Complementing Amino Acid Substitutions within Loop 6 of the α/β-Barrel Active Site Influence the CO_2/O_2 Specificity of Chloroplast Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

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ABSTRACT: Photosynthesis-deficient mutant 45-3B of the green alga *Chlamydomonas reinhardtii* contains a chloroplast mutation that causes valine-331 to be replaced by alanine within the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. This amino acid substitution occurs in loop 6 of the α/β-barrel active site, three residues distant from catalytic lysine-334. The mutation reduces the specific activity of the enzyme and also reduces its CO_2/O_2 specificity factor by 42%, but the amount of holoenzyme is unaffected. In a previous study, an intragenic-suppressor mutation, named S40-9D, was selected that causes threonine-342 to be replaced by isoleucine, thereby increasing the CO_2/O_2 specificity of the mutant enzyme by 36%. To determine which other residues might be able to complement the original mutation, nine additional genetically independent revertants have now been analyzed. Another intragenic suppressor, represented by mutation S61-2J, causes glycine-344 to be replaced by serine. This change increases the CO_2/O_2 specificity of the mutant enzyme by 25%. Of the revertants recovered and analyzed, the mutant enzyme was improved only due to true reversion or by intragenic suppression mediated by substitutions at residues 342 or 344. Changes in the physical properties of the two pairs of complementing substitutions indicate that steric effects within loop 6 are responsible for the observed changes in the CO_2/O_2 specificity of the enzyme.

Ribulose-1,5-bisphosphate (RuBP)[1] carboxylase/oxygenase (EC 4.1.1.39) is a bifunctional enzyme that catalyzes either the carboxylation or oxygenation of RuBP as the first step in either photosynthetic carbon assimilation or photorespiration, respectively [reviewed by Andrews and Lorimer (1987)]. Since CO_2 and O_2 are mutually competitive in catalysis (Ogren & Bowes, 1971), a relative increase in carboxylation or decrease in oxygenation would increase photosynthetic CO_2 fixation and, in turn, plant productivity [reviewed by Ogren (1984)]. The ratio of carboxylation to oxygenation is determined by the enzyme's CO_2/O_2 specificity factor (V_CK_O/V_OK_C), such that an increased specificity denotes relatively improved carboxylation (Laing et al., 1974; Jordan & Ogren, 1981). Kinetic studies have indicated that the CO_2/O_2 specificity of RuBP carboxylase/oxygenase is controlled precisely at the partial reaction between the enzyme-bound 2,3-enediolate of RuBP and the unbound gaseous substrate CO_2 or O_2 (Pierce et al., 1986). Thus, it appears that one could engineer the CO_2/O_2 specificity factor only by changing the way in which the enzyme differentially stabilizes the transition states of the carboxylation and oxygenation partial reactions (Chen & Spreitzer, 1991). However, no exact relationship has yet been established between the structure of RuBP carboxylase/oxygenase and its ability to stabilize these transition states.

The RuBP carboxylase/oxygenase holoenzyme of plants is comprised of eight copies each of a nuclear-encoded small subunit and a chloroplast-encoded large subunit [reviewed by Andrews and Lorimer (1987)]. X-ray crystallography has resolved a classic α/β-barrel active site within each large subunit (Chapman et al., 1988), and loops between the α-helices and β-strands of the barrel contain residues that coordinate with the six-carbon transition-state analogue CABP (Andersson et al., 1989). Whereas much is now known about the structure of the active site (Knight et al., 1990), directed mutagenesis has failed to identify amino acid substitutions that can influence the CO_2/O_2 specificity factor.

Attempts at expressing and assembling the plant hexadecameric holoenzyme in *Escherichia coli* have so far been unsuccessful [reviewed by Roy (1989)]. Therefore, directed mutagenesis aimed at influencing catalysis has been largely limited to the homodimeric *Rhodospirillum rubrum* enzyme [reviewed by Gutteridge (1990)], which has an active-site crystal structure quite similar to that of the plant enzyme (Schneider et al., 1986). The CO_2/O_2 specificity has been altered (decreased) in one case, but this was achieved only by restoration of partial activity via chemical modification to a directed-mutant enzyme that lacked activity (Smith et al., 1990). In the absence of a heritable change, it is still difficult to determine which amino acids could be replaced to improve catalysis.

The main limitation of the directed mutagenesis approach is that one needs to know which amino acid might be important before attempting to substitute it. In contrast, a genetic approach can be pursued in which a mutant enzyme is first identified, and then, only subsequently, its molecular basis is determined. Work of this nature is being performed with the eukaryotic green alga *Chlamydomonas reinhardtii* (Spreitzer

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[1] Abbreviations: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; V_C, V_{max} for carboxylation; V_O, V_{max} for oxygenation; K_C, Michaelis constant for CO_2; K_O, Michaelis constant for O_2; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; *mt*, mating type.

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& Ogren, 1985), and three mutations have already been defined within the chloroplast large-subunit gene (*rbc*L) that influence the CO_2/O_2 specificity of RuBP carboxylase/oxygenase (Chen et al., 1988, 1990; Chen & Spreitzer, 1989). These mutations cause amino acid substitutions within loop-regions 5 and 6 of the α/β-barrel active site.

In a previous study (Chen & Spreitzer, 1989), *rbc*L mutation 45-3B was identified that causes valine-331 to be replaced by alanine within large-subunit loop 6. This substitution was found to reduce the specific activity and CO_2/O_2 specificity factor of RuBP carboxylase/oxygenase. A second *rbc*L mutation, named S40-9D, was recovered by selecting for restored photosynthesis, and this mutation complemented the original defect by replacing threonine-342 with isoleucine on the opposite side of the same loop (Chen & Spreitzer, 1989). Whereas our observations clearly identified loop 6 as a determinant of enzyme specificity, it was still difficult to deduce exactly how the physical and chemical properties of the complementing residues influenced CO_2/O_2 specificity. In the present study, we have addressed this problem by selecting additional photosynthesis-competent "revertant" strains from the original valine-to-alanine mutant.

Experimental Procedures

Strains and Culture Conditions. All *C. reinhardtii* strains were grown at 25 °C in the dark with medium containing 10 mM sodium acetate (Spreitzer & Mets, 1981). Strain 2137 mt^+ was the wild-type strain, and the *pf*-2 (paralyzed flagella) mt^- centromere-marker strain was employed for genetic analysis (Spreitzer & Mets, 1981; Spreitzer et al., 1988). Photosynthesis-deficient mutant 45-3B mt^+ was recovered as a light-sensitive, acetate-requiring strain in a previous study (Chen & Spreitzer, 1989). The photosynthesis-competent R40-9D revertant strain, which contains an *rbc*L intragenic-suppressor mutation named S40-9D, was selected from the 45-3B mutant strain (Chen & Spreitzer, 1989).

Selection for Revertants and Genetic Analysis. Photosynthesis-competent revertant colonies were recovered spontaneously according to standard methods (Chen & Spreitzer, 1989). Independent clones of the 45-3B mutant strain were employed in these reversion experiments to ensure the independent origin of each revertant strain. Gamete induction in nitrogen-free acetate medium, zygote maturation, tetrad dissection, and phenotype scoring were performed as described previously (Spreitzer & Mets, 1981; Spreitzer et al., 1988).

Molecular Biology. DNA was purified and *rbc*L large-subunit genes were cloned as described previously (Spreitzer et al., 1985). DNA sequencing was performed with the aid of various synthetic oligonucleotides, U.S. Biochemical Corp. Sequenase, and [^{35}S]dCTP. The *rbc*L-containing *Eco*RI R15 fragment (Dron et al., 1982) from revertant strain R61-2J was cloned in pBR329 (Covarrubias & Bolivar, 1982), and this new plasmid was designated pLSR61-2J.

Assay of Enzyme Activity and Kinetic Constants. RuBP carboxylase activity was routinely measured in sonicated cell extracts under an atmosphere of 100% N_2 as the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ at 25 °C (Spreitzer & Chastain, 1987; Chen et al., 1990). The RuBP carboxylase/oxygenase holoenzyme was purified from sucrose gradients (Spreitzer & Chastain, 1987), and protein was determined by the method of Bradford (1976). The CO_2/O_2 specificity factor of purified enzyme was determined by simultaneously assaying the carboxylase and oxygenase activities at 25 °C in a reaction mixture containing 2 mM $NaH^{14}CO_3$ (1.8 Ci/mol), 30 μM [1-^{3}H]RuBP (30 Ci/mol), 10 mM $MgCl_2$, and 50 mM Bicine (pH 8.3) according to the standard procedure (Jordan & Ogren, 1981; Spreitzer et al., 1982). Kinetic constants of purified enzyme were measured by following the previous procedures (Chen et al., 1988; Chen & Spreitzer, 1989).

Assay of Transition-State Analogue Binding. Tightness of binding of the CABP transition-state analogue to purified enzyme was measured as described previously (Chen & Spreitzer, 1991). Unlabeled CABP and [^{14}C]CABP (20 Ci/mol) were provided by Dr. Raymond Chollet (Department of Biochemistry, University of Nebraska, Lincoln, NE).

Results

Recovery of Revertants. Photosynthesis-competent revertant colonies were recovered spontaneously at a frequency of about 3×10^{-8} when 45-3B mutant cells were plated on minimal medium (without acetate) in the light. Three independent revertant strains were chosen for further study from each of three distinct phenotypic classes. Revertants R33-8A, R64-7B, and R66-12A grew as well as wild type on minimal medium and had normal levels of RuBP carboxylase activity in crude extracts. Thus, these revertants have a phenotype like that of revertant R40-4A, which was previously shown to arise by true reversion (Chen & Spreitzer, 1989). All of the remaining revertants grew somewhat less well than wild type on minimal medium. However, revertants R21-3, R61-2J, and R66-8A had only about 20% of the normal level of RuBP carboxylase activity, whereas revertants R33-7A, R61-3E, and R64-6A had about 35% of the normal level of activity. The R40-9D revertant, analyzed previously (Chen & Spreitzer, 1989), would be a member of this latter group.

When each mt^+ revertant strain was crossed with *pf*-2 mt^-, all of the progeny had revertant (wild-type) phenotypes. This uniparental pattern of inheritance indicated that all of the mutations responsible for the revertant phenotypes reside within the chloroplast genome. In contrast, the nuclear *pf*-2 centromere marker was inherited in the expected Mendelian pattern.

Gene Cloning and Sequencing. Revertants R33-8A, R64-7B, and R66-12A, like revertant R40-4A studied previously (Chen & Spreitzer, 1989), arose from true reversion of the original 45-3B *rbc*L mutation, thus accounting for their wild-type phenotypes and normal levels of RuBP carboxylase activity. The other six revertants resulted from intragenic suppression. Revertants R33-7A, R61-3E, and R64-6A each arose from the same type of second-site mutation that was previously identified in the R40-9D revertant *rbc*L gene (Chen & Spreitzer, 1989). These mutations cause threonine (ACT) to be replaced by isoleucine (ATT) at large-subunit residue 342 (Figure 1, panel c). On the other hand, revertants R21-3, R61-2J, and R66-8A each arose from a common second-site mutation, but this mutation was different from the second-site mutation that is present in *rbc*L of the other revertant strains. In this case, the mutations cause glycine (GGT) to be replaced by serine (AGT) at large-subunit residue 344 (Figure 1, panel d). Thus, the original V331A substitution caused by the 45-3B mutation was found to be complemented only by either of two different amino acid substitutions (T342I or G344S), and these substitutions are separated by only one residue within the large-subunit primary structure (Figure 2).

Biochemical Properties. We previously showed that the T342I suppressor mutation present in R40-9D (and also present in R33-7A, R61-3E, and R64-6A) not only enhanced the activity but also altered the kinetic properties of the original 45-3B (V331A) mutant enzyme (Chen & Spreitzer, 1989). It was clear that the R21-3, R61-2J, and R66-8A revertants, which result from a different type of *rbc*L suppressor mutation

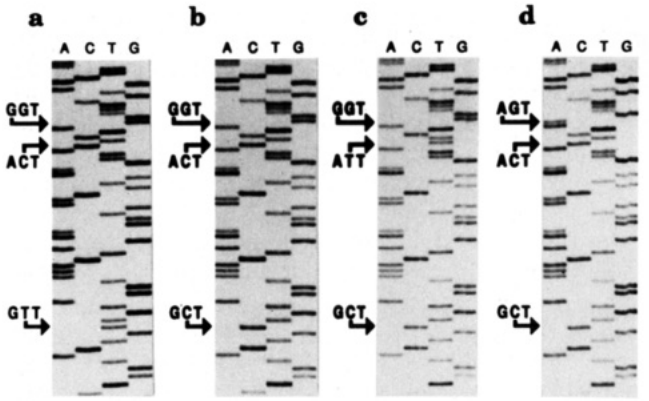

FIGURE 1: DNA sequencing of the chloroplast *rbc*L genes from wild type (a), mutant 45-3B (b), revertant R40-9D (c), and revertant R61-2J (d). The 45-3B *rbc*L gene has a T-A to C-G transition at nucleotide position 2182 that changes valine (GTT) to alanine (GCT) at large-subunit residue 331 (V331A). The R40-9D gene contains the same mutation but also has a C-G to T-A transition at nucleotide 2215 that changes threonine (ACT) to isoleucine (ATT) at residue 342 (V331A/T342I). The R61-2J *rbc*L gene contains, in addition to the 45-3B mutation, a G-C to A-T transition at nucleotide 2220 that changes glycine (GGT) to serine (AGT) at residue 344 (V331A/G344S). The active site lysyl residue occurs at position 334 (Soper et al., 1988). Nucleotide positions within *rbc*L are defined relative to the 5′-end of the R15 fragment (Dron et al., 1982).

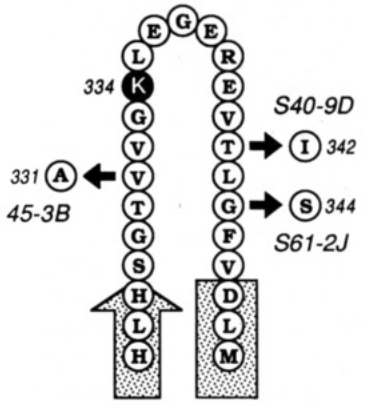

FIGURE 2: Amino acid substitutions within loop 6 of the α/β-barrel active site. The valine-331-to-alanine substitution (V331A), caused by the 45-3B mutation, can be complemented by either the threonine-342-to-isoleucine (T342I) or glycine-344-to-serine (G344S) substitution, caused by the S40-9D or S61-2J mutation, respectively. The active site lysyl residue occurs at amino acid position 334 (Soper et al., 1988). Loop 6 is flanked on the amino-terminal side by β-strand 6 (vertical arrow) and on the carboxyl-terminal side by α-helix 6 (vertical box). The diagram is based on the crystal structure of spinach RuBP carboxylase/oxygenase (Andersson et al., 1989). However, α-helix 6 may extend up through residue 339 (Knight et al., 1990).

(G344S), must also have altered RuBP carboxylase/oxygenase catalytic properties relative to the 45-3B mutant enzyme. Since these three revertant strains arose from identical mutations, only one of them, revertant R61-2J, was chosen for further study.

We routinely use the ratio of RuBP carboxylase activities measured under 100% N_2 versus 100% O_2 as a criterion for screening for enzymes with altered kinetic properties (Spreitzer & Chastain, 1987; Chen et al., 1990). This N_2/O_2 ratio is a function of the K_C and K_O kinetic parameters of RuBP carboxylase/oxygenase when determined at limiting CO_2 concentrations (Chen et al., 1988). Crude extract of mutant 45-3B (V331A) had an N_2/O_2 carboxylase ratio decreased to 1.2, whereas wild-type extract had a ratio of 2.9. Extracts of revertants R40-9D (V331A/T342I) and R61-2J (V331A/G344S) also had decreased N_2/O_2 carboxylase ratios

Table I: Kinetic Properties of RuBP Carboxylase/Oxygenase Purified from Wild-Type and Revertant R61-2J[a]

kinetic constant	wild type	R61-2J (V331A/G344S)
V_CK_O/V_OK_C[b]	62 ± 2	45 ± 1
K_C (μM CO_2)	33 ± 4	78 ± 8
K_O (μM O_2)	419 ± 5	1453 ± 45
V_C [μmol h^{-1} (mg of protein)$^{-1}$]	109 ± 5	28 ± 2
K_O/K_C[c]	13	19
V_C/V_O[c]	4.9	2.4

[a] The values are the means of three separate enzyme preparations with sample ($n - 1$) standard deviations. V_CK_O/V_OK_C is the CO_2/O_2 specificity factor (Jordan & Ogren, 1981; Laing et al., 1974). [b] Also determined in this study, the specificity values for enzymes purified from mutant 45-3B (V331A) and revertant R40-9D (V331A/T342I) were 36 ± 1 and 49 ± 2, respectively. [c] Calculated values.

(1.7 and 1.5, respectively), but these values were higher than that of the 45-3B mutant extract. Thus, the difference in the ratio indicated that the suppressor mutation, named S61-2J (G344S), present in the R61-2J *rbc*L gene not only increased the specific activity but also changed the kinetic properties of the original 45-3B (V331A) mutant enzyme.

CO_2/O_2 Specificity. On the basis of the preliminary observations noted above, we further analyzed the kinetic properties of RuBP carboxylase/oxygenase purified from revertant R61-2J and wild type (Table I). Whereas wild-type enzyme had a CO_2/O_2 specificity factor of 62 ± 2, the enzyme purified from R61-2J (V331A/G344S) had a CO_2/O_2 specificity factor of only 45 ± 1. However, this represents a 25% increase relative to the specificity factor of the 45-3B (V331A) mutant enzyme, which was found to have a value of 36 ± 1 (Table I; Chen & Spreitzer, 1989). Thus, either the S40-9D (T342I) or S61-2J (G344S) intragenic-suppressor mutation is able to enhance the CO_2/O_2 specificity factor of the 45-3B (V331A) mutant enzyme. Further analysis of the kinetic constants of the R61-2J (V331A/G344S) enzyme showed that the ratio of K_O to K_C is increased relative to that of the wild-type enzyme but that the V_C and V_C/V_O ratio are decreased (Table I). Nevertheless, the R61-2J enzyme is improved relative to the original 45-3B mutant enzyme primarily due to an increase in the value of V_C/V_O (Chen & Spreitzer, 1989). Since enzymes from mutant 45-3B (V331A), revertant R40-9D (V331A/T342I), and revertant R61-2J (V331A/G344S) all have the same value of 19 for the K_O/K_C ratio (Chen & Spreitzer, 1989; Table I), the differences in CO_2/O_2 specificity between these three enzymes must result from differences in their values for V_C/V_O.

Tightness of CABP Binding. We previously showed that the original 45-3B (V331A) mutant enzyme could not bind the carboxylation transition-state analogue CABP tightly primarily due to an impairment in the irreversible second binding step of the two-step process (Chen & Spreitzer, 1991). Thus, the mutant enzyme appears to be defective in stabilizing the transition state of the carboxylation step, thereby contributing to the change in the CO_2/O_2 specificity of the enzyme (Chen & Spreitzer, 1991). Furthermore, the tightness of CABP binding is improved for the R40-9D (V331A/T342I) revertant enzyme, and this increase in the tightness of binding is accompanied by an increase in the CO_2/O_2 specificity factor relative to that of the original mutant enzyme (Chen & Spreitzer, 1989, 1991). In the present study, we also measured the tightness of CABP binding to the R61-2J (V331A/G344S) revertant enzyme in comparison with that of the original 45-3B (V331A) mutant and R40-9D (V331A/T342I) revertant enzymes. As shown in Table II (fractionation 1), when purified

Table II: Stoichiometries of CABP Binding to Wild-Type and Mutant RuBP Carboxylase/Oxygenase Holoenzymes

strain	V_CK_O/V_OK_C[a]	[^{14}C]CABP/large subunit[b] fractionation 1	fractionation 2
wild type	62 ± 2	0.95	0.90
mutant 45-3B (V331A)	36 ± 1	0.53	0.05
revertant R40-9D (V331A/T342I)	49 ± 2	0.75	0.24
revertant R61-2J (V331A/G344S)	45 ± 1	0.70	0.21

[a] Values for the CO_2/O_2 specificity factor (V_CK_O/V_OK_C) were determined in this study as noted and shown in Table I. [b] Purified and CO_2/Mg^{2+}-activated RuBP carboxylase/oxygenase holoenzyme was incubated with 0.1 mM [^{14}C]CABP for 15 min and then fractionated on a Sephadex G-75 column during a 15-min period to obtain fractionation 1. This protein fraction was then mixed with a 10000-fold excess of unlabeled CABP and fractionated again (fractionation 2).

and activated RuBP carboxylase/oxygenase was incubated with [^{14}C]CABP for 15 min and then fractionated on a Sephadex G-75 column, wild-type enzyme retained CABP in a molar ratio of 0.95 per large subunit. Whereas the enzyme from mutant 45-3B (V331A) showed substantially reduced binding (a molar ratio of only 0.53), RuBP carboxylase/oxygenase enzymes from revertants R40-9D (V331A/T342I) and R61-2J (V331A/G344S) displayed enhanced binding (0.75 and 0.70, respectively) relative to the mutant enzyme. When the peak holoenzyme fractions were isolated from the first fractionation, incubated with an excess of unlabeled CABP, and fractionated again (Table II, fractionation 2), only about 5% of the bound [^{14}C]CABP was released from the wild-type enzyme but virtually all of the [^{14}C]CABP was lost from the 45-3B (V331A) mutant enzyme. In contrast, enzymes from the R40-9D (V331A/T342I) and R61-2J (V331A/G344S) revertants retained about 30% of their bound [^{14}C]CABP, indicating that the tightness of CABP binding was improved for the revertant enzymes relative to that of the original 45-3B (V331A) mutant enzyme.

Discussion

We previously identified an *rbc*L chloroplast mutation, named 45-3B, within *C. reinhardtii* that causes valine (GTT) to be replaced by alanine (GCT) at residue 331 within the large subunit of RuBP carboxylase/oxygenase (Figure 1, panel b; Chen & Spreitzer, 1989). This change reduces the specific activity of the enzyme by 95% and the CO_2/O_2 specificity factor by 42% (Table I), but the photosynthesis-deficient mutant strain maintains a normal level of RuBP carboxylase/oxygenase holoenzyme (Chen & Spreitzer, 1989). We previously characterized two revertants of mutant 45-3B and found that one arose from true reversion (revertant R40-4A, GCT → GTT) and one arose from intragenic suppression (Chen & Spreitzer, 1989). This latter revertant strain, named R40-9D, was found to contain a second-site *rbc*L mutation, named S40-9D, that causes threonine (ACT) to be replaced by isoleucine (ATT) a large-subunit position 342 (Figure 1, panel c). The R40-9D (V331A/T342I) revertant RuBP carboxylase/oxygenase enzyme, resulting from the complementing 45-3B (V331A) and S40-9D (T342I) mutations, was found to have a 36% increase in the CO_2/O_2 specificity factor relative to the original mutant enzyme (Table I; Chen & Spreitzer, 1989). Since the complementing amino acid substitutions flank a catalytic lysyl residue within loop 6 of the α/β-barrel active site (Figure 2; Soper et al., 1988; Andersson et al., 1989), it is apparent that a change on one side of the loop lowers enzyme specificity, whereas a second change on the opposite side of the loop increases specificity. We noted that the substituted amino acids could complement each other on the basis of the size of side chains and/or the hydropathy index and proposed that their interaction within loop 6 might influence CO_2/O_2 specificity by affecting the placement of lysine-334 within the active site. However, we could not determine from the enzyme crystal structure (Chapman et al., 1988; Andersson et al., 1989) whether these residues interacted directly with each other.

In the present study, we recovered and studied additional revertants of mutant 45-3B (V331A) to determine the number and nature of other amino acid substitutions that could complement the original substitution. Of the nine genetically independent revertants analyzed in the present study, three arose from true reversion (GCT → GTT), three arose from loop-6 mutations identical with the S40-9D (T342I) intragenic-suppressor mutation present in revertant R40-9D (Figure 1, panel c), and three arose from identical loop-6 suppressor mutations that are different from S40-9D. This latter group of intragenic-suppressor mutations is represented by the S61-2J mutation (Figure 1, panel d), which causes glycine (GGT) to be replaced by serine (AGT) at large-subunit residue 344 (Figure 2). RuBP carboxylase/oxygenase purified from revertant R61-2J (V331A/G344S) was found to have a CO_2/O_2 specificity factor 25% greater than the value for the original mutant enzyme (Table I). Thus, there are at least two ways in which second-site mutations can complement the original 45-3B (V331A) mutation to improve the CO_2/O_2 specificity factor. If there are other mutations that could complement the original mutation, they are too rare to be detected within the practical limits of genetic selection and screening.

The fact that the 45-3B valine-to-alanine substitution has been found to be complemented in only two ways, and by amino acid substitutions separated by only one residue (Figure 2), indicates that the complementing substitutions must be fulfilling some rather specific structural requirement within loop 6. A reduction in both the size of the side chain and the hydropathy index caused by replacing valine-331 with alanine in the 45-3B mutant enzyme was complemented by opposite changes in these two parameters when threonine-342 was replaced by isoleucine in the R40-9D (V331A/T342I) revertant enzyme (Chen & Spreitzer, 1989). However, when glycine-344 was replaced by serine in the R61-2J (V331A/G344S) revertant enzyme, the size of the complementing amino acid side chain is, again, increased, but the hydropathy index remains essentially unchanged (Gly = −0.4, Ser = −0.8; Kyte & Doolittle, 1982). Thus, steric interactions between the complementing residues would appear to be primarily responsible for the observed changes in the CO_2/O_2 specificity factor. On the basis of the X-ray crystal structure of spinach RuBP carboxylase/oxygenase, Knight et al. (1990) have noted that valine-331 interacts with threonine-342 and that the alanine-331 and isoleucine-342 amino acid substitutions would complement each other by filling in the space of the loop-6 hydrophobic core. Since glycine-344 is also buried within the hydrophobic core (Knight et al., 1990), its replacement by serine may complement the original valine-to-alanine substitution on the basis of similar structural features.

Directed mutagenesis of *R. rubrum* RuBP carboxylase/oxygenase has shown that the lysyl residue within loop 6 is essential for catalysis (Soper et al., 1988; Hartman & Lee, 1989), and X-ray crystallography of the spinach enzyme has revealed that the analogous loop-6 lysine-334 interacts directly with the 2-carboxyl group of the CABP transition-state analogue (Andersson et al., 1989). Since the 2-carboxyl group of CABP represents the key feature of the transition state of

the partial reaction of carboxylation (Pierce et al., 1986), lysine-334 may play a direct role in the differential stabilization of the carboxylation and oxygenation transition states (Chen & Spreitzer, 1991). The replacement of valine-331 by alanine in the 45-3B mutant enzyme would disrupt these interactions and account for the fact that the binding of CABP to the mutant enzyme is no longer irreversible (Table II; Chen & Spreitzer, 1991). Furthermore, complementing steric interactions that arise from the amino acid substitutions at residues 342 and 344 (Figure 2) may differentially restore the interactions between lysine-334 and the carboxylation and oxygenation transition states, as indicated by increases in enzyme CO_2/O_2 specificity and tightness of CABP binding (Tables I and II).

The CO_2/O_2 specificity of RuBP carboxylase/oxygenase is determined by the ratio of the rate constants (k_C/k_O) for the irreversible partial reactions between the 2,3-enediolate of RuBP and the gaseous substrates CO_2 and O_2 (Pierce et al., 1986). Therefore, one can calculate the difference between the activation energies of the oxygenation and carboxylation partial reactions ($\Delta G_O^* - \Delta G_C^*$) by replacing k_C/k_O with the CO_2/O_2 specificity factor values from Table I in the following equation (Chen & Spreitzer, 1991): $RT \ln (k_C/k_O) = \Delta G_O^* - \Delta G_C^*$, where $R = 8.315$ J mol^{-1} deg^{-1} and $T = 298.15$ K. Whereas wild-type RuBP carboxylase/oxygenase has a $\Delta G_O^* - \Delta G_C^*$ value of 10.23 kJ mol^{-1}, that of the 45-3B (V331A) mutant enzyme is reduced to 8.88 kJ mol^{-1}. The R40-9D (V331A/T342I) and R61-2J (V331A/G344S) revertant enzymes have $\Delta G_O^* - \Delta G_C^*$ values restored to 9.65 and 9.44 kJ mol^{-1}, respectively. Although these differences in the differential stabilization of the carboxylation and oxygenation transition states are small in thermodynamic terms, they further indicate that subtle changes within the structure of RuBP carboxylase/oxygenase can have profound effects on CO_2/O_2 specificity.

The altered steric interactions caused by the 45-3B (V331A), S40-9D (T342I), and S61-2J (G344S) mutations would change the structure of loop 6 and, in turn, may affect the placement of lysine-334 within the active site. On the basis of recent crystallographic analysis, Knight et al. (1990) have suggested that loop 6 is a flexible flap that folds over the active site during catalysis and that a variety of observed intrasubunit and intersubunit interactions may be involved in keeping the flap closed. Whether it is "movement" or conformation of loop 6 that ultimately determines the placement of lysine-334 within the active site is a subject for future investigations. Nevertheless, out of a total of 11 independent reversion events, described here and elsewhere (Chen & Spreitzer, 1989), a primary defect within loop 6 is found to be complemented only by either of two amino acid substitutions also within loop 6 (Figure 2). There is always a possibility that amino acid substitutions in other holoenzyme domains might eventually be discovered that can complement substitutions within loop 6. However, in light of the findings presented here, one must also consider that steric interactions within loop 6 may be more important for determining the CO_2/O_2 specificity of RuBP carboxylase/oxygenase than are interactions between loop 6 and other structural domains.

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References

Andersson, I., Knight, S., Schneider, G., Lindqvist, Y., Lundqvist, T., Brändén, C. I., & Lorimer, G. H. (1989) *Nature 337*, 229–234.

Andrews, T. J., & Lorimer, G. H. (1987) in *The Biochemistry of Plants* (Hatch, M. D., & Boardman, N. K., Eds.) Vol. 10, pp 131–218, Academic Press, New York.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Chapman, M., Suh, S. W., Curmi, P. M. G., Cascio, D., Smith, W. W., & Eisenberg, D. S. (1988) *Science 241*, 71–74.

Chen, Z., & Spreitzer, R. J. (1989) *J. Biol. Chem. 264*, 3051–3053.

Chen, Z., & Spreitzer, R. J. (1991) *Planta 183*, 597–603.

Chen, Z., Chastain, C. J., Al-Abed, S. R., Chollet, R., & Spreitzer, R. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4696–4699.

Chen, Z., Green, D., Westhoff, C., & Spreitzer, R. J. (1990) *Arch. Biochem. Biophys. 283*, 60–67.

Covarrubias, L., & Bolivar, F. (1982) *Gene 17*, 79–89.

Dron, M., Rahirē, M., & Rochaix, J. D. (1982) *J. Mol. Biol. 162*, 775–793.

Gutteridge, S. (1990) *Biochim. Biophys. Acta 1015*, 1–14.

Hartman, F. C., & Lee, E. H. (1989) *J. Biol. Chem. 246*, 11784–11789.

Jordan, D. B., & Ogren, W. L. (1981) *Plant Physiol. 67*, 237–245.

Knight, S., Andersson, I., & Brändén, C. I. (1990) *J. Mol. Biol. 215*, 113–160.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Laing, W. A., Ogren, W. L., & Hageman, R. H. (1974) *Plant Physiol. 54*, 678–685.

Ogren, W. L. (1984) *Annu. Rev. Plant Physiol. 35*, 415–442.

Ogren, W. L., & Bowes, G. (1971) *Nature, New Biol. 230*, 159–160.

Pierce, J., Andrews, T. J., & Lorimer, G. H. (1986) *J. Biol. Chem. 261*, 10248–10256.

Roy, H. (1989) *Plant Cell 1*, 1035–1042.

Schneider, G., Lindqvist, Y., Brändén, C. I., & Lorimer, G. (1986) *EMBO J. 5*, 3409–3415.

Smith, H. B., Larimer, F. W., & Hartman, F. C. (1990) *J. Biol. Chem. 265*, 1243–1245.

Soper, T. S., Mural, R. J., Larimer, F. W., Lee, E. H., Machanoff, R., & Hartman, F. C. (1988) *Protein Eng. 2*, 39–44.

Spreitzer, R. J., & Mets, L. (1981) *Plant Physiol. 67*, 565–569.

Spreitzer, R. J., & Ogren, W. L. (1985) in *Molecular Biology of the Photosynthetic Apparatus* (Steinback, K. E., Bonitz, S., Arntzen, C. J., & Bogorad, L., Eds.) pp 355–360, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Spreitzer, R. J., & Chastain, C. J. (1987) *Curr. Genet. 11*, 611–616.

Spreitzer, R. J., Jordan, D. B., & Ogren, W. L. (1982) *FEBS Lett. 148*, 117–121.

Spreitzer, R. J., Goldschmidt-Clermont, M., Rahirē, M., & Rochaix, J. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5460–5464.

Spreitzer, R. J., Al-Abed, S. R., & Huether, M. J. (1988) *Plant Physiol. 86*, 773–777.