The loop between α-helix 6 and β-strand 6 in the αβ-barrel active site of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) plays a key role in discriminating between gaseous substrates CO₂ and O₂. Based on numerous x-ray crystal structures, loop 6 is either closed or open depending on the presence or absence, respectively, of substrate ligands. The carboxyl terminus folds over loop 6 in the closed conformation, prompting speculation that it may trigger or latch loop 6 closure. Because an x-ray crystal structure of tobacco Rubisco revealed that phosphate is located at a site in the open form that is occupied by the carboxyl group of Asp-473 in the closed form, it was proposed that Asp-473 may serve as the latch that holds the carboxyl terminus over loop 6. To assess the essentiality of Asp-473 in catalysis, we used directed mutagenesis and chloroplast transformation of the green alga *Chlamydomonas reinhardtii* to create D473A and D473E mutant enzymes. The D473A and D473E mutant strains can grow photoautotrophically, indicating that Asp-473 is not essential for catalysis. However, both substitutions caused 87% decreases in carboxylation catalytic efficiency (Vₘ₅₀/Kₘ₅₀) and 16% decreases in CO₂/O₂ specificity. If the carboxyl terminus is required for stabilizing loop 6 in the closed conformation, there must be additional residues at the carboxyl terminus/loop 6 interface that contribute to this mechanism. Considering that substitutions at residue 473 can influence CO₂/O₂ specificity, further study of interactions between loop 6 and the carboxyl terminus may provide clues for engineering an improved Rubisco.

Like many αβ-barrel-domain enzymes (1), the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) has a loop (i.e., between β-strand 6 and α-helix 6) that folds over substrate during catalysis (reviewed in Refs. 2–4). Numerous studies have indicated that loop 6 plays a major role in discriminating between CO₂ and O₂ in the competing RuBP carboxylation and oxygenation reactions of Rubisco (reviewed in Refs. 2 and 3), and Lys-334 at the apex of loop 6 interacts with the C-2 carboxyl group of the transition state analog CABP in various Rubisco x-ray crystal structures (5–9). However, Rubisco may be unique among αβ-barrel enzymes in that the carboxyl terminus folds over loop 6 and appears to stabilize its closed conformation. This arrangement of loop 6 and the carboxyl terminus is also observed in the crystal structure of unactivated Rubisco that contains RuBP in the active site (10). In vivo, Rubisco activase is responsible for facilitating the opening of the closed structure to release this RuBP (reviewed in Refs. 2 and 11), thereby allowing spontaneous carboxylation of an active site Lys-201 and introduction of Mg²⁺ to produce the active form of the enzyme (reviewed in Ref. 12). The change from closed to open conformation in the carboxyl-terminal, αβ-barrel domain of the large subunit is accompanied by movement of the amino-terminal domain of a neighboring large subunit (reviewed in Ref. 4), which also contributes residues to the active site and may contain the primary site of interaction with Rubisco activase (13, 14). In the absence of RuBP or CABP, the carboxyl terminus is disordered, and loop 6 is usually disordered or misfolded into an open conformation depending on the source or treatment of the Rubisco used for crystallization (15–19). An open crystal structure of tobacco Rubisco was solved in which phosphate was observed to reside at a site normally occupied by the carboxyl group of Asp-473 in the closed structure (18). This prompted speculation that Asp-473 may serve as a latch that holds the carboxyl terminus over loop 6 in the closed conformation (18). In plant-like Rubisco enzymes, comprised of eight ~55-kDa large and eight ~16-kDa small subunits, Asp-473 is 100% conserved, but the large subunit carboxyl terminus is quite variable in length and sequence identity (Fig. 1). Some bacterial Rubisco enzymes exist as only large subunit dimers (reviewed in Ref. 20), and without electron density for the carboxyl terminus (21), it is more difficult to assign a residue comparable with Asp-473 in their divergent sequences (i.e., *Rhodospirillum rubrum*, Fig. 1). In previous studies, the role of the large subunit carboxyl terminus in Rubisco function was investigated by using biochemical or genetic methods that deleted or added residues (22–26) or by using directed mutagenesis to replace cyanobacterial carboxyl-terminal residues with those characteristic of land plants (25). Although some alterations were observed in carboxylation catalytic efficiency or holoenzyme stability (22–26), only the deletion of as many as 10 residues from the carboxyl terminus of the *Synechococcus* enzyme caused a substantial reduction in CO₂/O₂ specificity (Fig. 1, residues 466–475) (25). If the carboxyl terminus plays a role in stabilizing loop 6 during catalysis, and loop 6 determines the differential stabilization of the carboxylation and oxygenation transition states (27, 28), one might expect that specific carboxyl-terminal residues may influence CO₂/O₂ specificity.

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However, the function of individual residues was not assessed by directed-mutagenesis substitutions in previous studies. The green algae *Chlamydomonas reinhardtii* serves as a useful model organism for the study of Rubisco because photosynthesis-deficient mutants can be maintained with acetate as an alternative source of carbon and energy. Mutant strains that lack functional genes for the large (chloroplast rbcL) or small (nucleus rbcS) subunit can be complemented by transformation (reviewed in Ref. 29), and the wild-type rbcL gene can be replaced with a mutant rbcL gene regardless of whether the mutant large subunit can produce a functional holoenzyme (e.g. Ref. 30). Furthermore, because mutant genes exist in *vivo* and evolutionary conservation and potential critical function of mutations as a means for identifying complementing structural competence transformants were selected on minimal medium in the light (80 microeinsteins/m²/s) by standard methods (36, 39). Successive rounds of selection, single-colony isolation, and restriction enzyme analysis were performed to ensure the homoplasmicity of the D473A and D473E mutant genes (40). The mutant rbcL genes were PCR-amplified and completely sequenced in order to confirm that only the intended mutations were present (30, 36, 39). The mutant *Chlamydomonas* strains were named D473A and D473E.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions—** *C. reinhardtii* 2137 mt− is the wild-type strain (32). Mutant MX3312, which lacks the rbcL gene that encodes the Rubisco large subunit, was obtained from Dr. Genhai Zhu (Maxygen, Inc., Redwood City, CA) and used as the host for chloroplast transformation. This mutant was created via chloroplast transformation. The resultant mutant plasmids, named pLS-D473A and pLS-D473E, were transformed into the chloroplast of rbcL mutant MX3312 by using a helium-driven biostatic device (38), and photosynthesis-competent transformants were selected on minimal medium in the light (80 microeinsteins/m²/s) by standard methods (36, 39). Successive rounds of selection, single-colony isolation, and restriction enzyme analysis were performed to ensure the homoplasmicity of the D473A and D473E mutant genes (40). The mutant rbcL genes were PCR-amplified and completely sequenced in order to confirm that only the intended mutations were present (30, 36, 39). The mutant *Chlamydomonas* strains were named D473A and D473E.

**Gel Electrophoresis and Western Analysis—** Rubisco holoenzyme was purified from cell extract by sucrose gradient centrifugation in 50 mM Bicine (pH 8.0), 10 mM NaH14CO3 (58 Ci/mol), 10 mM MgCl2, and 1 mM dithiothreitol was incubated at each temperature for 20 min. The samples were then cooled on ice and assayed for RuBP carboxylase activity at 25 °C (36, 44). Activities for each enzyme were normalized against the level of activity measured after the 35 °C incubation (wild type, 1.5 pmol/min/mg; mutant D473A, 0.2 pmol/min/mg; mutant D473E, 0.2 pmol/min/mg).

**Enzyme Analysis—** Rubisco holoenzyme was purified from cell extract by sucrose gradient centrifugation in 50 mM Bicine (pH 8.0), 10 mM NaHCO3, 10 mM MgCl2, and 1 mM dithiothreitol (40). The thermal stability of purified Rubisco was determined as described previously (36, 44).

RubP carboxylase and oxygenase activities were measured via the incorporation of acid-stable 14C from NaH14CO3 (45). The ratio of carboxylation to oxygenation at any given concentrations of CO2 and O2 is determined by the CO2/O2 specificity factor $V_c/K_c/V_o/K_o$, where $V_c$ and $V_o$ are the $V_{max}$ values for carboxylation and oxygenation, and $K_c$ and $K_o$ are the $K_{m}$ values for CO2 and O2 (46). Thus, $V_{max}$ of purified and activated Rubisco (20 μg/reaction) was determined by assaying carboxylase and oxygenase activities simultaneously in 25 μl [1-14H]RuBP (15.8 Ci/mol) and 2 mM NaH14CO3 (0.5 Ci/mol) in 30-min reactions at 25 °C (47, 48). [1-14H]RuBP and phosphoglycerate phosphatase were synthesized/purified by standard methods (47, 49). $K_{cat}$ (RubBP) was determined by adding 1–5 μg of activated Rubisco to 1-m1 reaction mix-
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| Kinetic properties of Rubisco purified from wild type and large-subunit mutants D473A and D473E |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Enzymes                         | $V^a_c$ (V/K/Kc) | $V^a_c$ (V/K/Kc) | $K_v^a$ | $K_v^a$(RuBP)$^a$ | $V/K^b$ | $K_v/K^b$ | $V/N^b$ (V/K/Kc) |
| Wild type                      | 64 ± 2          | 113 ± 6         | 36 ± 2 | 520 ± 52        | 16 ± 8 | 3.1    | 14       |
| D473A                          | 53 ± 2          | 38 ± 7          | 90 ± 3 | 2700 ± 220      | 40 ± 11| 0.4    | 30       |
| D473E                          | 55 ± 1          | 36 ± 6          | 101 ± 14| 2657 ± 138     | 35 ± 7 | 0.4    | 26       |

$^a$ Values are the means ± S.D. (n = 1) of three separate enzyme preparations.

RESULTS

Directed Mutagenesis, Transformation, and Mutant Phenotypes—To test the functional significance of Asp-473, directed mutagenesis was used to eliminate its charged side group by replacement with Ala or to alter its size without affecting charge by replacement with Glu. When rbcL mutant plasmids pLS-D473A and pLS-D473E were transformed into the chloroplast of the rbcL deletion mutant MX3312, photosynthesis-competent transformants were recovered on minimal medium in the light at frequencies similar to that of transformation with the wild-type rbcL gene (36, 39). Furthermore, phenotypic analysis of a number of independent transformants via spot tests (32) at 25 or 35 °C on minimal medium or acetate medium (in the light or in the darkness) revealed no obvious differences in growth relative to that of wild type. Such analysis has previously proved useful for identifying rbcL mutants with various defects in Rubisco catalysis, assembly, or stability (reviewed in Ref. 29). Thus, despite the critical role proposed for Asp-473 in Rubisco catalysis, substitution with Ala or Glu caused no major alterations in holoenzyme function or stability in vivo.

Holozyme Levels and Thermal Stability in Vitro—When extracts of mutants D473A and D473E were fractionated on sucrose gradients, no significant differences in the amount of Rubisco holoenzyme were observed relative to that of wild type (data not shown). SDS-polyacrylamide gel electrophoresis and Western analysis also showed that the mutants have wild-type levels of Rubisco subunits (Fig. 2). To further confirm that the D473A and D473E mutants do not affect the structural stability of Rubisco, thermal inactivation experiments were performed (36, 44). As shown in Fig. 3, the D473A and D473E mutant enzymes are not different from wild-type Rubisco with respect to thermal stability in vitro. Thus, removing the Asp-473 side group or increasing its size has no obvious effect on Rubisco holoenzyme stability in vivo or in vitro.

Catalytic Properties—The purified D473A and D473E mutant enzymes were found to have 17 and 14% decreases in $V/K_v$, respectively (Table I). Despite ~2-fold beneficial increases in $K_v/K$, the 52–59% decreases in $V/K_v$ and 87% decreases in carboxylation catalytic efficiency ($V/K_v$) are ultimately responsible for the decreased $\Delta \Omega$ values (Table I). The D473A and D473E mutant enzymes also have decreases in the binding affinity for CABB (Table II), which is anticipated from the decreased carboxylation $V/K_v$ and $\Omega$ values, the latter of which is indicative of a change in the relative stabilities of the carboxylation and oxygenation transition states (27, 28). However, $K_v$(RuBP) is increased ~2-fold for the mutant enzymes (Table I). This indicates that the conversion of RuBP to the 2,3-enediol(ate), a step common to both carboxylation and oxygenation, is also altered by the mutant substitutions.

DISCUSSION

Rubisco catalyzes the rate-determining step of photosynthesis, but it has a carboxylation $k_{cat}$ of only a few per second and is competitively inhibited by $O_2$. Oxygenation of RuBP generates phosphoglycolate, which is the first intermediate in the nonessential photosynthetic pathway that leads to the loss of $CO_2$. These properties make Rubisco an obvious target for genetic engineering aimed at improving agricultural productivity (reviewed in Refs. 2 and 3). However, despite the variation in the $V_v$ and $\Omega$ values of Rubisco enzymes from divergent species (52), Rubisco catalysis depends on a conserved set of active site residues (reviewed in Refs. 2, 4, 12). Thus, attempts to design a better Rubisco will likely depend on our depth of understanding of the structure-function relationships of the enzyme some distance from the active site. With 16 subunits, ~4,800 amino acids, and ~76,000 atoms arranged in strikingly similar x-ray crystal structures (reviewed in Ref. 4), it is a daunting task to identify regions of structure far from the active site that may influence catalysis. Only by genetic screening and selection in vivo (29), random or scanning mutagenesis (53–55), bioinformatic analysis of sequence divergence (56), and/or detailed comparative analysis of divergent x-ray crystal structures (18) may it be possible to identify regions worthy of experimental analysis. Based on an extensive comparison of Rubisco crystal structures, Duff et al. (18) proposed that Asp-473 in the carboxyl-terminal region of the large subunit may serve as a critical latch residue that accounts for the folding of the carboxyl terminus over loop 6 during catalysis. Because the large subunit carboxyl terminus is one of the most variable regions of Rubisco structure with respect to both length and sequence identity (4, 26) (Fig. 1), Asp-473 might serve as a critical connection by which the variation in the carboxyl terminus could influence loop 6 catalysis.

Although we had intended to exploit chloroplast transforma-
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Fig. 4. Stereo image of the relationship between the large subunit carboxyl terminus (residues 466–475, blue) and the active site loop 6 (residues 328–342, yellow) in the x-ray crystal structure of Chlamydomonas Rubisco (Protein Data Bank code 1GKS) (9). The carboxyl-terminal residue Asp-473 (red) interacts with several loop 6 residues (Glu-336, Glu-338, Val-341) but is also in contact with other large subunit residues (green) that are at the interface between the carboxyl terminus and loop 6 (Arg-134, Asp-302, Arg-303, His-310). In a previous study with Synechococcus Rubisco (57), substitutions at Lys-128 (orange) caused decreases in CO₂/O₂ specificity. Lys-128 is in the amino-terminal domain of a neighboring large subunit and forms hydrogen bonds with residues in both the carboxyl terminus (Phe-467) and loop 6 (Val-331) (58). Lys-334 is the active site residue that interacts with the C-2 carboxyl group of the transition state analog CABP (black).

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Substitutions at the Asp-473 Latch Residue of *Chlamydomonas* Ribulosebisphosphate Carboxylase/Oxygenase Cause Decreases in Carboxylation Efficiency and CO$_2$/O$_2$ Specificity

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