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Reduced CO_2/O_2 specificity of ribulose-bisphosphate carboxylase/oxygenase in a temperature-sensitive chloroplast mutant of *Chlamydomonas*

(photosynthesis/photorespiration/chloroplast genetics/plant productivity/genetic engineering)

ZHIXIANG CHEN, CHRIS J. CHASTAIN*, SOUHAIL R. AL-ABED, RAYMOND CHOLLET, AND ROBERT J. SPREITZER†

Department of Biochemistry, University of Nebraska, Lincoln, NE 68583-0718

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ABSTRACT **The *Chlamydomonas reinhardtii* chloroplast mutant 68-4PP is phenotypically indistinguishable from wild type at 25°C but fails to grow photosynthetically at 35°C. It had about 30% of the wild-type level of ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) holoenzyme and carboxylase activity when grown at 25°C, but less than 15% when grown at 35°C. Pulse-labeling with ^{35}S showed that the decrease in enzyme level at the restrictive temperature was not a result of reduced synthesis of enzyme subunits. The CO_2/O_2 specificity factor (V_CK_O/V_OK_C, where V_C and V_O are V_{max} values for carboxylation and oxygenation and K_C and K_O are K_m values for CO_2 and O_2) of the mutant enzyme was found to be significantly less than that of the wild-type enzyme (54 ± 2 and 62 ± 1, respectively), and this alteration was accompanied by increases in K_O and K_C and a decrease in V_C/V_O. DNA sequencing revealed a single missense mutation in the 68-4PP chloroplast large-subunit gene. This mutation causes leucine to be replaced by phenylalanine at position 290 in the large-subunit polypeptide sequence. These results (*i*) support previous studies that implicated this region of the large subunit as an important structural component of the enzyme's function and (*ii*) demonstrate that chloroplast genetic modification of the CO_2/O_2 specificity factor of a plant-type carboxylase/oxygenase is feasible.**

Ribulose-1,5-bisphosphate (Rbu-P_2) carboxylase/oxygenase (EC 4.1.1.39) is a key enzyme in photosynthetic CO_2 fixation that is assembled from eight chloroplast-encoded large subunits and eight nuclear-encoded small subunits in all plants and most algae (1). It catalyzes both the carboxylation and the oxygenation of Rbu-P_2 in the chloroplast and thus initiates the competitive metabolic pathways of photosynthesis and photorespiration, respectively (2). Photorespiration is a nonessential process in plants (3), and the competition of O_2 with CO_2 at the large-subunit active site (4) ultimately leads to the loss of fixed carbon and a reduction in photosynthetic efficiency (5). Thus, chemical or genetic manipulation that reduces oxygenase activity or increases carboxylase activity may increase photosynthetic efficiency and plant productivity.

The relative rates of Rbu-P_2 carboxylation and oxygenation are determined by the CO_2/O_2 specificity, V_CK_O/V_OK_C [where V_C and V_O are the maximal velocities (V_{max}) for carboxylation and oxygenation and K_C and K_O are the Michaelis constants (K_m) for CO_2 and O_2], at any given concentrations of CO_2 and O_2 (5). A higher enzyme specificity factor indicates a greater specificity for carboxylation. Previous studies showed that the specificity of Rbu-P_2 carboxylase/oxygenase could be reduced by incubating the enzyme at elevated temperature (6) or by substituting Mn^{2+} for the Mg^{2+} that is required for catalysis and/or activation by CO_2 and divalent metal cation (7, 8). Furthermore, the specificity factor is known to vary among species (9), suggesting that Rbu-P_2 carboxylase/oxygenase has evolved toward a higher efficiency with respect to carboxylation. These observations indicate that enzyme specificity is not immutable. Genetic modification of the CO_2/O_2 specificity factor, either by directed mutagenesis *in vitro* (10–14) or by random mutagenesis *in vivo* (15–17), could lead to the development of rational approaches for the genetic engineering of Rbu-P_2 carboxylase/oxygenase.

We recently recovered a temperature-sensitive Rbu-P_2 carboxylase/oxygenase mutant from the unicellular green alga *Chlamydomonas reinhardtii* (18). This mutant, designated 68-4PP, is unable to grow photosynthetically at 35°C, but it is phenotypically indistinguishable from wild type at 25°C. In the present study, we found that the mutant Rbu-P_2 carboxylase/oxygenase holoenzyme had a significant reduction in CO_2/O_2 specificity when compared with the wild-type enzyme. Furthermore, DNA sequencing revealed a missense mutation in the chloroplast large-subunit gene that replaced leucine with phenylalanine at position 290 in the large-subunit protein sequence. This amino acid substitution occurs in a region of the large-subunit polypeptide that may be important in determining the carboxylation efficiency of the holoenzyme.

MATERIALS AND METHODS

Strains and Culture Conditions. All *C. reinhardtii* strains were routinely maintained on 10 mM acetate medium (19) in the dark at 25°C. For experimental procedures, wild-type 2137 mating type (*mt*) + (19) and the temperature-sensitive, acetate-requiring 68-4PP *mt*+ mutant (18) were grown on a rotary shaker in the dark with 50 to 500 ml of acetate medium at 25°C or 35°C.

Biochemical Analysis. Light-dependent CO_2 fixation was measured in whole cells as the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$, as described (20). Photosynthetic electron transport (O_2 uptake with methyl viologen) was determined in dark-grown intact cells with a Hansatech O_2 electrode (18). Chlorophyll was assayed in sonicated cell lysates or whole cells (21). Clarified lysates were assayed to determine the amount of total soluble cell protein (22).

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Abbreviations: Rbu-P_2, ribulose 1,5-bisphosphate; K_C, K_m for CO_2; K_O, K_m for O_2; V_C, V_{max} for carboxylation; V_O, V_{max} for oxygenation; *mt*, mating type.

*Present address: Department of Biology, Texas A & M University, College Station, TX 77843.
†To whom reprint requests should be addressed.

Sucrose gradients were used to purify and measure the amount of Rbu-P_2 carboxylase/oxygenase holoenzyme (20). Proteins were fractionated by NaDodSO$_4$/PAGE (23) or isoelectric focusing under denaturing conditions (24). For pulse labeling, 35°C-grown cells were incubated at 35°C with carrier-free $H_2{}^{35}SO_4$ for 5 min and then "chased" by incubation with 10 mM Na_2SO_4 for 1 hr, and samples were prepared as described (25).

Rbu-P_2 carboxylase activity was measured by standard methods (18) as the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ by sonicated lysates. To determine the CO_2/O_2 specificity factor, holoenzyme was purified from 25°C-grown cells by centrifugation in sucrose gradients (20), and carboxylation and oxygenation were assayed simultaneously at 25°C with 63 μM [1-^{3}H]Rbu-P_2 and 2 mM $NaH^{14}CO_3$ in a 1-hr reaction according to a modified method (26) of the procedure of Jordan and Ogren (8). K_C and K_O were determined from assays of purified enzyme performed at both 100% N_2 (i.e., without O_2) and 100% O_2. The assays were initiated with 10 μg of activated enzyme in 1-ml reaction mixtures containing 0.4 mM Rbu-P_2, 10 mM $MgCl_2$, 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine (pH 8.0), and 0.4, 0.8, 1.4, 2.4, or 4.4 mM $NaH^{14}CO_3$. Reactions were stopped after 1 min with 0.5 ml of 3 M formic acid in methanol. K_C was computed from the 100% N_2 assays. K_O was derived from the ratio (R) of carboxylase activities at 100% N_2 versus 100% O_2, according to the relationships of kinetic constants presented by Laing *et al.* (5): $R = 1 + K_C[O_2]/K_O(K_C + [CO_2])$. K_O was calculated from the intercept of $1/(R - 1) = K_O/[O_2] + K_O[CO_2]/K_C[O_2]$.

Large-Subunit Gene Cloning and Sequencing. DNA purification, cloning in bacteriophage λ, and subcloning of the R15 large-subunit gene fragment (27) into a plasmid were performed as described (25), except that the pBR329 plasmid (28) was used to construct the pLS68-4PP plasmid containing the large-subunit gene. A *Hae* III restriction fragment containing the Rbu-P_2 carboxylase/oxygenase large-subunit gene was isolated from pLS68-4PP and digested with restriction endonucleases *Hin*dIII and *Hin*cII. The resulting fragments were cloned in both orientations in bacteriophage M13 vectors (29). Sequencing was performed by the dideoxynucleotide chain-termination method (30).

RESULTS

Biochemical Properties of Mutant 68-4PP. In a previous study (18), the 68-4PP mutant was recovered as a strain that required acetate for growth at 35°C but displayed normal photosynthetic growth at 25°C. Preliminary results obtained at 35°C indicated that the mutant had a specific reduction in Rbu-P_2 carboxylase activity, and its uniparental mode of inheritance suggested that it resulted from a mutation in the chloroplast large-subunit gene (15, 18). In the present study, we investigated the biochemical properties of the 68-4PP mutant at both the permissive (25°C) and restrictive (35°C) temperatures (Table 1). When grown at 25°C, the mutant had 30% of the wild-type rate of whole-cell CO_2 fixation, and Rbu-P_2 carboxylase activity was also reduced to about 30%. In contrast, 35°C-grown mutant cells had only 13% of the wild-type CO_2-fixation rate, and carboxylase activity was negligible. However, there was no significant difference in the levels of whole-chain electron-transport activity or chlorophyll when 68-4PP was compared with wild type at each of the two temperatures (Table 1). Thus, pleiotropic alterations in photosynthesis were not apparent, suggesting that the temperature-sensitive phenotype of 68-4PP was related directly to the level of Rbu-P_2 carboxylase activity.

The 68-4PP mutant also had reduced levels of Rbu-P_2 carboxylase/oxygenase holoenzyme protein at 25°C and 35°C, and these levels were correlated with the decreased amounts of carboxylase activity (Table 1). Both large and small subunits were found to be reduced proportionately when soluble cell proteins were analyzed by NaDodSO$_4$/PAGE (Fig. 1). The mutant subunits were of normal size (Fig. 1), and, when purified holoenzyme from 25°C-grown cells was subjected to isoelectric focusing under denaturing conditions (20, 24), the mutant subunits had normal isoelectric points (data not shown).

Table 1. Biochemical analysis of mutant 68-4PP *mt*+ grown and assayed at the permissive (25°C) and restrictive (35°C) temperatures

	Biochemical properties, % of control*				
				Rbu-P_2 carboxylase	
Temperature	CO_2 fixation	Whole-chain e$^-$-transport activity	Chl	Activity	Protein
25°C	30	126	98	32	36
35°C	13	123	79	1	10

*Wild-type control values at 25°C (or 35°C) were as follows: CO_2 fixation, 119 (or 134) μmol of CO_2 per hr per mg of chlorophyll (Chl); whole-chain electron (e$^-$)-transport activity, 14 (or 25) μmol of O_2 per hr per mg of Chl; Chl, 134 (or 66) μg per mg of total soluble protein; Rbu-P_2 carboxylase activity, 5 (or 8) μmol of CO_2 per hr per mg of soluble protein; Rbu-P_2 carboxylase/oxygenase protein, 45 (or 34) μg per mg of soluble protein.

The dramatic reduction in mutant-enzyme subunits at 35°C (Fig. 1, lane 4) could have resulted from reduced synthesis of one or both subunit types (25) or from degradation before or after holoenzyme assembly. To investigate these possibilities, 35°C-grown cells were pulse-labeled with $^{35}SO_4^{2-}$ for 5 min and chased with Na_2SO_4 for 1 hr at 35°C. Cell proteins were extracted, and samples of equal radioactivity were subjected to NaDodSO$_4$/PAGE and fluorography. There was no difference in the amount of labeling of either large or small subunits when mutant 68-4PP and wild type were compared after the 5-min pulse (Fig. 2, lanes 1 and 2), indicating that large and small subunits were synthesized at normal rates in the mutant. After the 1-hr chase (Fig. 2, lanes 3 and 4), some degradation of both large and small subunits was observed in the mutant, but the amount of labeled subunits was much

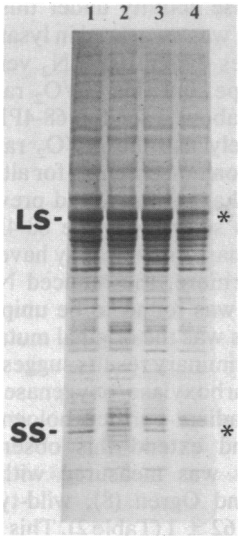

FIG. 1. NaDodSO$_4$/PAGE of total soluble cell proteins from wild type and mutant 68-4PP. Cells were grown in the dark at 25°C (lanes 1 and 2) or 35°C (lanes 3 and 4) prior to extraction. Each lane received 60 μg of protein, and the gel was stained with Coomassie blue after electrophoresis. Lanes: 1 and 3, wild type; 2 and 4, mutant 68-4PP. The 55,000-dalton large subunit (LS) and 16,500-dalton small subunit (SS) are indicated (*).

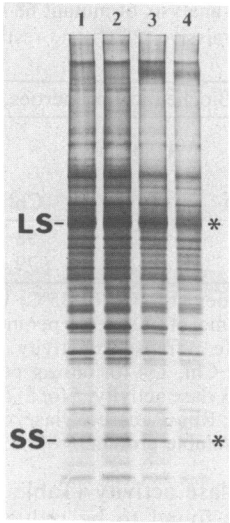

FIG. 2. Pulse labeling of soluble cell proteins in wild type and mutant 68-4PP grown in the dark at 35°C. Cells were labeled with $^{35}SO_4^{2-}$ for 5 min (lanes 1 and 2) and chased with 10 mM Na_2SO_4 for 1 hr (lanes 3 and 4) at 35°C. The cells were extracted, and equal amounts of radioactivity were subjected to $NaDodSO_4$/PAGE followed by fluorography. Lanes: 1 and 3, wild type; 2 and 4, mutant 68-4PP. The large subunit (LS) and small subunit (SS) are indicated (*).

greater in the mutant than expected from $NaDodSO_4$/PAGE (Fig. 1, lanes 3 and 4) or from previous observations of large-subunit nonsense mutants (25). Thus, the slow rate of mutant-subunit degradation at 35°C suggests that the holoenzyme assembles prior to its degradation to the low level observed in sucrose gradients (Table 1) or by $NaDodSO_4$/PAGE (Fig. 1).

CO_2/O_2 Specificity of Rbu-P_2 Carboxylase/Oxygenase. We have begun recovering temperature-sensitive, acetate-requiring mutants so that the partially defective Rbu-P_2 carboxylase/oxygenase enzymes can be purified and studied from cells grown at the permissive temperature (18). As noted above (Table 1), lysates of 68-4PP had more than 30% of wild-type carboxylase activity under this condition. When carboxylase activity was measured in lysates at limiting CO_2, the ratio of activities under 100% N_2 versus 100% O_2 was about 3 for wild type, but this N_2/O_2 ratio of carboxylase activities was only about 2 for the 68-4PP mutant (data not shown). We routinely use the N_2/O_2 ratio of carboxylase activities as a criterion for screening for altered enzymes (16), but no significant change was found previously. Given this decrease in oxygen sensitivity, the 68-4PP mutant Rbu-P_2 carboxylase/oxygenase enzyme may have had an increase in K_O/K_C (5). Furthermore, the reduced N_2/O_2 ratio of carboxylase activities was found to be uniparentally inherited (data not shown), as was the original mutant phenotype (18).

Although our preliminary results suggested that the K_O/K_C ratio of Rbu-P_2 carboxylase/oxygenase was increased in 68-4PP, sucrose-gradient-purified holoenzyme was investigated to verify and extend this observation. When the CO_2/O_2 specificity was measured with the dual-labeling assay of Jordan and Ogren (8), wild-type enzyme had a specificity factor of 62 ± 1 (Table 2). This value is in excellent agreement with previous studies of the *Chlamydomonas* enzyme (9, 26). In contrast, the specificity of the mutant enzyme was only 54 ± 2. This 13% reduction in enzyme specificity was accompanied by a 29% increase in K_O/K_C, but both the K_C and the K_O of the mutant enzyme were significantly greater than in the wild-type enzyme; K_C (37 μM) was about 30% higher, whereas K_O (664 μM) was about 60% higher (Table 2). Thus, by calculation, the mutant enzyme must also have a decreased V_C/V_O.

Table 2. Kinetic properties of Rbu-P_2 carboxylase/oxygenase purified from 25°C-grown mutant 68-4PP *mt*+ and wild-type 2137 *mt*+

Strain	Kinetic constants				
	V_CK_O/V_OK_C*†	K_C,* μM	K_O,* μM	K_O/K_C	V_C/V_O
68-4PP	54 ± 2	37 ± 2	664 ± 26	18	3.0
2137	62 ± 1	29 ± 1	412 ± 25	14	4.4

*Values were obtained from at least three different enzyme preparations and are reported with the sample (n − 1) SD.
†CO_2/O_2 specificity factor (8, 9).

Gene Cloning and Sequencing. Since the 68-4PP mutant phenotype was temperature-conditional and uniparentally inherited (18) and the mutant Rbu-P_2 carboxylase/oxygenase enzyme had a significantly altered CO_2/O_2 specificity (Table 2), it seemed likely that 68-4PP contained a missense mutation in the chloroplast large-subunit gene. The large-subunit genes were cloned and completely sequenced from the 68-4PP mutant and wild type. A single transition from C to T was found at nucleotide position 2058 within the R15 fragment (27) of the 68-4PP mutant. This mutation would cause a leucine (CTT) residue to be replaced by phenylalanine (TTT) at amino acid position 290 within the 68-4PP large-subunit protein.

DISCUSSION

Chloroplast Molecular Genetics. We have been investigating photosynthesis-deficient mutants of *C. reinhardtii* that contain mutations in the chloroplast large-subunit gene of Rbu-P_2 carboxylase/oxygenase (15–17). Since these mutants are recovered randomly from large collections of acetate-requiring mutants (18, 19), they may provide information about enzyme structure and function that is different from the information that can be obtained by directed mutagenesis *in vitro*. However, of four mutations identified within the *C. reinhardtii* large-subunit gene, two were nonsense mutations (25) and two were missense mutations that caused amino acid substitutions only several residues away from the active-site lysine at position 175 (17, 20, 31). It thus appeared that the mutations were not distributed randomly within the large-subunit gene. This observation suggested that many mutations occur in the large-subunit gene, but few of them are severe enough to produce an acetate-requiring phenotype (18).

The nonrandom distribution of particularly lethal mutations within the large-subunit gene could be useful for identifying essential structural or functional domains of the holoenzyme. However, to investigate more subtle alterations in enzyme catalysis, a phenotypically different class of mutants appeared to be required. It is for this reason that we have begun to recover temperature-conditional, acetate-requiring mutants (18). These mutants require acetate for growth at 35°C, but they do not require acetate and are phenotypically wild-type at 25°C. They would not have been recovered as acetate-requiring strains in previous experiments performed at 25°C (19).

In the present study, a temperature-sensitive, acetate-requiring mutant (68-4PP) was investigated that appeared to be specifically deficient in Rbu-P_2 carboxylase activity as a result of a chloroplast gene mutation (18). The 68-4PP mutant was found to have reduced levels of carboxylase activity and holoenzyme protein when it was grown at 25°C, but these levels were reduced even more at 35°C (Table 1, Fig. 1). The decrease in Rbu-P_2 carboxylase/oxygenase holoenzyme was not due to a reduction in the rate of synthesis of the large or

small subunits (Fig. 2), and the slow degradation of the recently synthesized subunits suggested that the holoenzyme assembled prior to its degradation (Fig. 2). It is thus likely that the 68-4PP mutation affects holoenzyme stability and that the subsequent degradation of enzyme subunits is primarily responsible for the temperature-conditional, acetate-requiring phenotype.

DNA sequencing showed that the 68-4PP mutant resulted from a mutation in the chloroplast large-subunit gene that would cause phenylalanine to be substituted for leucine at position 290 in the large-subunit polypeptide sequence. This amino acid substitution is far removed from the amino acid substitutions that occurred near lysine-175 in the two large-subunit missense mutants investigated previously (17, 20, 31). Thus, temperature-sensitive Rbu-P_2 carboxylase/oxygenase mutants can be recovered that appear to define a separate class of large-subunit gene mutations. Since these mutants must retain a significant level of carboxylase activity and holoenzyme at the permissive temperature, they will be invaluable for investigating the catalytic functions of the enzyme.

Rbu-P_2 Carboxylase/Oxygenase Specificity and Structure. The change of a leucine to a phenylalanine at residue 290 in the large-subunit protein of the 68-4PP mutant is responsible for a 13% reduction in the enzyme's CO_2/O_2 specificity factor (Table 2). The K_C and K_O of the mutant enzyme increased, with the major change occurring in K_O (Table 2). These changes would increase the enzyme's affinity for substrate CO_2 relative to O_2 (i.e., increased K_O/K_C), but any potential benefit conferred by this change was apparently negated by a decrease in V_C/V_O. Even though the specificity of Rbu-P_2 carboxylase/oxygenase is reduced by the 68-4PP mutation, our results demonstrate that chloroplast genetic modification of the specificity factor of a plant-type holoenzyme is possible. This observation should encourage novel genetic approaches for increasing the CO_2/O_2 specificity of the enzyme.

A number of investigators have been using *in vitro* directed mutagenesis to examine the importance of large-subunit amino acid residues (10–14). Since the chloroplast Rbu-P_2 carboxylase/oxygenase holoenzyme cannot be expressed in *Escherichia coli*, such work has focused on the homodimeric large-subunit-like enzyme of the photosynthetic bacterium *Rhodospirillum rubrum*. In only one case was a change in CO_2/O_2 specificity reported, but one could not determine whether the magnitude of the change was experimentally significant (12). The random recovery of mutations in the *Chlamydomonas* large-subunit gene may be useful for identifying potentially interesting regions for the application of directed mutagenesis.

Although there is only about 30% sequence identity between the *R. rubrum* Rbu-P_2 carboxylase/oxygenase subunit and the large subunit of eukaryotes (32), the three-dimensional structures of the corresponding subunit dimers are remarkably similar (33, 34). Furthermore, the *R. rubrum* crystal structure has been resolved to 2.9 Å, allowing the majority of amino acid residues to be located (33). The phenylalanine-for-leucine substitution at position 290 in the 68-4PP mutant large subunit occurs in or between a β-strand and an α-helix of carboxyl-terminal loop 5. The α-helix in this region may form part of a phosphate binding site for Rbu-P_2 (33). A histidine at position 298, also in this region, was initially identified as an essential active-site residue (35), but when the corresponding *R. rubrum* residue (His-291) was replaced with alanine, the mutant enzyme retained a significant level of catalytic activity (13). However, the K_m of the mutant enzyme for Rbu-P_2 was increased 15-fold and the tight binding of 2-carboxyarabinitol 1,5-bisphosphate, a transition-state analogue, was reduced markedly (13). The instability and altered specificity of Rbu-P_2 carboxylase/oxygenase in mutant 68-4PP support the idea that conformational changes in the region surrounding residue 290 have significant effects on the active site of the enzyme.

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