A MUTANT STRAIN OF CHLAMYDOMONAS REINHARDI LACKING RIBULOSE DIPHOSPHATE CARBOXYLASE ACTIVITY*

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Mutant strains of the unicellular green alga *Chlamydomonas reinhardi* which have impaired photosynthesis are characterized by their inability to fix carbon dioxide in the light at the wild-type rate.[1] The incapacity of these mutant strains to fix carbon dioxide by photosynthesis can be attributed to one of three possibilities: (1) the loss of a step in the photosynthetic electron transport chain; (2) the loss of the capacity to carry out photosynthetic phosphorylation; or (3) the loss of one of the steps in photosynthetic carbon dioxide fixation associated with the reductive pentose phosphate cycle.

The mutant strains of *C. reinhardi* described to date[2-4] fall into the first category mentioned above. In this paper we shall describe *ac-20,* a mutant strain which lacks the capacity for photosynthetic carbon dioxide fixation as a consequence of the loss of RuDP carboxylase activity.

Organisms and the Methods.—The organisms used in the experiments described below were the wild-type strain of *C. reinhardi,* 137c, and the mutant strain *ac-20,* derived from wild type by ultraviolet irradiation followed by a screening test[5] for carbon dioxide fixation.

Cells, in the logarithmic phase of growth, were harvested from shake cultures grown at 25°C in high salt minimal medium[6] supplemented with 0.2% sodium acetate. Light (2500 lux) was provided by daylight fluorescent lamps.

Carbon dioxide fixation by whole cells was measured as previously described.[7] The light intensity was 60,000 lux. The water-soluble products of carbon dioxide fixation by whole cells in the light were also examined. A cell suspension in minimal medium (30 ml, 10 mg/ml wet weight) was placed in a "lollipop" and illuminated with 20,000 lux. Temperature was maintained at 25°C. The suspension was aerated, and after 10 min C^{14}-labeled sodium bicarbonate (8 μmoles, 50 μc/μmole) was introduced into the "lollipop." Sixty sec later the reaction was terminated by emptying the contents of the "lollipop" into a hot methanol-chloroform mixture (12:5 v/v). The water-soluble products were then extracted according to the method of Bieleski and Young.[8] The products were separated on a Dowex-1 chloride column (1 × 90 cm) using two stages of linear HCl gradient elution. The gradient was as follows: tubes 1–35, water; tubes 36–155, a linear gradient from 0.028 *N* to 0.041 *N* HCl; tubes 156–265, a linear gradient from 0.070 *N* to 0.130 *N* HCl. Aliquots of 100 lambda from each tube were plated on planchets and counted. Twelve peaks were observed.

Chloroplast fragments were prepared according to the method of Levine and Volkmann.[1] Crude extracts for enzyme assays were obtained from cells which had been washed once in 0.02 *M* Tris buffer, pH 7.5, and then resuspended in 5 ml of the same buffer. The cells were disrupted by sonic oscillation at 0°C for 2$^1/_2$ min using a Mullard 20-kc ultrasonic disintegrator. The disrupted cell preparations were then centrifuged at 20,000 × *g* for 20 min at 0°C. The green supernatant was used as the crude extract.

The activity of the photosynthetic electron transport chain was measured by assaying the rate of TPN photoreduction by chloroplast fragments using the method described by Levine and Smillie.[2]

Carbon dioxide fixation by crude extracts, as measured by the activity of RuDP carboxylase and of the R-5-P to 3-PGA sequence of enzymes (i.e., phosphoriboisomerase, phosphoribulokinase, and RuDP carboxylase), was assayed by a modification of the technique of Mendiola and Akazawa.[9] The reactions were run in 150 × 15-mm tubes, rather than in Warburg flasks, and the reactions were allowed to proceed without having been gassed with 5% carbon dioxide in nitrogen.

Phosphoriboisomerase activity was assayed according to the method of Axelrod,[10] and the reaction was allowed to proceed for 10 min at 38°C.

Phosphoribulokinase activity was measured according to the method of Fuller *et al.*[11] However, because it was found that crude extracts had considerable ATPase activity, it was necessary to purify them partially. This was achieved by passing each crude extract through a polyacrylamide gel (Bio-Gel P-300). Bio-Gel (obtained from Bio-Rad Laboratories) was equilibrated overnight with Tris buffer (pH 7.5 and 0.02 *M*) and poured into a chromatographic column (1.9 × 60 cm) as a thick suspension. Both the inlet and the outlet of the tube were then closed, leaving a large air bubble at the top. The tube was inverted until the gel suspension was even and the air bubble could move freely from one end of the column to the other. All the following procedures were then carried out at 4°C. The inlet was connected to a buffer reservior placed one meter above the top of the column, the outlet was opened, and the gel was allowed to settle to a constant bed height. After this a 1-cm layer of Bio-Gel P-10 and a circular piece of "Kemwipe" paper were placed on top of the gel bed to prevent the disturbance of the gel surface. The final bed height was 23 cm. One ml of crude extract was applied and eluted at the rate of 10 ml per hour. A fraction was obtained from the column having phosphoribulokinase activity essentially free of ATPase activity.

Purified phosphoriboisomerase was prepared from spinach according to the method of Hurwitz *et al.*[12] Chlorophyll was determined by a modification[13] of the procedure of MacKinney.[14] Protein was measured by the Biuret method.[15]

Results and Discussion.—Carbon dioxide fixation and TPN photoreduction: The rates of carbon dioxide fixation by cells of wild type and of *ac-20* are given in Table 1. It can be seen that the mutant strain fixed carbon dioxide in the light at a rate approximately 16 times less than that of the wild-type strain. This low rate of carbon dioxide fixation was also reflected in the profile of the soluble products of carbon dioxide fixation (Fig. 1), in which it was found that all of the products of normal short-term carbon dioxide fixation, with the exception of

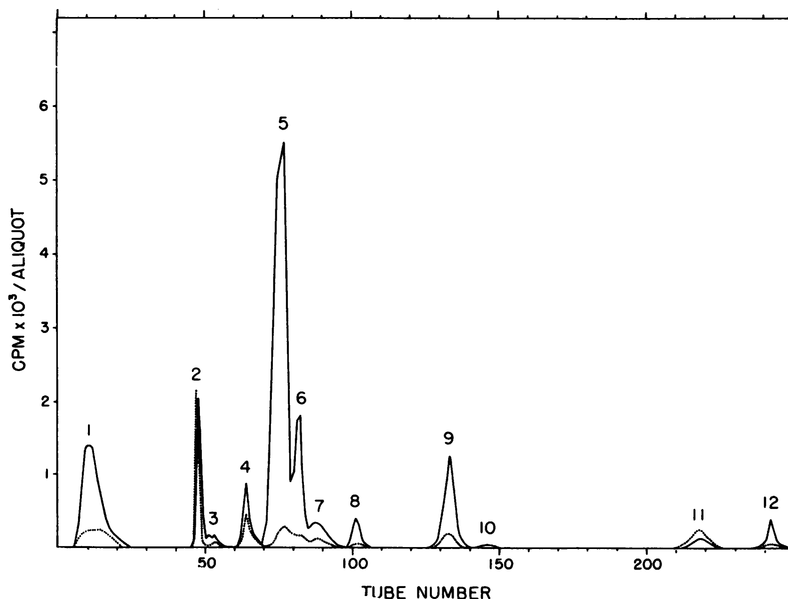

FIG. 1.—Dowex-1 chloride separation of water-soluble products of photosynthetic carbon dioxide fixation by wild-type cells (solid line) and cells of *ac-20* (dotted line). The peaks correspond to the following carrier compounds: peak *1*, alanine; peak *2*, aspartate; peak *5*, glucose-6-phosphate; peak *6*, fructose-6-phosphate; peak *7*, ribose-5-phosphate; peak *8*, dihydroxyacetone phosphate; peak *9*, 3-phosphoglyceric acid; and peak *11*, phosphoenolpyruvate. Peaks *3*, *4*, *10*, and *12* were unidentified. Carrier fructose-1,6-diphosphate was eluted between tubes 195 and 200.

aspartate (peak no. 2), phosphoenolpyrvuate (peak no. 11), and one unknown peak (no. 4), were very low.

The photoreduction of TPN was assayed to determine whether or not the photosynthetic electron transport chain was operative in *ac-20*. Table 1 shows that isolated chloroplast fragments of the mutant strain carried out TPN photoreduction at a rate that was at least half of that found for chloroplast fragments obtained from wild type. As will be reported later,[16] chloroplast fragments of this mutant strain can carry out cyclic photosynthetic phosphorylation with phenazine methosulfate and noncyclic photosynthetic phosphorylation to TPN reduction.

The activity of enzymes of the R-5-P to 3-PGA sequence: The specific activities of the R-5-P to 3-PGA sequence of enzymes as a whole, as well as those of the three component enzymes, are given in Table 2. It can be seen for *ac-20* that the specific activity of the sequence of enzymes is negligible. Of the three component enzymes, RuDP carboxylase is essentially inactive, whereas the specific activities of phosphoriboisomerase and phosphoribulokinase are similar to those of the wild-type strain.

TABLE 1

CARBON DIOXIDE FIXATION AND TPN PHOTOREDUCTION BY WILD TYPE AND *ac-20*

Strain	μmoles CO_2 Fixed/Hr/mg Chlorophyll: Light	Dark	Light minus dark	μmoles TPN photoreduced/hr/mg chlorophyll
Wild type	47.4	5.4	42.0	69
ac-20	3.51	0.86	2.65	37

For CO_2 fixation, the reaction mixtures contained cells equivalent to 0.25 mg chlorophyll and the following in μmoles: $KHCO_3$, 400; $NaH^{14}CO_3$, 6 (50 μc/μmole). Water was added to a final volume of 2.5 ml.

For TPN photoreduction, the reaction mixtures (0.8 ml) in the test and control cuvettes contained chloroplast fragments (10–15 μg chlorophyll) and the following in μmoles: TPN, 0.2; $MgCl_2$, 0.015; and Tris, pH 7.5, 31. An excess of purified PPNR from wild-type *C. reinhardi* was included in the test cuvette only.

TABLE 2

SPECIFIC ACTIVITIES OF ENZYMES OF THE R-5-P TO 3-PGA SEQUENCE IN EXTRACTS OF WILD TYPE AND *ac-20*

Enzyme	Wild Type	*ac-20*	Wild Type	*ac-20*
	μMoles CO_2 Fixed/Hr			
	per mg Protein		per mg Chlorophyll	
The R-5-P to 3-PGA sequence	0.24	0.001	2.06	0.01
RuDP carboxylase	0.28	0.001	2.41	0.01
	μMoles DPNH Oxidized/Hr			
	per mg Protein		per mg Chlorophyll	
Phosphoribulokinase	96.8	71.2	832	823
	μMoles Ru-5-P Formed/Hr			
	per mg Protein		per mg Chlorophyll	
Phosphoriboisomerase	123	158	1058	1849

The reaction mixture for the R-5-P to 3-PGA sequence of enzymes contained crude extract of either wild type or *ac-20* equivalent to 0.5–1.0 mg of protein and the following in μmoles: Tris buffer, pH 7.5, 100; $MgCl_2$, 5; reduced glutathione, 10; ATP, 5; R-5-P, 5; $KHCO_3$, 20; and $NaH^{14}CO_3$, 1 (specific activity 50 μc/μmole). Water was added to a final volume of 1.5 ml. Control reactions were identical except for the absence of ATP. The reaction was run for 20 min at 23°C.

The reaction mixture for the assay of phosphoriboisomerase was as above except that there was no added ATP or $NaH^{14}CO_3$. Control reactions were run in the presence of equivalent amounts of boiled extract and in the absence of extract. The reaction was run for 10 min at 38°C.

The reaction mixture for RuDP carboxylase was as above, except that RuDP (0.1 μmole) was present and R-5-P and ATP were not added. Control reactions were run in the absence of RuDP. The reaction was run for 10 min at 23°C.

The reaction mixture for the assay of phosphoribulokinase contained partially purified extract of either wild type or *ac-20* equivalent to 0.02–0.05 mg protein, an excess of purified spinach phosphoriboisomerase, and the following in μmoles: Tris buffer, pH 7.5, 20; $MgCl_2$, 10; reduced glutathione, 5; ATP, 0.33; PEP, 0.33; DPNH, 0.1; and R-5-P, 5. The mixtures also contained pyruvate kinase, 3.3 μg of protein per ml, and lactic dehydrogenase, 0.5 unit. Water was added to a final volume of 1 ml. The oxidation of DPNH was followed at 340 mμ in a Cary model 14 recording spectrophotometer after addition of extract and R-5-P. The control cuvette had the identical reaction mixture except for the absence of DPNH. The temperature during the reaction was 23°C.

In order to determine whether the negligible RuDP carboxylase activity of *ac-20* resulted from the presence of an inhibitor in the extract, a mixing experiment was done in which the activity of the wild-type extract was assayed in the presence of an equal amount of *ac-20* extract. The activity was found to be identical in the presence and absence of *ac-20* extract.

The inability of *ac-20* to carry out normal photosynthesis can be ascribed most simply to its lack of RuDP carboxylase activity. This conclusion is supported by the fact (Table 1) that isolated chloroplast fragments of the mutant strain have the capacity to carry out reactions of the photosynthetic electron transport chain. The results presented here provide *enzymic* evidence for the participation of RuDP carboxylase in photosynthetic carbon dioxide fixation *in vivo*. In this sense they are in accord with the findings of others[17] based upon kinetic analysis of soluble products of *in vivo* photosynthetic carbon dioxide fixation.

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Abbreviations: ATP, adenosine 5′-triphosphate; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; PEP, phosphenolpyruvate; 3-PGA, 3-phosphoglyceric acid; PPNR, photosynthetic pyridine nucleotide reductase; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; RuDP, ribulose diphosphate; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide, respectively; and Tris, tris (hydroxymethyl) aminomethane.

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