Biochemical and genetic analysis of an RuBP carboxylase/oxygenase-deficient mutant and revertants of *Chlamydomonas reinhardii*

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1. INTRODUCTION

RuBP carboxylase/oxygenase (EC 4.1.1.39) is a chloroplast localized enzyme consisting of 2 non-identical subunits, each present in 8 copies [1]. The large subunit is coded by a chloroplast gene in *Chlamydomonas reinhardii* [2,3] and contains the active site of the enzyme [4]. The function of the nuclear-encoded [5] small subunit remains unknown. The gaseous substrates of the enzyme, CO_2 and O_2, compete at the same active site [6]. Carboxylation of RuBP initiates C_3 photosynthesis. Oxygenation of RuBP yields phosphoglycolate, which leads to the release of CO_2 via photorespiration. Enhanced rates of CO_2 fixation may be achieved if oxygenase activity could be reduced or eliminated [7].

The function of photorespiration is unknown. However, mutants of *Arabidopsis* that lack photorespiration have been recovered as CO_2-requiring strains [8]. These mutants demonstrate that photorespiration is not essential, and indicate that attempts to abolish photorespiration at ambient levels of CO_2 must focus on reducing the oxygenase activity of RuBP carboxylase/oxygenase. The ratio of carboxylase to oxygenase activity varies in phylogenetically diverse species, apparently increasing during evolution [9]. This observation suggests that the ratio of the two activities may be amenable to genetic alteration within a species.

In [10], a light-sensitive, acetate-requiring chloroplast mutant of *Chlamydomonas reinhardii* was described that lacks RuBP carboxylase activity and produces an enzyme with an altered large subunit isoelectric point. Here, we describe the isolation of photosynthetically competent revertants of this mutant in studies designed to explore the possibility of recovering enzymes with altered ratios of carboxylase/oxygenase activity. All revertants possess an enzyme restored to wild-type function. These results show that the defective RuBP carboxylase/oxygenase protein can be changed back to its original state, indicating that genetic approaches to modifying the enzyme are feasible.

2. MATERIALS AND METHODS

Chlamydomonas reinhardii wild-type strain 2137 *mt*+ [11] and centromere-marker strain *pf*-2 (paralyzed flagellae) *mt*– were used. The uniparentally inherited, non-photoautotrophic mutant *rcl-u*-1–10-6C *mt*+ was selected as a light-sensitive, acetate-requiring mutant in [10,11]. All strains were maintained in the dark on 10 mM sodium acetate medium [11]. Cultures were grown in the dark at 25°C on a rotary shaker or on medium solidified with 15 g/l Bacto agar. Cells were counted with a hemacytometer.

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Abbreviations: RuBP, ribulose-1,5-bisphosphate; CABP, 2-carboxyarabinitol-1,5-bisphosphate; *mt*, mating type

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RuBP carboxylase/oxygenase was purified from 250 ml cultures by centrifugation in a sucrose density gradient [10]. Enzyme preparations were dialyzed overnight at 4°C against 20 mM bicine (pH 8.0) and 0.1 mM EDTA. The CO_2/O_2 specificity of the enzyme [12] was determined by simultaneous assay of the 2 activities at 25°C in reaction mixtures containing ~10 μg protein, 2.5 mM $NaH^{14}CO_3$ (2 Ci · mol^{-1}), 10 mM $MgCl_2$, 50 mM bicine (pH 8.3) and 0 or 1.23 mM O_2. Reactions (0.5 ml final vol.) were initiated with 10 nmol of [1-^{3}H]RuBP (30 Ci · mol^{-1}) and terminated after 10 min with 0.1 ml of a solution containing 0.5 N H_2SO_4 and 50 mM $ZnSO_4$. Carboxylase/oxygenase reaction products were separated and quantitated as in [12]. In determining $V_{max}(CO_2)$ and $K_M(CO_2)$, enzymes were preincubated for 4 h at 4°C in 10 mM $NaH^{14}CO_3$ (2 Ci · mol^{-1}), 10 mM $MgCl_2$ and 50 mM bicine (pH 8.5). Assays were initiated by adding ~10 μg of activated enzyme in 20 μl to reaction mixtures at 25°C containing 0.4 mM RuBP, 10 mM $MgCl_2$ and 50 mM bicine (pH 8.3) in 1.0 ml final vol. [$NaH^{14}CO_3$] was 0.7, 1.2, 2.2, 3.2, 5.2, 8.2 and 15.2 mM. The reactions, assayed under a N_2 atmosphere, were terminated after 30 s with 0.5 ml 3 M formic acid in methanol. $K_M(CO_2)$ and $V_{max}(CO_2)$ were estimated from Scatchard plots. Unlabeled and ^{14}C-labeled CABP were prepared as in [13]. Protein was determined by the method in [14]. Relative isoelectric points of the subunits of RuBP carboxylase/oxygenase were determined by non-equilibrium pH gradient electrophoresis using pH 3.5–10 ampholines [15]. Enzyme was mixed to 8 M urea directly from a sucrose gradient, loaded on gels, and electrophoresed for 4.5 h at 400 V and 25°C.

Using cloned isolates of *rcl-u*-1–10-6C *mt*+, 5-fluorodeoxyuridine treatment and ethyl methanesulfonate mutagenesis, followed by an expression time, were performed in the dark exactly as in [16]. Following this treatment, 1×10^6 cells were plated on each of several 100 mm acetate medium plates at 4000 lux (cool-white fluorescent light), a light intensity at which the *rcl-u*-1 mutant does not survive. Non-light-sensitive colonies, described in [16], were replica-plated to minimal medium (without sodium acetate) at 4000 lux. Colonies capable of photosynthesis survived under these conditions and were saved from independent experiments for further analysis. Gamete induction, zygote maturation, zygote germination and tetrad analysis were performed as described [11].

3. RESULTS AND DISCUSSION

RuBP carboxylase/oxygenase isolated from the *rcl-u*-1 mutant possessed negligible but detectable activity (table 1). The rates of carboxylation and oxygenation were <0.003% of that observed for the wild-type enzyme. Carboxylase activity in the mutant enzyme was found to be higher at 40°C than at 25°C and was inhibited by O_2 when assayed at 30 μM CO_2 (table 1).

Further evidence that the mutant protein possessed enzyme characteristics was obtained from binding studies. The protein was capable of forming a stable complex with CO_2, Mg^{2+} and CABP, an analogue of the 6-carbon intermediate of the carboxylase reaction [13,17]. Mutant enzyme bound CABP in a molar ratio of 0.9 CABP/large subunit monomer (fig.1). When the enzyme was incubated in the presence of CABP and $^{14}CO_2$, $^{14}CO_2$ was tightly bound to the enzyme (fig.2A). These experiments indicate that CO_2 was able to bind at the CO_2 activation site [18]. The ratio of bound $^{14}CO_2$ to large subunit monomer was found to be 0.55. The same ratio was found for wild-type enzyme treated in the same way (fig.2B). Therefore, the low value may have resulted from isotope dilution rather than non-stoichiometric binding of CO_2 at the activator site. Since Mg^{2+} is required for CABP binding with spinach RuBP carboxylase/oxygenase [13], it is likely that the mutant *Chlamydomonas* enzyme binds Mg^{2+}. These results indicate that the *rcl-u*-1 mutation does not greatly alter the conformation of the enzyme at the active site, but the nature of the defect is not known.

No photoautotrophic colonies were found after plating 1×10^{10} dark-grown cells of the *rcl-u*-1 mutant clones on minimal medium following mutagenesis treatment. Previous results had shown that non-light-sensitive colonies can be recovered by plating the light-sensitive *rcl-u*-1 cells on acetate medium at 4000 lux [16]. These non-light-sensitive strains were found to contain nuclear suppressor mutations which relieved photosensitivity without affecting RuBP carboxylase/oxygenase [16]. Nevertheless, the non-light-sensitive, acetate-requiring revertants may have included revertants with re-

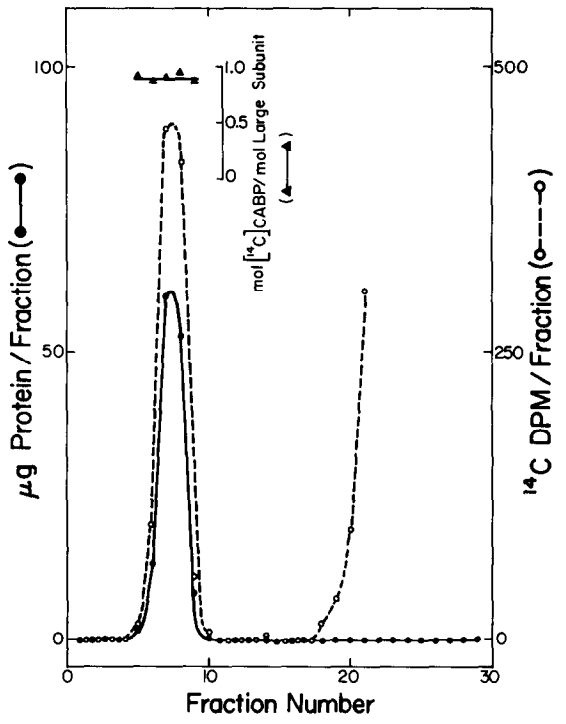

Fig.1. Binding of CABP by mutant RuBP carboxylase/oxygenase. Protein (300 μg/ml) was incubated for 15 min at 30°C in 0.1 mM [^{14}C]CABP (575 dpm/nmol), 10 mM $NaHCO_3$, 10 mM $MgCl_2$ and 50 mM bicine at pH 8.0. Following incubation, 0.4 ml mixture was loaded onto a Sephadex G-75 column (1×30 cm) equilibrated at 25°C with 50 mM bicine (pH 8.0) and 0.1 mM EDTA. The column was eluted with the same buffer and 0.5 ml fractions were collected for protein and ^{14}C dpm determinations.

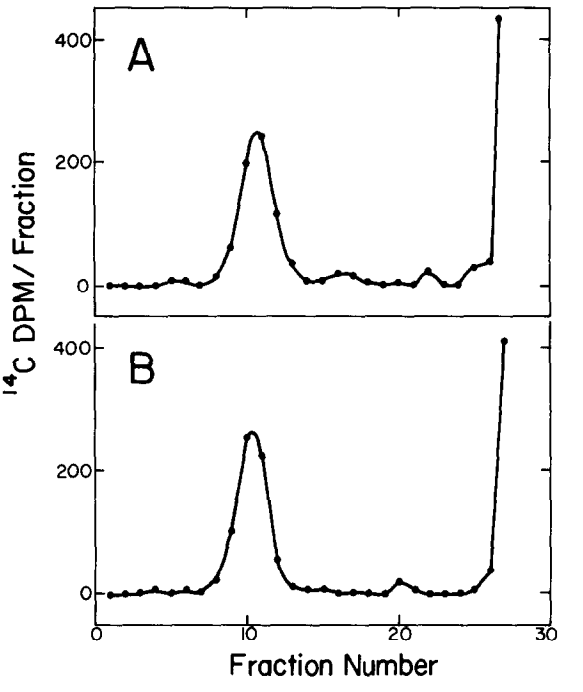

Fig.2. Binding of activator CO_2 by mutant (A) and wild-type (B) RuBP carboxylase/oxygenase. Proteins (200 μg/ml) were incubated for 15 min at 30°C in 1 mM CABP, 10 mM $NaH^{14}CO_3$ (1280 dpm/nmol), 10 mM $MgCl_2$, and 50 mM bicine at pH 8.0. Following incubation, 0.4 ml mixture was loaded onto a Sephadex G-75 column (1×30 cm) equilibrated at 25°C with 50 mM bicine (pH 8.0) and 0.1 mM EDTA. The column was eluted with the same buffer and 0.4 ml fractions were collected for ^{14}C dpm determination.

stored ability to carry out sufficient photosynthesis to relieve photosensitivity, but not enough to survive photoautotrophically. Since only a few non-light-sensitive revertants were analyzed, revertants with restored RuBP carboxylase activity that occurred at a low frequency may have been overlooked. To screen the non-light-sensitive colonies that arose from mutagenized *rcl-u*-1–10-6C *mt*+ cells, they were replica-plated to minimal medium at 4000 lux. We reasoned that revertants with partially restored RuBP carboxylase activity would survive more easily from colonies than from single cells. The results from one of these experiments are presented in table 2. Photoautotrophic revertants were recovered at a frequency of $\sim 1/1 \times 10^7$ cells; 9 independent revertants were recovered from several experiments.

The photoautotrophic revertants were found to be phenotypically indistinguishable from wild-type. Genetic analysis of crosses between each revertant and *pf*-2 *mt*–, in which over 25 tetrads were analyzed per cross, revealed no re-expression of the *rcl-u*-1 acetate-requiring phenotype. This indicates that the revertants did not arise from suppression by nuclear mutations, since such mutations would show 2:2 segregation against the uniparentally inherited *rcl-u*-1 phenotype. Biochemical analysis of the revertants indicated that RuBP

Table 1

Effect of temperature and oxygen on mutant RuBP carboxylase activity μmol CO_2 . mg protein^{-1} . min^{-1}

Expt.	Variable	Rate
I[a]	25°C	5.3×10^{-5}
	40°C	8.4×10^{-5}
II[b]	100% N_2	16.6×10^{-5}
	100% O_2	7.9×10^{-5}

[a] Enzyme (25 μg) was incubated for 10 min at the indicated temperature in 950 μl of a solution containing 20 μmol $NaH^{14}CO_3$, 10 μmol $MgCl_2$ and 50 μmol bicine at pH 8.3; 10 min reactions were initiated by the addition of 0.5 μmol RuBP in 50 μl

[b] Enzyme (20 μg) was incubated for 10 min at 25°C in 480 μl of a solution containing 1.25 μmol $NaH^{14}CO_3$, 5 μmol $MgCl_2$ and 25 μmol bicine at pH 8.3; 20 min reactions were initiated by the addition of 0.01 μmol RuBP in 20 μl

Table 3

Biochemical analysis of photoautotrophic revertants of *rcl-u*-1–10-6C *mt*+

Strain	$K_m(CO_2)$	$V_{max}(CO_2)$	Specificity factor
	% Control[a]		
R4-7	98	99	100
R6-2	101	96	101
R6-3	96	98	100
R7-2	107	105	95
R7-4	98	102	98
R7-5	101	104	99
R7-6A	99	105	98
R7-6B	100	103	97
R1-1B	[b]	[b]	106

[a] Control values equal: $K_m(CO_2)$, 29 μM; $V_{max}(CO_2)$, 2.03 μmol . mg protein^{-1} . min^{-1}; specificity factor, 61

[b] Not determined

carboxylase/oxygenase was indistinguishable from wild-type with regard to $K_M(CO_2)$, $V_{max}(CO_2)$ and the carboxylase/oxygenase ratio (table 3). Non-equilibrium pH gradient electrophoresis of the RuBP carboxylase/oxygenase subunits from the revertants revealed that the isoelectric point of the large subunit had returned to that found in the wild-type enzyme. These results suggest that all of the photoautotrophic revertants arose from true reversion. The existence of these revertants demonstrates that the *rcl-u*-1–10-6C mutation is not a deletion in the *rcl-u*-1 chloroplast gene, since such mutations are not expected to revert at the frequency reported here. Furthermore, since revertants of the *rcl-u*-1–10-6C mutation return the mutant strain to wild-type in every respect examined, the results demonstrate that the near absence of carboxylase and oxygenase activities, altered large subunit isoelectric point, photosensitivity and acetate requirement all result from mutation in the single *rcl-u*-1 chloroplast gene.

Table 2

Recovery of photoautotrophic revertants of *rcl-u*-1–10-6C *mt*+

Expt. 7	Control	Mutagenesis
Cells plated ($\times 10^{-7}$)	2.6	4.2
Non-light-sensitive colonies ($\times 10^{-3}$)	1.4	12.3
Photoautotrophic revertants	0	3
Revertants/cells plated ($\times 10^{7}$)	0	0.7

Although the photoautotrophic revertants were indistinguishable from wild-type, it is not clear why they could not be recovered directly on minimal medium. Reconstruction experiments, in which mixtures of mutant and wild-type cells were plated on minimal medium, indicated that there was no cooperative death of wild-type cells. Considering that the *C. reinhardii* chloroplast contains as many as 80 genomes (review [19]), a longer expression time may be required for mutant and revertant genes to sort out. This expression time would be provided if several cell divisions occurred on acetate medium in the light. Furthermore, such a result would significantly enrich photoautotrophic revertant colonies relative to the number of cells plated on acetate medium in the light.

4. CONCLUSIONS

We have succeeded in recovering revertants of a chloroplast mutation that specifically affects the large subunit of RuBP carboxylase/oxygenase in

Chlamydomonas reinhardii. Although these revertants regain complete photoautotrophy and possess RuBP carboxylase/oxygenase that is indistinguishable from wild-type, it is evident that the genetic manipulations possible with *C. reinhardii* chloroplast DNA may permit the modification of RuBP carboxylase/oxygenase, as has apparently occurred through evolution [9].

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