of resuspending medium. All operations were at 4°C.

The chloroplasts were illuminated in a water bath at 25°C. White light with an intensity of 30000 lux was supplied from a Leitz Prado projector. At the times indicated chloroplasts were removed in 0.1 ml samples and broken by direct addition to reaction mixtures containing 100 µmoles Tris-HCl buffer pH 7.8 or 100 µmoles MES-KOH buffer pH 6.8, 10 µmoles $MgCl_2$, 20 µmoles KCl, 1 µmole ATP, 0.6 µmole PEP, 0.38 µmole NADH, 2.8 units lactate dehydrogenase, 1 unit pyruvate kinase and 2 µmoles ribose-5-phosphate. The final volume was 1 ml. Three min after starting the reaction with ribose-5-phosphate, 20 µl of 0.5 M dithiothreitol were added to the reaction mixture. Phosphoribulokinase activity was calculated from the change in absorbance at 366 nm measured with an Eppendorf 1101 M photometer at 25°C.

Thylakoid membranes were obtained by homogenising 1 ml of the suspension of isolated intact chloroplasts with a glass Ten Broeck homogeniser. The broken chloroplasts were centrifuged at 13000 x g for 10 min. For phosphoribulokinase extraction, the pellet was resuspended in 1 ml of 50 mM HEPES-KOH buffer (pH 6.8) containing 10 mM dithiothreitol. The suspension was centrifuged after 10 min at 13000 x g for 10 min and the enzyme activity in the supernatant was determined.

RESULTS: The activity of phosphoribulokinase from freshly ruptured chloroplasts held in darkness was virtually zero when measured at pH 6.8 (Fig. 1A). At pH 7.8, however, enzyme acti-

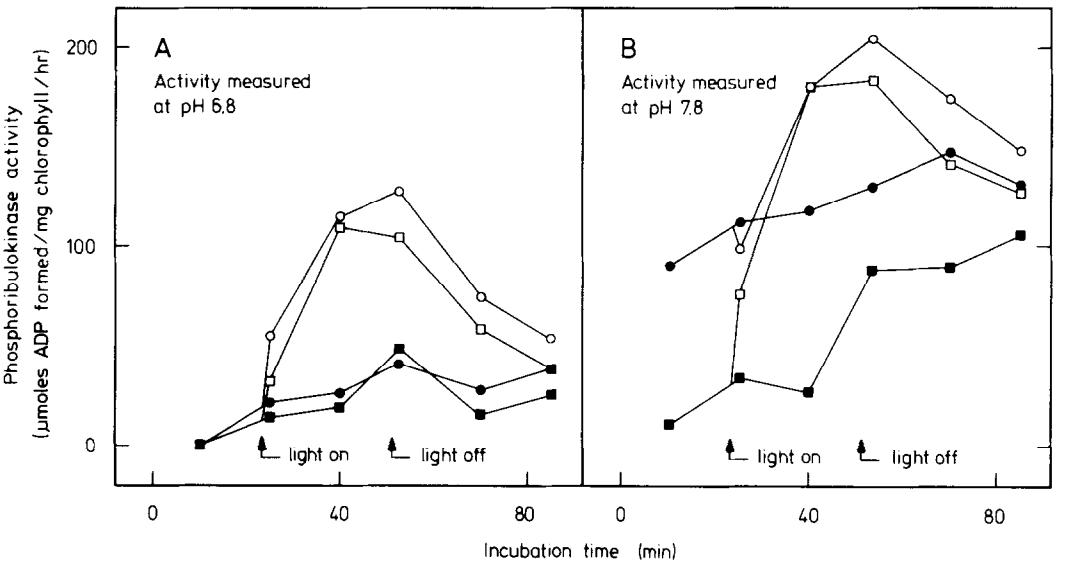

Figure 1: Light activation of phosphoribulokinase in intact chloroplasts. The chloroplasts were illuminated between 24 min and 52 min of incubation time. The chlorophyll concentration was 0.12 mg/ml; phosphoribulokinase activity was measured at pH 6.8 (A) and pH 7.8 (B). Activity of control (continuous darkness) without (■—■) and with (●—●) 10 mM dithiothreitol; and activity after illumination without (□—□) and with (○—○) 10 mM dithiothreitol.

vity was clearly detected (Fig. 1B). At pH 6.8, dithiothreitol (10 mM) only slightly activated phosphoribulokinase from darkened chloroplasts but at pH 7.8 it increased activity over 7-fold.

After illumination of chloroplasts, the activity of phosphoribulokinase increased (whether determined at pH 6.8 or 7.8); the highest activity achieved at pH 6.8 was about 50 % of that measured at pH 7.8. The substantial activation by dithiothreitol observed at pH 7.8 before illumination disappeared almost immediately after the beginning of illumination, thus making the response to dithiothreitol similar to that seen when measurements were made at pH 6.8.

The light induced activation is quite rapid. After only 1 min illumination of chloroplasts, the activity increased clearly at both pH's (Fig. 1), reaching the highest level after 16 min. In some experiments the activity measured at pH 7.8 increased much more rapidly. There was often 50% of maximal activity after a

Table 1: Velocity of light activation of phosphoribulokinase in intact chloroplasts, measured at pH 7.8.

time of illumination (sec)	0	5	55	840
activity measured at pH 7.8 without dithiothreitol	53	227	340	435
activity measured at pH 7.8 with 10 mM dithiothreitol	77	368	340	435

Activity in µmoles ADP formed/mg chlorophyll/hr.

few seconds illumination (Table 1). Even without illumination a slow increase in activity was seen, mainly at pH 7.8; an explanation of this increase is not yet available, but it may represent a slow release of enzyme from chloroplast membranes (see below).

After darkening chloroplasts previously illuminated, the enzyme activity decreases (Fig. 1). A complete and rapid inactivation of phosphoribulokinase from illuminated chloroplasts was achieved by the addition of 10 mM cystamine to the reaction mixture (either at pH 6.8 or pH 7.8); 50% inactivation was observed after 1-2 min.

Some of the phosphoribulokinase activity of spinach chloroplasts can be pelleted by centrifugation after chloroplast breakage. This result was obtained with preparations of both darkened and illuminated chloroplasts. The pelleted enzyme was easily solubilised by suspending the pellet in 50 mM HEPES-KOH buffer pH 6.8 or 7.8, and the activity released was often equal to that originally in soluble form, (i.e. to that present in the supernatant after centrifugation of the broken chloroplasts) (Fig. 2A). The potential activity of phosphoribulokinase therefore, was as much as twice the activity measured directly with

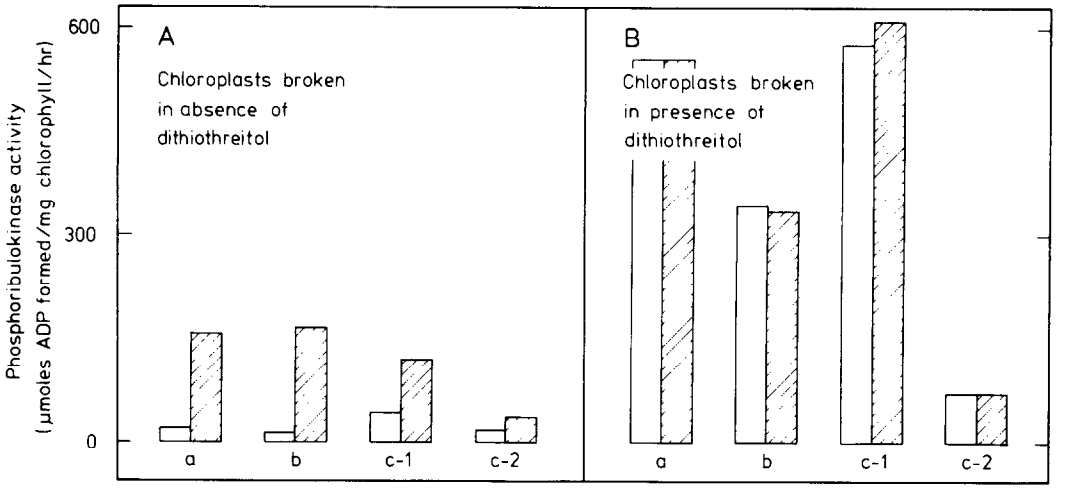

Figure 2: Phosphoribulokinase activity in chloroplasts. Chloroplasts previously held in darkness were ruptured in 50 mM HEPES-KOH buffer (pH 6.8) without (A) and with (B) 10 mM dithiothreitol. (a) activity in broken chloroplasts before centrifugation, (b) activity in supernatant after centrifugation, (c-1) and (c-2) activity in pellet from centrifugation: first and second extractions. Measurements were made at pH 7.8 without (non-hatched) and with (hatched) 10 mM dithiothreitol.

broken chloroplasts. Total recovery of the pelleted phosphoribulokinase could be achieved by repeated resuspensions of the pellet. The presence of either 1 to 10 mM $MgCl_2$, 2 mM ribose-5-phosphate plus 3 units phosphoriboisomerase/ml, 1 mM ATP plus 10 mM $MgCl_2$, 0.1 mM NADH, or 0.1 mM NADPH in the extraction medium did not noticeably influence the ratio of soluble to membrane-associated phosphoribulokinase activity.

Incubation of broken chloroplasts with 10 mM dithiothreitol leads to a higher activity of phosphoribulokinase than can be measured with the same concentration of dithiothreitol in the reaction mixture; both the soluble and membrane associated enzyme activities were increased by this preincubation with dithiothreitol. Under these circumstances the total phosphoribulokinase activity (measured in the presence of dithiothreitol) was up to 5-times that found with broken chloroplasts not treated with dithiothreitol (cf. Figs. 2A and 2B).

DISCUSSION: These investigations have demonstrated that, during darkness, almost no chloroplast phosphoribulokinase activity can

be demonstrated after isolating chloroplasts under a very low light intensity and measuring enzyme activity at pH 6.8 immediately after rupturing the chloroplasts. This pH value of 6.8 is close to the in vivo pH of the chloroplast stroma in darkness (11). Determination of the "dark activity" of phosphoribulokinase at pH 7.8 [the pH-optimum for phosphoribulokinase activity (12)] does not seem suitable, since this pH corresponds to that of the stroma in the light (11).

The rapid increase of phosphoribulokinase activity after illumination of chloroplasts confirms earlier results (2). In addition, the present results show clearly an increase of phosphoribulokinase activity from zero in the dark to values in the light corresponding to the rate of CO_2 fixation (5).

Dithiothreitol has an effect on phosphoribulokinase activity similar to that induced by light: presumably it activates by reduction of enzyme disulfide bonds (3). The behaviour of phosphoribulokinase activity towards dithiothreitol, the rapid increase of its activity following illumination, and its dependence on pH in a manner which corresponds well to the rate of the pH change in the chloroplast stroma (13), all suggest that inactive phosphoribulokinase in darkened chloroplasts is effectively activated by simultaneous reduction of the enzyme and alkalization of the chloroplast stroma following illumination.

An explanation is still being sought for the ability of dithiothreitol to increase the enzyme activity in incubations of broken chloroplasts to values much higher than when the chloroplasts are broken into reaction mixtures containing the same concentration of dithiothreitol (Fig. 2).

The observed association between phosphoribulokinase and thylakoid membranes suggests that a direct interaction between thylakoid sulfhydryl groups exposed after illumination (3, 14) and the bound enzyme might be facilitated; reduction of phosphoribulokinase by these sulfhydryl groups is not inconceivable, and may constitute, at least in part, the mechanism of light-mediated activation. The observed enzyme-membrane association makes a mediation of reduction equivalents between thylakoids and phosphoribulokinase by soluble compounds (15) seem less necessary than would otherwise be the case.

ACKNOWLEDGEMENTS: We thank Dr. G.J. Kelly for helpful discussions and assistance in preparation of the manuscript.

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