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Properties of Phosphoribulokinase of Whole Chloroplasts[1]

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ABSTRACT

The ability of intact spinach (*Spinacia oleracea*) choloroplast preparations to catalyze CO_2 fixation and photophosphorylation was examined. Under conditions optimal for CO_2 fixation, only poor photophosphorylation was observed. Conditions optimal for photophosphorylation were found to be highly inhibitory to the CO_2-fixing capacity of the intact chloroplast preparation.

A method for following the activity of phosphoribulokinase in the intact chloroplast preparation was developed, and conditions for optimal activity were defined. The enzyme was found to be activated 2- to 4-fold by preillumination with a half-time of less than 15 seconds. Activation was inhibited by magnesium ions and selectively by inhibitors of photosynthetic electron transport. We concluded that activation was due to the effect of a photoproduced reductant in a site preceding ferredoxin in the electron transport chain. The photoactivated state of the enzyme decayed in the dark with a half-time of about 8 minutes.

During the last decade, the introduction of methods for the isolation of relatively intact chloroplasts (9, 20), made it possible to study the complete photosynthetic complex at the cell-free level. Such chloroplast preparations fix CO_2 at reasonably high rates into the normal array of photosynthetic products, and seem subject to many of the regulatory processes which operate at the level of the whole cell.

However, the "envelope" of such intact preparations, in contrast to that of chloroplast membrane preparations, has been reported to be rather impermeable to many of the substrates of photoinduced electron transport and photophosphorylation, notably nucleotides such as ADP, ATP, and NADP. Thus, intact chloroplasts are rather poor catalysts of photophosphorylation, presumably because ADP cannot easily penetrate the envelope (9, 13, 20).

We attempted to measure the capacity of intact chloroplasts to catalyze photophosphorylation directly and indirectly by introducing R5P[3] as the final phosphate acceptor. In contrast to ADP, R5P is considered to be rather freely permeable through the chloroplast envelope (9, 12). As will be shown, under optimal conditions for CO_2 fixation, intact chloroplasts esterify phosphate rather poorly with either ADP or R5P as the final phosphate acceptor. Conditions optimal for ATP synthesis with either phosphate acceptor have been found, but such conditions are highly inhibitory to over-all CO_2 fixation.

Phosphoribulokinase is considered to be a major user of ATP in the intact chloroplast. It has been previously shown (14, 15, 16) to be subject to light activation. We confirmed this observation and describe here, further features of the activation phenomenon.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*) was obtained either from a local farm or grown in a growth chamber. Intact chloroplast preparations were made by blending 10 g of freshly picked, fully expanded leaves cut into small pieces in 40 ml of a solution containing: sorbitol, 0.3 M; HEPES, 50 mM; EDTA, 2 mM; KCl, 20 mM; $MgCl_2$, 2 mM; Pi, 0.2 mM; pH 6.7. Blending was carried out in a VirTis homogenizer Model 45 for 3 sec at 0 C. The suspension was filtered through gauze and centrifuged at 2000*g* for 50 sec. The pellet was resuspended in 2.0 ml of the same solution, but at pH 7.8, and this suspension served as the chloroplast preparation.

Reaction mixtures were prepared in small test tubes, placed in a water bath at 25 C, and allowed to equilibrate for 2 min in the dark. Illumination was with 20,000 lux of white light. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 3%. The suspension was centrifuged, and a sample of the supernatant was used to analyze for $^{14}CO_2$ incorporated into acid-stable products or ^{32}Pi esterified. $^{14}CO_2$ fixed was determined by first placing the 0.5-ml samples in a dessicator under aspirator vacuum for 1 hr, followed by addition of 8.0 ml of scintillation fluid (100 g of naphthalene, 5 g of PPO, and dioxane to a total volume of 1 liter) and measuring the radioactivity in a Beckman Model LS-150 scintillation counter using the $^3H + {}^{14}C$ window. The total radioactivity (cpm) introduced into the reaction mixture was determined by placing an appropriate small sample of the reaction mixture in a solution containing 0.3 M sorbitol, 0.2 M unbuffered tris, and taking a 0.5-ml sample of the latter and 8.0 ml of scintillation fluid for radioactivity determination. ^{32}P inorganic phosphate esterified was assayed by the isobutanol-benzene extraction procedure, as previously described (1).

RESULTS

Under conditions optimal for CO_2 fixation, little or no phosphorylation could be observed (Table I). In absolute terms, the rate of CO_2 fixation far exceeds the rate of phosphate esterification, a difference first noted and discussed by Baldry *et al.* (4). This difference is observed despite the fact that a large proportion of the products of this CO_2 fixation is reduced to the level of sugar phosphates (9, 10, 20) and also directly measured in this experiment. Addition of ADP, R5P, or both has

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[3] Abbreviations: R5P: D-ribose-5-phosphate; PMS: phenazine methosulfate; DSPD: disalicylidenepropanediamine; FCCP: carboxyl cyanide *p*-trifluoromethoxyphenylhydrazone.

no significant effect on either process. Addition of EDTA abolishes phosphorylation without affecting CO_2 fixation. The missing components for active phosphorylation seem to be free magnesium ions and an electron transport catalyst, such as PMS, as in the case of envelope-lacking chloroplasts (3). However, these components were found to be highly inhibitory for CO_2 fixation (Table I, see also ref. 2).

Since the addition of R5P did not stimulate phosphorylation under any of the conditions tested, it is clear that it was not the rate of entry of ADP which was rate-limiting in this case. This is even more evident in Figure 1A, where limiting amounts of ADP were added in the presence or absence of R5P. In the absence of added R5P, phosphorylation of the 0.05 μmole of ADP added proceeds rapidly until the ADP is fully converted to ATP; this is followed by a slower rate, most likely due to phosphorylation of endogenous internal phosphate acceptors, presumably R5P and its precursors. This second rate is accelerated by the addition of R5P, presumably indicating the activity of phosphoribulokinase. The phosphorylation of the endogenous internal phosphate acceptors can be slowed down considerably by azide (Fig. 1B), previously shown to inhibit CO_2 fixation in intact chloroplasts (2, 8), or by eliminating the major osmotic component, sorbitol, from the reaction mixture and thus converting the intact chloroplast preparation to a chloroplast membrane preparation (Fig. 1C). Neither procedure affects the activity of phosphoribulokinase.

Table II illustrates some of the properties of the phosphoribulokinase as measured by the above procedure. As can be seen, ATP could substitute for ADP, allowing for an even clearer detection of the activity of phosphoribulokinase. The presence of EDTA was required for phosphoribulokinase activity. The requirement for magnesium, PMS, and ADP is, of course, due to their participation in photophosphorylation. DCMU did not inhibit either PMS cyclic phosphorylation or phosphoribulokinase, but did inhibit O_2 evolution at 1 μM (not shown). Arsenite, which was previously shown to inhibit CO_2 fixation in isolated chloroplasts (11) and the activation and activity of phosphoribulokinase under some conditions (15), was only partially inhibitory even at 5 mM.

Glycerate-3-P and fructose-1,6-diP were poor substitutes for R5P in causing increased phosphate esterification (Table II). This difference is noteworthy when other properties of the three compounds are compared with respect to their effect on CO_2 fixation in the chloroplast. Thus, the three intermediates of the reductive pentose phosphate cycle stimulate CO_2 fixation (5, 17), remove the well known lag in CO_2 fixation (5, 19), and

Table I. *Comparison of CO_2 Fixation and Photophosphorylation in Intact Chloroplasts*

The reaction mixture (2.0 ml) contained: sorbitol, 0.3 M; HEPES, pH 7.8, 50 mM; Pi, 0.3 mM (containing 5×10^5 cpm ^{32}P in the phosphorylation experiments); $NaHCO_3$, 5 mM (containing 1×10^7 cpm $NaH^{14}CO_3$ in the CO_2 fixation experiments); ascorbate 5 mM and chloroplasts containing 34 μg of Chl. The reaction was run for 4 min for photophosphorylation and 10 min for CO_2 fixation in 20,000 lux of white light. Rates were calculated from the linear portion of the time course curves. Other details are described under "Materials and Methods."

Additions	Specific Activity	
	CO_2 fixed	Pi esterified
	μmoles/mg Chl·hr	
None	43	3
ADP, 1 mM	41	4
R5P, 0.1 mM	62	3
ADP, 1 mM; R5P, 0.1 mM	60	4
ADP, 1 mM; $MgCl_2$, 4 mM	11	18
ADP, 1 mM; $MgCl_2$, 4 mM; PMS, 3 μM	9	72
ADP, 1 mM; $MgCl_2$, 4 mM; PMS, 10 μM	...	126
ADP, 1 mM; $MgCl_2$, 4 mM; PMS, 30 μM	0	160
ADP, 1 mM; $MgCl_2$, 4 mM; PMS, 30 μM, R5P, 0.1 mM	0	162
ADP, 1 mM; PMS, 10 μM	20	9
EDTA, 2 mM	43	0

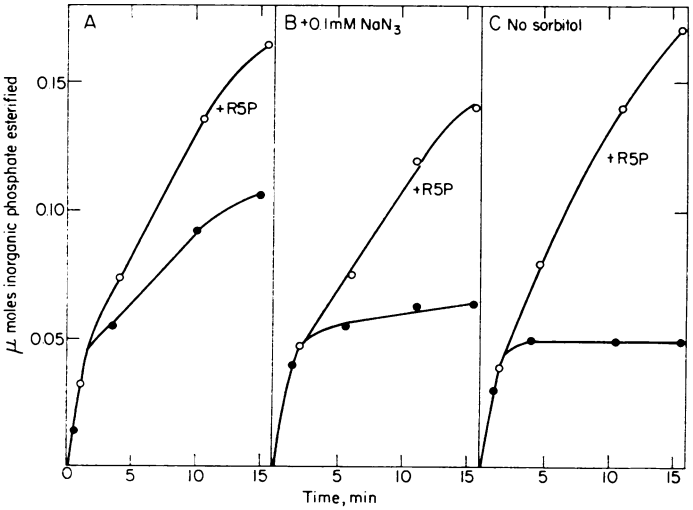

FIG. 1. Phosphorylation in the presence and absence of ribose-5-phosphate. The reaction mixture (2.0 ml) contained: sorbitol, 0.2 M; HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $MgCl_2$, 4 mM; ADP, 25 μM; Pi, 0.3 mM (containing 1.5×10^6 cpm ^{32}P); PMS, 10 μM; R5P, 0.5 mM, where indicated; ascorbate, 5 mM; $NaHCO_3$, 5 mM; and chloroplasts containing 45 μg of Chl. Other details as described under "Materials and Methods."

Table II. *Phosphoribulokinase and Photophosphorylation in Intact Chloroplasts*

The reaction mixture (2.0 ml) contained: sorbitol, 0.2 M; HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $MgCl_2$, 4 mM; PMS, 10 μM; ADP, 25 μM; Pi, 0.3 mM (containing 9×10^5 cpm ^{32}P); $NaHCO_3$, 5 mM; ascorbate, 5 mM and chloroplasts containing 71 μg of Chl. The reaction was run for 15 min.

Changes in Reaction Mixture	Inorganic Phosphate Esterified	
	Control	+ 0.5 mM R5P
	% of control	
None	100[1]	168
No ADP	10	15
No EDTA	78	76
No $MgCl_2$	15	46
No PMS	90	130
No sorbitol	66	182
+ ATP, 25 μM	36	122
Glycerate-3-P, 0.5 mM, in place of R5P	...	120
Fructose-1,6-diP, 0.5 mM, in place of R5P	...	116
+ DCMU, 1 μM	91	152
+ Arsenite, 5 mM	60	105
+ NaN_3, 0.5 mM	67	120

[1] 0.09 μmole of Pi esterified.

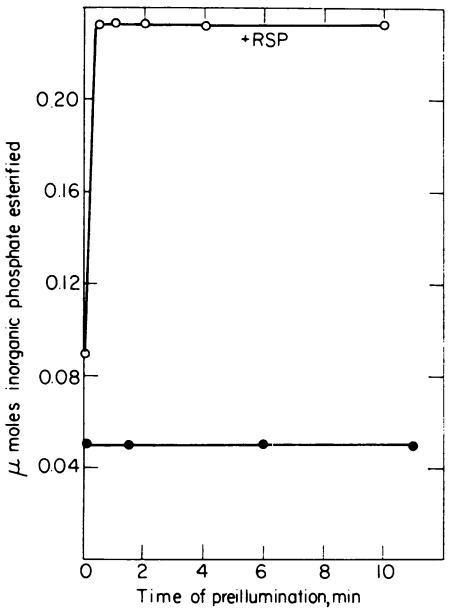

FIG. 2. Time course of activation of phosphoribulokinase. The preillumination reaction mixture (0.4 ml) contained: sorbitol, 0.2 M; HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 5 mM; ascorbate, 5 mM; Pi, 0.3 mM, and chloroplasts containing 41 μg of Chl. The mixture was illuminated for the period indicated, and 1.6 ml containing the following was added: HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 3 mM; ascorbate, 5 mM; Pi, 0.3 mM (containing 4.4×10^5 cpm ^{32}P); $MgCl_2$, 5 mM; ADP, 31 μM; PMS, 12.5 μM, and R5P, 0.5 mM (where indicated). Illumination was continued for 15 min.

Table III. *Requirements for Activation of Phosphoribulokinase*

The preillumination reaction mixture (0.4 ml) contained: sorbitol, 0.2 M; HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 5 mM; ascorbate, 5 mM; and chloroplasts containing 27 μg of Chl. The mixture was illuminated with 20,000 lux of white light or kept in darkness, as indicated, for 2 to 3 min and 1.6 ml containing the following was added: HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 5 mM; ascorbate, 5 mM; Pi 0.38 mM (containing 4×10^5 cpm ^{32}P). Illumination was continued for another 15 min. In addition to the above, the components mentioned in the table at the final concentration indicated (for the 2.0 ml final reaction mixture) were added either to the preillumination reaction mixture (within the 0.4 ml) or to the second mixture (within the 1.6 ml). During the reaction stage all tubes in this experiment had identical reaction mixtures.

Components Added during Preincubation	Inorganic Phosphate Esterified	
	Dark preincubation	Preillumination
	% of control	
None	100[1]	310
$MgCl_2$, 4 mM	85	162
R5P, 0.5 mM	105	300
ATP, 25 μM	108	296
Pi, 0.27 mM	100	314
PMS, 10 μM	92	88
$MgCl_2$, 4 mM; R5P, 0.5 mM; ATP, 25 μM; Pi, 0.27 mM	90	166
$MgCl_2$, 4 mM; R5P, 0.5 mM; ATP, 25 μM; Pi, 0.27 mM; PMS, 10 μM	96	108

[1] 0.04 μmole of Pi esterified.

restore CO_2 fixation in the presence of uncouplers and certain inhibitors (7, 17). Furthermore, each compound, with a *Km* of about 10 μM, overcomes the inhibition of CO_2 fixation by O_2 (personal communication from M. Harvey and J. M. Robinson). While these observations indicate that glycerate-3-P and fructose-1,6-diP can readily penetrate the chloroplastic envelope to carry out functions at catalytic concentrations, their quantitative conversion to R5P may be relatively slow.

Phosphoribulokinase was reported previously to be activated by light (15, 16). We attempted to follow this activation using the assay described in Table II. As can be seen in Figure 2, we could easily observe the activation, which amounted to 2- to 4-fold in different experiments and had a half-time of less than 15 sec. No activation was observed in the rate of photophosphorylation by the same chloroplasts (not shown). Some of the properties of the activation phenomenon are shown in Table III. In this experiment, all tubes had identical content during the assay period, and differed only by which component was added before and which after the preillumination period. Magnesium ions, and even more potently PMS, inhibited the activation and thus had to be added only after the preillumination period.

Figure 3 presents the results of studies with several inhibitors in an attempt to define the nature of the activation reaction. Here the extent of inhibition of activation by several inhibitors

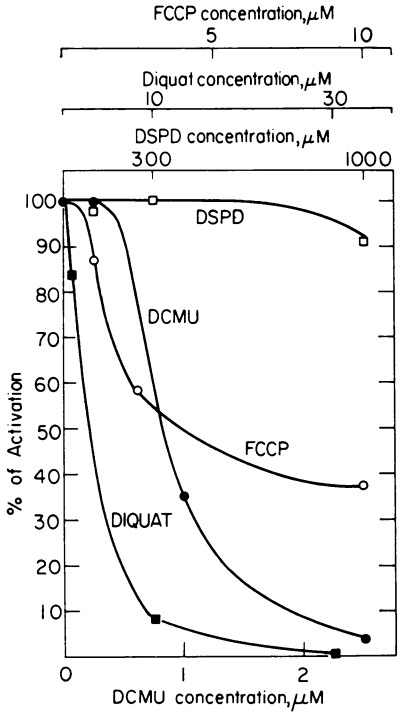

FIG. 3. Inhibition of the activation of phosphoribulokinase. The preillumination reaction mixture (0.4 ml) was as described under Figure 2, except for containing the inhibitor at the concentration indicated. Preillumination was for 2 to 4 min. The second reaction mixture (1.6 ml) described under Fig. 2 (+R5P) was added and illumination continued for 15 min. The inhibitor was therefore 5-fold more dilute during the reaction phase. As a control, these same 5-fold diluted concentrations of all inhibitors tested were added in a parallel experiment only in the second reaction mixture. When added to the second reaction mixture, no inhibition was observed with DCMU up to 30 μM; with DSPD up to 300 μM; with diquat up to 10 μM and FCCP up to 1 μM. In the case of FCCP, a correction was applied for calculating the point at 10 μM (2 μM after dilution) for the small inhibition (about 15%) observed during the reaction phase by 2 μM FCCP.

Table IV. *Decay in the Dark of the Activation of Phosphoribulokinase*

The preillumination reaction mixture (0.4 ml) contained: sorbitol, 0.2 M; HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 5 mM; ascorbate, 5 mM; Pi, 0.3 mM, and chloroplasts containing 41 μg of Chl. The mixture was illuminated for 2 min with 20,000 lux of white light. Light was turned off for the indicated period, followed by addition of 1.6 ml of the following reaction mixture and reilluminated for 15 min: HEPES, pH 7.8, 50 mM; EDTA, 2 mM; $NaHCO_3$, 5 mM; ADP, 31 μM; Pi, 0.3 mM (containing 4×10^5 cpm ^{32}P); $MgCl_2$, 5 mM; PMS 12.5 μM; ascorbate, 5 mM. Where indicated, R5P was added within the 1.6 ml reaction mixture.

Pretreatment	Inorganic Phosphate Esterified	
	No R5P	+ 0.5 mM R5P
	% of control	
No preillumination	100[1]	153
Preilluminated, 2 min	103	320
Preilluminated, 2 min; followed by dark incubation, 2 min	...	274
Preilluminated, 2 min; followed by dark incubation, 5 min	98	252
Preilluminated, 2 min; followed by dark incubation, 10 min	100	210
Preilluminated, 2 min; followed by dark incubation, 30 min	102	148

[1] 0.051 μmole of Pi esterified.

is plotted against the inhibitor concentration. DCMU inhibited the activation at 1 μM. DCMU at this concentration had no effect on PMS photosphosphorylation or the phosphoribulokinase reaction (Table II). However, DSPD, another electron transport inhibitor acting at the level of ferredoxin (6, 18) was without effect. It seems, therefore, that the activation factor was either a reduced product located prior to the level of ferredoxin or a high energy intermediate. FCCP, an uncoupler, was only partially inhibitory at concentrations which completely inhibited the high energy state. Diquat, which was previously shown to divert the electron flow from NADP to O_2 at the photosystem I electron acceptor level without affecting the coupled phosphorylation (21), was inhibitory at 10 μM. It is concluded, therefore, that the activation is due to the effect of a reduced product formed by the photosynthetic electron transport chain but located prior to ferredoxin in the electron transport path. This conclusion is consistent with the observation by Latzko and Gibbs (15) on the activation of phosphoribulokinase by reducing agents in the dark.

Table IV presents data on the decay of the activation phenomenon. The decay, in contrast to the activation of phosphoribulokinase, was relatively slow with a half-time of about 8 min.

DISCUSSION

Present concepts of the photosynthetic carbon reduction cycle require the synthesis of 3 ATP molecules per CO_2 molecule reduced to the level of carbohydrate. It is curious, therefore, that preparations which have the highest capacity for CO_2 fixation are notoriously poor in catalyzing photophosphorylation. The data presented make it unlikely (Tables I and II) that this is due to, as generally assumed, the poor penetration ability of the phosphate acceptors into the intact chloroplast. Rather, in view of the recently discovered intricate control mechanisms which seem to operate within the intact chloroplasts (9), the answer to this apparent contradiction may lie therein.

Phosphoribulokinase seems to be a regulated enzyme within the photosynthetic carbon reduction cycle. It provides the immediate CO_2 acceptor and, therefore, the control of its activity by a photoreduced reducing agent is a logical site of regulation. Since it is activated very rapidly, the kinase cannot, by itself, account for the much longer lag periods encountered in the kinetics of CO_2 fixation in isolated chloroplasts (9, 20).

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