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Characterization of a Photosynthetic Mutant Strain of *Chlamydomonas reinhardi* Deficient in Phosphoribulokinase Activity[1]

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ABSTRACT

A mutant strain of the unicellular green alga, *Chlamydomonas reinhardi*, is unable to fix carbon dioxide by photosynthesis because it is deficient in phosphoribulokinase activity. The absence of light-dependent carbon dioxide fixation in cells of the mutant strain supports the operation of the Calvin-Benson scheme of photosynthetic carbon dioxide fixation in this organism. No deficiency other than low phosphoribulokinase activity was found which would account for the inability of cells of the mutant strain to fix carbon dioxide by photosynthesis. Activities comparable to those in the wild-type strain were found for eight other enzymes of the Calvin cycle and two enzymes associated with the C_4 dicarboxylic acid pathway. The normal rates of nicotinamide adenine dinucleotide phosphate photoreduction and of photosynthetic phosphorylation observed in chloroplast fragments prepared from cells of the mutant strain indicated that the photosynthetic electron transport chain in the mutant is intact.

The kinetics of labeling of carbohydrates in *Chlorella* performing photosynthesis in the presence of sodium-^{14}C-bicarbonate indicate a role for phosphoribulokinase in photosynthetic carbon dioxide fixation (3). The localization of phosphoribulokinase activity exclusively in the chloroplasts of spinach leaves (22) constitutes additional evidence for the role played by this enzyme in photosynthesis. Studies described below on a mutant strain of the unicellular green alga *Chlamydomonas reinhardi* that is deficient in phosphoribulokinase activity confirm the role played by this enzyme in photosynthetic carbon dioxide fixation.

METHODS

Strain F-60 was isolated from the wild-type strain of *C. reinhardi* (137c) as a strain that requires acetate for growth. It was derived by Mr. Pierre Bennoun by treatment of the wild-type strain with the chemical mutagen methyl methanesulfonate (25, 4).

Both the wild-type and mutant strains were grown on tris-acetate-phosphate (TAP) medium (13) under conditions previously described (23). The light intensity from daylight fluorescent lamps was 2000 lux. For studies of whole cells or chloroplast fragments, 300-ml shake cultures were harvested in the logarithmic phase of growth. Cultures of 1.5 liters, grown in 2-liter flasks and agitated with magnetic stirrers, were used to prepare crude extracts.

Carbon dioxide fixation by photosynthesis and by photoreduction under hydrogen was measured at 25 C by the method of Togasaki as described in Reference 11. Cells were washed once and resuspended in minimal (34) medium. The cells were agitated with a stream of air throughout the experiment. The reaction vessel for measuring CO_2 fixation contained 10 μmoles of $NaH^{14}CO_3$, specific activity 0.5 μc/μmole; cells equivalent to 0.09 to 0.1 mg of chlorophyll; and minimal medium to a total volume of 2.2 ml. The cells were incubated for 5 min in the light from a 150-w GE projector flood lamp (intensity 60,000 lux) before $NaH^{14}CO_3$ was injected. Aliquots were taken every minute for 5 min in the light followed by samples taken every minute for 5 min in the dark. Each aliquot was added to an equal volume of concentrated HCl–glacial acetic acid (4:1, v/v) to terminate the reaction. In certain experiments, the reaction was terminated by a procedure similar to that of Hatch and Slack (15). Aliquots of 0.2 ml were added to 0.1 ml of 80% ethanol (v/v) containing 0.2 N HCl and 0.4 mg/ml of 2,4-dinitrophenylhydrazine at −50 C. The tubes were stored at −15 C for 18 hr before samples were plated. The two different procedures for terminating the reaction gave the same results.

For the measurement of CO_2 fixation by photoreduction, the vessel contained 0.02 ml of 1×10^{-3} M DCMU[2] in addition to the contents for CO_2 fixation described above. The cells were agitated by a stream of hydrogen gas. They were incubated in a hydrogen atmosphere for 30 min in the dark and then for 10 min in the light before $NaH^{14}CO_3$ was injected. Samples were taken as described above for CO_2 fixation.

The photoreduction of NADP by chloroplast fragments was measured by the method of Gorman and Levine (13) as modified by Chua and Levine (7). Photosynthetic phosphorylation was measured as described by Gorman and Levine (13). Chlorophyll was determined by a modification (1) of the method of MacKinney (27). Protein was determined by either the biuret reaction (14) or the Folin phenol reaction (26).

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[2] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1, 1-dimethylurea; PMS, phenazine methosulfate; FeCy, potassium ferricyanide; FDP, fructose 1,6-diphosphate; PEP, phosphoenol pyruvic acid; 3-PGA, 3-phosphoglycerate; R-5-P, ribose 5-phosphate; Ru-5-P, ribulose 5-phosphate; RuDP, ribulose 1,5-diphosphate; LDH, lactic acid dehydrogenase; PK, pyruvate kinase; αGP DH, α-glycerol 1-phosphate dehydrogenase; G-3-P DH, glyceraldehyde 3-phosphate dehydrogenase; G-6-P DH, glucose 6-phosphate dehydrogenase; DTT, dithiothreitol.

Phosphoriboisomerase product was made by the method of De la Haba *et al.* (8) using spinach phosphoriboisomerase prepared by the method of Hurwitz *et al.* (20). The final product contained R-5-P and Ru-5-P in a ratio of 6.7:1. RuDP was made by the method of Horecker *et al.* (19). All other chemicals were obtained from commercial sources. Enzymes were purchased from either Sigma Chemical Corp. or from Calbiochem Corp.

PREPARATION OF CRUDE EXTRACTS

All operations were carried out at 0 to 4 C. Cells were harvested as previously described (13). They were washed once with and resuspended in 0.05 M tris-HCl buffer, pH 7.5. In earlier experiments, 5 ml of buffer were used to resuspend the cells. The stability of phosphoribulokinase was found to be increased when DTT was present and when the extract was more concentrated in protein. In later experiments cells were resuspended by the addition of 1 ml of tris buffer containing 0.01 M DTT per gram of cell pellet. The cells were disrupted by sonic oscillation for three 1-min intervals using a Mullard 20-kc ultrasonic disintegrator. The preparation was centrifuged at 138,000*g* for 90 min. Unless otherwise indicated, enzymes were assayed using the resulting yellow-orange supernatant.

The activities of PEP carboxylase, malic enzyme, and RuDP carboxylase were tested with sonicated preparations which had been centrifuged at 480*g* for 5 min to remove whole cells.

For the assay of pyruvate phosphate dikinase, the cells were resuspended in tris buffer containing 0.01 M DTT, 0.005 M $MgCl_2$, and 0.005 M Na pyruvate. Sonic oscillation was carried out at 0 C. Crude extract was prepared by centrifugation at 480*g* for 5 min at 22 C rather than at 0 C.

For the assay of ferredoxin activity, cell fragments were prepared as described for NADP photoreduction (13). The resulting supernatant was centrifuged at 20,000*g* for 20 min, and the green supernatant was used as the crude extract.

REACTION MIXTURES FOR ENZYME ASSAYS

Reaction mixtures contained crude extract equivalent to 0.05 to 0.2 mg of protein in a total volume of 1.0 ml. RuDP carboxylase, PEP carboxylase, malic enzyme, pyruvate phosphate dikinase, and phosphoriboisomerase were assayed at room temperature (21–24 C). All other assays were measured as the change in absorbance at 340 nm using a Cary model 14 recording spectrophotometer. The reference cuvettes lacked NADH (or NADPH or NADP), and the reaction was initiated by the addition of substrate. The temperature during the assays was 25 C. References to the methods used are given below.

Phosphoribulokinase and ATPase (28). The reaction mixture contained 100 μmoles of tris-HCl buffer, pH 7.5; 10 μmoles of $MgCl_2$; 0.5 μmole of PEP; 0.5 μmole of ATP; 10 μmoles of GSH; 3 units each of LDH and PK; and 0.1 μmole of NADH. Crude extract was added, and the rate of the reaction resulting from ATPase activity was recorded for 1.5 min. Isomerase product containing 0.5 μmole of Ru-5-P was added, and the rate of the reaction was recorded. The phosphoribulokinase rate was then corrected for ATPase activity.

The assay procedure described above could be used for either wild-type or mutant extracts. However, a modified procedure proved more convenient for the assay of the low phosphoribulokinase activity present in mutant extracts. It was observed that water, if added in place of isomerase product, caused a slight apparent stimulation of the ATPase activity. This apparent stimulatory effect was negligible compared to the high phosphoribulokinase activity present in wild-type extracts but was about equal to the low phosphoribulokinase activity present in extracts of the mutant strain. In this modified procedure, the reference cuvette as well as the test cuvette contained NADH. Extract was added to both the reference and test cuvettes, and the reaction was initiated by the addition of isomerase product to the test cuvette and water to the reference cuvette. Since the test cuvette contained the substrates for both ATPase and phosphoribulokinase whereas the reference cuvette contained the substrate for ATPase only, the phosphoribulokinase rate measured by this modified procedure was already corrected for ATPase activity. Rates obtained by the modified procedure equaled those obtained by the original procedure when the latter rates were corrected for the apparent stimulation of ATPase activity by dilution.

RuDP Carboxylase (24). The reaction mixture contained 100 μmoles of tris buffer, pH 7.5; 5 μmoles of $MgCl_2$; 10 μmoles of GSH; 0.1 μmole of RuDP; 20 μmoles of $KHCO_3$; and 5 μmoles of $NaH^{14}CO_3$, specific radioactivity 0.5 μc/μmole. The reaction was terminated by treating aliquots with an equal volume of glacial acetic acid.

PEP Carboxylase. The mixture was the same as for RuDP carboxylase but lacking $KHCO_3$ and contained 10 μmoles of PEP in place of RuDP.

Malic Enzyme. The mixture was the same as for RuDP carboxylase but contained 15 μmoles of Na pyruvate and 0.4 μmole of NADPH in place of RuDP.

Ru-5-P to PGA Sequence. The mixture was the same as for RuDP carboxylase but contained 5 μmoles of ATP and 0.5 μmole of phosphoriboisomerase product in place of RuDP.

Phosphoriboisomerase (24). The mixture contained 100 μmoles of tris buffer, pH 7.5; 5 μmoles of $MgCl_2$; 10 μmoles of GSH; 5 μmoles of R-5-P. The reaction was run for 5 min at room temperature, and the Ru-5-P formed was assayed at 37 C by the method of Axelrod and Yang (2).

G-3-P DH (22). The mixture contained 100 μmoles of tris buffer, pH 7.8; 10 μmoles of $MgCl_2$; 5 μmoles of ATP; 0.1 μmole of NADH or NADPH; 10 μmoles of GSH; 0.9 unit of 3-PGA kinase; and 5 μmoles of 3-PGA.

3-PGA Kinase (22). The mixture contained 100 μmoles of tris buffer, pH 7.5; 10 μmoles of $MgCl_2$; 10 μmoles of GSH; 5 μmoles of ATP; 0.1 μmole of NADH; 0.2 unit of G-3-P DH; and 5 μmoles of PGA.

Triosephosphate Isomerase. The mixture contained 100 μmoles of tris buffer, pH 7.5; 0.1 μmole of NADH; 0.4 unit of αGP DH; and 1.5 μmoles of G-3-P.

FDP Aldolase (30). The mixture contained 100 μmoles of tris buffer, pH 7.5; 0.1 μmole of NADH; 10 μg each of triosephosphate isomerase and αGP DH; and 10 μmoles of FDP.

Alkaline FDPase (29). The mixture contained 100 μmoles of tris buffer, pH 8.5; 5 μmoles of $MgCl_2$; 1 μmole of EDTA; 0.1 μmole of NADP; 1.7 units of G-6-P isomerase; 0.1 unit of G-6-P DH; and 10 μmoles of FDP.

Malate Dehydrogenase (18). This mixture contained 50 μmoles of tris buffer, pH 7.8; 1 μmole of EDTA; 0.5 μmole of oxaloacetate; and 0.1 μmole of NADPH.

Ferredoxin. The mixture was the same as that for NADP photoreduction with green extract (1–2 mg of protein) substituted for purified ferredoxin and ferredoxin-NADP reductase.

Pyruvate Phosphate Dikinase (17). The mixture contained 100 μmoles of tris buffer, pH 8.3; 10 μmoles of $MgCl_2$; 10 μmoles of GSH; 15 μmoles of Na pyruvate; 5 μmoles of ATP; 2 μmoles of Na orthophosphate. The reaction was run 30 min at room temperature and terminated by boiling for 2 min. The PEP formed was then determined. Tubes containing boiled extract or lacking pyruvate served as controls.

RESULTS

Absence of Photosynthetic CO_2 Fixation in Cells of the Mutant. Table I shows that cells of F-60 totally lack light-dependent CO_2 fixation. This deficiency in photosynthesis is consistent

Table I. *Photosynthetic Activities of Cells and Fragments of Wild Type and F-60*

Rates are expressed in μmoles/hr·mg chlorophyll. Values are taken from individual experiments. Contents of reaction mixtures are described in the "Methods" section.

	Wild Type			F-60		
	Light	Dark	Light-minus-dark	Light	Dark	Light-minus-dark
Intact cells						
CO_2 fixation by photosynthesis						
Control (no DCMU)	149	0	149	1.0	1.0	0
With 10^{-5} M DCMU present	0.9	0	0.9	1.0	0.6	0.4
Inhibition by 10^{-5} M DCMU, %	99	...	99	0	40	0
CO_2 fixation by photoreduction	35	0	35	0.4	0	0.4
Chloroplast fragments						
Hill reaction with NADP	103,82			125,78		
Photosynthetic phosphorylation						
Noncyclic (FeCy)	94			89		
Cyclic (PMS)	266,57			134,122		

Table II. *Specific Activities of Crude Extracts of Wild Type and F-60*

Rates are given in μmoles of substrate consumed/hr·mg of protein. For enzymes assayed on green extracts rates are also given in μmoles of substrate consumed/hr·mg of chlorophyll in parentheses. Contents of reaction vessels are described in the "Methods" section. Values are taken from individual experiments.

	Wild Type	F-60
Ru-5-P kinase	122	2.0
ATPase	3.6	2.6
R-5-P isomerase	52	59
Ru-5-P → PGA sequence	0.49	0.0
G-3-P DH NAD	8.4	5.1
G-3-P DH NADP	12.5	10.9
3-PGA kinase	75	70
Triosephosphate isomerase	111	110
FDP aldolase	3.5	7.5
Alkaline FDPase	0.82	0.52
Ferredoxin	31	35
PEP carboxylase	0.10 (1.04)	0.09 (1.35)
Malic enzyme	0.0 (0.0)	0.0 (0.0)
RuDP carboxylase	4.56 (47.8)	2.74 (42.4)
Pyruvate phosphate dikinase	0.0 (0.0)	0.0 (0.0)
Malate dehydrogenase (NADP)	0.72	0.62

with the inability of the cells to grow phototrophically. In seven other measurements of CO_2 fixation by cells of F-60 the light-minus-dark rate of CO_2 fixation was also zero, the light (and dark) rates ranging from 0.42 to 1.14 μmoles/hr·mg chlorophyll with a mean value of 0.81. The mean value for the light or dark CO_2 fixation in the mutant strain is approximately the same as that for cells of wild type in which photosynthesis is inhibited by DCMU. In contrast to its inhibitory effect on wild type, DCMU either had no effect on CO_2 fixation in the light or produced a slight stimulation in cells of F-60. In the presence of DCMU, the dark rate of CO_2 fixation in F-60 appeared to be inhibited or eliminated, thus increasing the apparent light-minus-dark rate. The reason for the depression of the dark rate of CO_2 fixation in the mutant strain by DCMU is not clear.

Carbon dioxide fixation by the mutant strain is higher in TAP medium than in minimal medium. However, at the maximum, total CO_2 fixation by the mutant strain even in TAP medium represents only 2.3% that of the wild-type strain. The highest rate observed for CO_2 fixation in TAP medium by cells of the mutant strain was 1.87 μmoles/hr·mg chlorophyll; the highest light-minus-dark rate was 0.74 μmole/hr· mg chlorophyll.

Low Phosphoribulokinase Activity in the Mutant. The activities of nine Calvin cycle enzymes are shown in Table II. Except for the low phosphoribulokinase activity in extracts of F-60, all other Calvin cycle enzyme activities assayed are comparable to those of wild type. The specific activity of phosphoribulokinase in F-60 is 1.6% that of wild type.

The low phosphoribulokinase activity in F-60 extracts is not due to an elevated rate of competing ATPase activity (Table II). Nor is the low phosphoribulokinase rate due to the presence of a dissociable inhibitor in the extract of the mutant. The rate of wild-type was depressed by only 8% in the presence of an equal volume of F-60 extract (data not shown). This depression of the wild-type rate is probably caused by the presence of ATPase activity in the F-60 extract. If the low rate of phosphoribulokinase present in F-60 were caused entirely by an inhibitor, an inhibition of at least 48% of the wild-type rate by the mutant extract would be expected.

The enzymes PEP carboxylase, NADP-dependent malate dehydrogenase, malic enzyme, and pyruvate phosphate dikinase are believed to be associated with the C_4 dicarboxylic acid pathway of photosynthetic CO_2 fixation (16, 17, 32). The first two enzymes were found in the mutant with activities comparable to those found in wild type (Table II). Activities of the latter two enzymes were not detected in extracts of either the wild-type or mutant strains.

Normal Operation of Photosynthetic Electron Transport in the Mutant. Examination of four partial reactions which contribute to photosynthetic CO_2 fixation indicates that the impaired photosynthesis in the mutant is due solely to the deficiency in the Calvin cycle enzyme phosphoribulokinase. The fixation of carbon dioxide by photoreduction is a process in which hydrogen rather than water serves as the electron donor and which is mediated by photosystem I independently of photosystem II (5, 6). The labeling pattern of products formed during photoreduction indicates that the CO_2 is fixed by the Calvin cycle (10, 31). The absence of photoreduction in cells of F-60 (Table I) is consistent with the location of the block in photosynthesis lying in the Calvin cycle (or in photosystem I) rather than in the photosynthetic electron transport chain.

The intactness of photosynthetic electron transport involving both photosystems I and II is implied by the combined results of experiments measuring NADP photoreduction and ferredoxin

activity. Table I shows that chloroplast fragments of F-60 are able to photoreduce NADP with water as the electron donor. An assay for ferredoxin (Table II) was necessary to complete the study of the electron transport chain since this protein is added in excess to the reaction mixture in the assay for NADP photoreduction.

Cyclic photosynthetic phosphorylation mediated by PMS and noncyclic photosynthetic phosphorylation associated with the photoreduction of ferricyanide are observed in sand-ground fragments of F-60 at rates comparable to those of wild type (Table I).

Normal Cell Structure in the Mutant. Electron micrographs revealed no detectable differences between cells of F-60 and wild type (12). In particular, chloroplast structure in the mutant appeared to be quite normal. The content of chlorophyll and the ratio of chlorophyll *a*/*b* in the mutant are not significantly different from the values found in wild type. The mutant is not deficient in protein, having 1.2 times as much protein per cell as wild type.

DISCUSSION

The low phosphoribulokinase activity found in the mutant is not due to a higher than usual activity of competing ATPase or to the presence of a dissociable inhibitor in the mutant extract. It is assumed that the low enzyme activity is caused by the production of a structurally altered protein with low enzyme activity or of a subnormal amount of the wild-type protein. The isolation of phosphoribulokinase from both the mutant and wild-type strains will allow a distinction to be made between these two alternatives.

The deficiency in photosynthesis in a mutant possessing low phosphoribulokinase activity indicates the importance of this enzyme for photosynthetic carbon dioxide fixation. The total absence of any light-dependent carbon dioxide fixation in the phosphoribulokinase-deficient mutant argues against even a minor contribution to photosynthetic carbon dioxide fixation by pathways that act independently of the Calvin cycle in *C. reinhardi*.

The review by Stiller (33) presents arguments to show that the carbon dioxide fixation step may involve the formation *de novo* of a 2-carbon fragment in a process independent of the Calvin cycle. In the detailed scheme of Tanner *et al.* (35) which involves the formation *de novo* of a 2-carbon fragment, Mn^{2+} and NADPH in the presence of light mediate the formation of a radical from CO_2. Two such radicals combine to form a 2-carbon precursor of glycolic acid. Since the NADPH required in this scheme appears to be produced in the mutant, the absence of photosynthetic CO_2 fixation indicates that the pathway proposed by Tanner *et al.* probably does not occur in *C. reinhardi*.

In the C_4 dicarboxylic acid pathway of photosynthetic CO_2 fixation, the carboxylation of PEP to form oxaloacetate precedes the transfer of the fixed carbon to an intermediate of the Calvin cycle (15, 21). The interaction between the C_4 dicarboxylic acid pathway and the Calvin cycle would predict that PEP could not be regenerated by the C_4 pathway in the mutant described in this paper. Hence, the absence of light-dependent CO_2 fixation in cells of the phosphoribulokinase-deficient mutant does not constitute evidence against the operation of the C_4 pathway in *C. reinhardi* unless it can be demonstrated that a pool of PEP comparable to that in wild type is available to the chloroplast in cells of the mutant strain. The low ratio of PEP carboxylase to RuDP carboxylase activity in extracts of both the wild-type and mutant strains is typical of plants possessing the Calvin cycle and not typical of plants in which the C_4 pathway operates (32). The low ratio of PEP carboxylase to RuDP carboxylase activity and the absence of light-dependent CO_2 fixation in cells of the phosphoribulokinase-deficient strain are consistent with the absence of the C_4 pathway in *C. reinhardi*.

Phosphoribulokinase is a protein localized in the chloroplasts of green plants (22). Hence the phosphoribulokinase-deficient mutant described in this paper would be excellent material for investigating the roles played by the nucleus and by the chloroplast in determining the structure of a chloroplast protein and in regulating its synthesis. Such studies are currently underway in this laboratory.

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