Deletion of nine carboxy-terminal residues of the Rubisco small subunit decreases thermal stability but does not eliminate function

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Abstract A recent X-ray crystal structure of ribulose-1,5-bisphosphate carboxylase/oxygenase from the green alga *Chlamydomonas reinhardtii* lacks 13 carboxy-terminal residues of the small subunit. To determine the importance of this divergent region, a non-sense mutation was created that removes nine residues. This engineered gene was transformed into a *Chlamydomonas* strain that lacks the small-subunit gene family. The resulting holoenzyme has a normal CO_2/O_2 specificity but decreased carboxylation V_{max}. Whereas wild-type enzyme retained most of its carboxylase activity after a 10-min incubation at 55°C, the mutant enzyme was inactivated. Thus, although disordered or divergent, the carboxy terminus is required for maximal activity and stability.

Key words: Chloroplast enzyme; Genetic engineering; Photosynthesis; Protein stability; Ribulose-1,5-bisphosphate carboxylase/oxygenase; *Chlamydomonas reinhardtii*

1. Introduction

The chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) controls the productivity of plants by catalyzing the initial reactions of both photosynthetic CO_2 fixation and photorespiration (reviewed in [1]). Because CO_2 and O_2 are mutually competitive for ribulose-1,5-bisphosphate (RuBP), an increase in the catalytic efficiency (V_{max}/K_m) for carboxylation (V_c/K_c) or decrease in the catalytic efficiency for oxygenation (V_o/K_o) would lead to an increase in net CO_2 fixation. Whereas the CO_2/O_2 specificity (Ω) of Rubisco is defined as $\Omega = V_cK_o/V_oK_c$, net CO_2 fixation equals the difference between the velocities of carboxylation and oxygenation [2]. The possibility for engineering Rubisco to increase the production of food or renewable energy is supported by the observation that Ω and the carboxylation and oxygenation kinetic constants vary between species [3].

In land plants and green algae, the Rubisco holoenzyme is comprised of eight, 55-kDa large subunits (coded by the polyploid chloroplast *rbcL* gene) and eight, 15-kDa small subunits (coded by a family of *rbcS* nuclear genes) (reviewed in [1]). It has been difficult to engineer the Rubisco enzyme of land plants because chloroplast transformation of the *rbcL* gene is established only for tobacco [4], and it has not been possible to eliminate the family of *rbcS* genes in the nucleus [5]. Furthermore, land plants require photosynthesis for survival. In contrast, the green alga *Chlamydomonas reinhardtii* has served as a good genetic model for eukaryotic Rubisco (reviewed in [6]). *Chlamydomonas* can survive in the absence of Rubisco when provided with acetate as an alternative source of carbon, its chloroplast is easy to transform, and a mutant has been recovered that lacks both members of the *rbcS* gene family [7]. The X-ray crystal structure of *Chlamydomonas* Rubisco has also been solved recently to the highest resolution of any Rubisco enzyme [8].

Although the large subunit contains the active site, small subunits are more divergent than large subunits. This observation supports the notion that small subunits may contribute to the differences in kinetic properties of Rubisco enzymes from different species (reviewed in [1]). In fact, genetic selection and Ala-scanning mutagenesis have revealed that specific amino-acid substitutions in the loop between β strands A and B of the *Chlamydomonas* small subunit can influence Ω and carboxylation catalytic efficiency [9,10]. This βA–βB loop is one of very few differences among the α-carbon backbones of Rubisco enzymes [8]. It contains 27 residues in *Chlamydomonas*, but only 22 residues in land plants or 12 residues in prokaryotes and non-green algae (reviewed in [1]).

A second region of substantial structural divergence involves the small-subunit carboxy terminus. The *Chlamydomonas* small subunit is 11 residues longer than the small subunits of many land plants (e.g. spinach) but 22 residues shorter than the small subunits of non-green algae (e.g. *Galdieria partita*) and some prokaryotes (e.g. *Alcaligenes eutrophus*) (Fig. 1). The very long carboxy-terminal extensions of these latter organisms form β loops that may compensate for the relatively small number of residues that comprise their βA–βB loops [14,15]. However, some prokaryotes have small βA–βB loops and short carboxy termini [13] whereas green algae, like *Chlamydomonas*, have large βA–βB loops and longer carboxy termini. In one X-ray crystal structure of *Chlamydomonas* Rubisco [8], 13 residues deduced from *rbcS* gene sequences are absent from the small-subunit carboxy terminus [8]. In a sec-

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Abbreviations: K_c, K_m CO_2; K_o, K_m O_2; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate; V_c, V_{max} of carboxylation; V_o, V_{max} of oxygenation; Ω, CO_2/O_2 specificity factor

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ond, more-recent crystal structure [16], these residues are present but located on the surface of the small subunit. This raises questions of whether the carboxy-terminal residues are labile or dynamic, and what role they may play in the structure and function of the holoenzyme.

To determine the significance of the longer carboxy terminus of the *Chlamydomonas* small subunit, a mutant was constructed by directed mutagenesis and nuclear transformation in which the deduced carboxy terminus would be shortened by nine residues. The mutant cells can survive photosynthetically, but the mutant Rubisco enzyme has a substantial decrease in holoenzyme thermal stability in vitro.

2. Materials and methods

2.1. Strains and culture conditions

C. reinhardtii 2137 mt^+ was the wild-type strain [17]. Mutant *rbcS*Δ mt^- was used as the host for nuclear transformation [7]. It lacks photosynthesis and requires acetate for growth due to deletion of the 13-kb locus that contains the two *rbcS* genes (*rbcS1* and *rbcS2*) [18]. All *Chlamydomonas* strains are maintained at 25°C in darkness on medium containing 10 mM acetate and 1.5% Bacto-agar [17]. For biochemical analysis, cells were grown in 250–500 ml of liquid acetate medium at 25°C on a rotary shaker at 120 rpm in darkness.

2.2. Directed mutagenesis and transformation

Plasmid pSS1 [7], which contains the entire *Chlamydomonas rbcS1* gene [18], was used for directed mutagenesis and transformation. Mutagenesis was performed with synthetic oligonucleotides and a kit from Pharmacia [19]. To create the carboxy-terminal deletion, the codon for Phe-132 (TTC) was changed to a non-sense codon (TAA). The plasmid (pSS1-F132UAA) was cloned and amplified in *Escherichia coli* XL1-Blue (Stratagene). Purified plasmid DNA was transformed into *Chlamydomonas* strain *rbcS*Δ by the glass-bead vortexing method [7,20]. Transformed cells were recovered by selecting for photosynthetic competence on minimal medium (without acetate) in the light (80 μmol photons m^{-2} s^{-1}) at 25°C [7]. DNA was isolated from one of the transformants [21], and the *rbcS1*-F132UAA gene was PCR amplified [7] and sequenced completely by the University of Nebraska DNA Sequencing Facility to ensure that only the correct mutation was present. This mutant strain was named F132UAA.

2.3. Biochemical analysis

About 1×10^9 cells were harvested by centrifugation, resuspended in 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine (pH 8.0), 10 mM $NaHCO_3$, 10 mM $MgCl_2$, and 1 mM dithiothreitol, and sonicated at 0°C for 3 min. Total soluble protein was quantified [22] and subjected to SDS–polyacrylamide gel electrophoresis [23]. After transfer to nitrocellulose membrane, the proteins were stained with Ponceau S to further confirm equal loading of proteins. The membrane was then probed with anti-large-subunit or anti-small-subunit Rubisco immunoglobulin G [24]. Antibodies were detected with goat anti-rabbit immunoglobulin G/alkaline phosphatase conjugate, and visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Rubisco holoenzyme was purified from the cell extracts by sucrose-gradient centrifugation [25]. The carboxylation and oxygenation kinetic constants of purified and activated enzyme were determined by measuring the incorporation of acid-stable ^{14}C from $NaH^{14}CO_3$ [26]. Ω was determined by assaying carboxylase and oxygenase activities simultaneously with 14.6 μM [1-^{3}H]RuBP (7.3 Ci mol^{-1}) and 2 mM $NaH^{14}CO_3$ (0.5 Ci mol^{-1}) in 30-min reactions at 25°C [27,28]. The [1-^{3}H]RuBP and phosphoglycolate phosphatase used in the assays were synthesized and purified according to standard methods [27,29].

Rubisco thermal stability was assayed by incubating purified and activated enzyme (approximately 5 μg) in 0.5 ml of 50 mM *N*,*N*-bis (2-hydroxyethyl)glycine (pH 8.0), 10 mM $NaH^{14}CO_3$ (2 Ci mol^{-1}), and 10 mM $MgCl_2$ at various temperatures for 10 min [10,30]. The samples were then cooled on ice for 5 min, and carboxylase activity was initiated at 25°C in air by adding 20 μl of 10 mM RuBP. After 1 min, the reactions were stopped with 0.5 ml of 3 M formic acid in methanol. The samples were dried, and incorporation of ^{14}C was measured by liquid scintillation spectrometry.

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                  120       130       140
                   .         .         .         .         .
Chlamydomonas     GFLVQRPKTARDFQPANKRSV
Spinach           SFIAYKPAGY
Tobacco           SFIAYKPEGY
Synechococcus     SFIVHRPGR
Alcaligenes       SFIVNRPADEPGFRLVRQEEPGRTLRYSIESYAVQARPEGSRY
Galdieria         SFIVNRPKHEPGFNLMRQEDKSRSIKYTIHSYESY-KPEDERY
                   .         .         .         .         .
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Fig. 1. Divergent sequences of the small-subunit carboxy terminus are observed in Rubisco enzymes for which the X-ray crystal structures have been solved [8,11–16]. Residues are numbered according to the mature small-subunit 1 of *Chlamydomonas*. Residues past Lys-127 are not visible in the 1.4-Å *Chlamydomonas* structure [8].

3. Results and discussion

Directed mutagenesis was used so as to create a UAA non-sense codon in place of the codon for small-subunit Phe-132 (Fig. 1). Rather than shortening the *Chlamydomonas* small-subunit carboxy terminus to the same length as the spinach small subunit, two charged residues were retained (Arg-130 and Asp-131). Because the *Chlamydomonas* crystal structure available at the time of this study lacks electron density for residues after Lys-127 [8], we could only assume that Arg-130 and Asp-131 may be important either for interacting with solvent or for forming ionic interactions in a hydrophobic environment. Their removal might have prevented the recovery of a stable holoenzyme and precluded functional studies of the small-subunit carboxy terminus.

When the *rbcS1*-F132UAA mutant gene was transformed into the photosynthesis-deficient *rbcS*Δ host strain, photosynthesis-competent colonies were recovered at a frequency of 2.5×10^{-8} cells, indicating that the carboxy-terminal residues are not essential for Rubisco function. However, because this frequency is more than 100 times lower than that obtained with the wild-type *rbcS1* gene [7], it seemed likely that the carboxy-terminal deletion was deleterious for holoenzyme expression, function, or structure. This was further supported by phenotypic comparisons [17]. The mutant strain was indistinguishable from wild-type *rbcS1* transformants when growth was compared on minimal medium at 25°C and acetate medium in darkness at 25 or 35°C, but it grew much more slowly

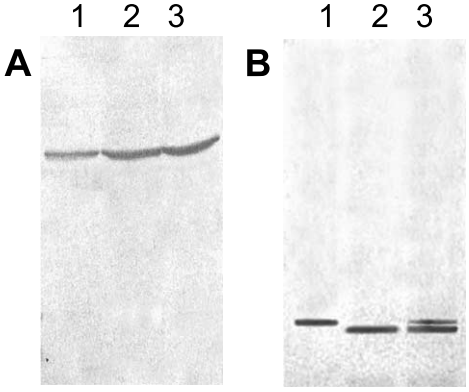

Fig. 2. SDS–polyacrylamide gel electrophoresis and immunoblotting indicates that the F132UAA mutant has a reduced molecular mass for the Rubisco small subunit. Total soluble proteins extracted from cells grown in darkness at 25°C were fractionated by gel electrophoresis (25 μg $lane^{-1}$), transferred to nitrocellulose, and detected with either Rubisco large-subunit (A) or small-subunit (B) antibodies. Protein samples were from wild-type (lane 1), mutant F132UAA (lane 2), or a mixture of wild-type and mutant-F132UAA extracts (lane 3).

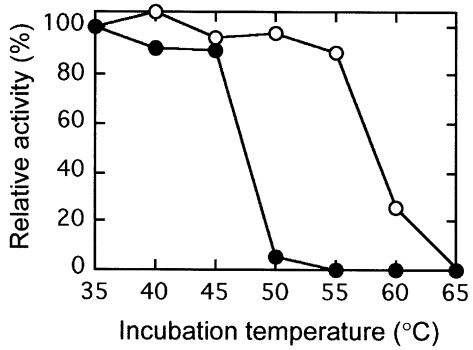

Fig. 3. Rubisco purified from small-subunit mutant F132UAA is thermally unstable in vitro. Rubisco enzymes from wild type (○) or mutant F132UAA (●) were incubated at each temperature for 10 min. The samples were then cooled on ice, and RuBP carboxylase activity was assayed at 25°C. Activities were normalized against activity measured after the 35°C incubation (wild-type, 96 μmol h^{-1} mg^{-1}; mutant F132UAA, 66 μmol h^{-1} mg^{-1}). In three separate experiments, the wild-type enzyme retained 89% or more activity than that retained by the mutant enzyme after the 50°C incubation.

on minimal medium in the light at 35°C. This phenotype was observed for the several F132UAA mutant strains recovered independently by transformation.

When total soluble cell proteins were fractionated by SDS–polyacrylamide gel electrophoresis and probed with small-subunit antibodies, the small subunit of mutant F132UAA was found to have a decreased molecular mass relative to that of the wild-type small subunit (Fig. 2). This result confirms that the engineered non-sense mutation leads to early termination of small-subunit synthesis. It also indicates that native small subunits do not lack these carboxy-terminal residues as a result of some hypothetical cellular mechanism or in vitro accident of proteolysis. Thus, it is most likely that the residues after small-subunit Lys-127 are absent from one X-ray crystal structure of *Chlamydomonas* Rubisco [8] but present in another [16] because they can become disordered and lack sufficient electron density for detection.

As noted above, preliminary observations indicated that the F132UAA mutant strain might have less Rubisco holoenzyme or activity when grown at 35°C. Because nuclear transformation of *Chlamydomonas* occurs via non-homologous recombination [7,20], it is difficult to assess small differences in expression or protein stability by examining in vivo levels of Rubisco holoenzyme [10]. One assumes that different recombination events and locations in the genome may influence the level of transcription of a transformed gene. Therefore, thermal stability experiments were performed with purified enzyme in vitro to see whether the removal of the small-subunit carboxy-terminal residues might affect holoenzyme stability (Fig. 3). Although the F132UAA-mutant and wild-type enzymes were not affected by 'growth' temperatures [31] up to 35°C, the mutant enzyme was substantially inactivated at temperatures above 45°C. After a 10-min incubation at 50°C, the mutant enzyme retained only 5% of its initial carboxylase activity (Fig. 3). In contrast, wild-type Rubisco showed no appreciable decline in activity after an incubation as high as 55°C, and retained more than 20% of its initial activity after a 10-min incubation at 60°C (Fig. 3). Thus, the carboxy-terminal residues may play a role in holoenzyme stability. Because the thermal instability of the F132UAA-mutant enzyme is observed only at temperatures far exceeding those that *Chlamydomonas* might encounter in nature, the contribution of these residues to stability appears be rather subtle. However, minor changes in protein folding or stability might be of greater significance in vivo when Rubisco is susceptible to limited proteolysis and may require specific structural features for the process of holoenzyme assembly. Longer small-subunit carboxy-terminal extensions are likely to be of even greater importance for Rubisco stability in thermophilic prokaryotes and algae.

Table 1
Kinetic parameters of Rubisco purified from wild-type and small-subunit mutant F132UAA

Kinetic constant[a]	Wild-type	Mutant F132UAA
$\Omega = V_c K_o / V_o K_c$	61 ± 2	60 ± 2
V_c (μmol h^{-1} mg^{-1})	154 ± 12	51 ± 8
K_c (μM CO_2)	30 ± 7	29 ± 4
K_o (μM O_2)	469 ± 8	470 ± 25
V_c/K_c (μmol h^{-1} mg^{-1} μM^{-1})[b]	5 ± 1	2 ± 1

[a]Values are from three separate enzyme preparations ± standard deviation ($n-1$).
[b]Calculated value.

To determine whether the carboxy-terminal residues might play a role in catalytic efficiency or CO_2/O_2 specificity, wild-type and mutant-F132UAA Rubisco enzymes were purified and compared (Table 1). Except for a decrease in V_c, the kinetic constants of the mutant enzyme were not different from those of the wild-type enzyme. Because Ω was also not affected, the decrease in V_c must be accompanied by a proportional decrease in V_o. These decreases may simply reflect a structural instability of the mutant-F132UAA holoenzyme (Fig. 3). Thus, although *Chlamydomonas* Rubisco has a lower Ω value than that characteristic of land plants ($\Omega \approx 80$), differences in the small-subunit carboxy terminus are unlikely to account for differences in Ω [3].

4. Conclusions

Removal of the divergent carboxy-terminal residues of the *Chlamydomonas* small subunit does not substantially affect Rubisco catalytic efficiency. Instead, these residues may be most important for their contribution to holoenzyme structural stability. In recent X-ray crystal structures of *Chlamydomonas* Rubisco [8,16], the carboxy-terminal residues were found to be either disordered or packed against amino-terminal residues of the same small subunit on the surface of the enzyme. In contrast, the very long carboxy-terminal extensions characteristic of non-green algae and some prokaryotes interact with small-subunit βA–βB loops at the interface between large and small subunits [14,15]. It may also be possible to examine the influence of these quite different structures on the function of Rubisco by exploiting *rbcS* transformation and genetic selection in *Chlamydomonas*.

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