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Structural and Functional Consequences of the Substitution of Proximal Residues Cys-172 and Cys-192 in the Large Subunit of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase from *Chlamydomonas reinhardtii*[†]

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[†] Coordinates deposited to the Protein Data Bank with the codes 2vdh (C172S) and 2vdi (C192S).

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Short Title: Mutations of proximal cysteines 172 and 192 of *Chlamydomonas* Rubisco

¹ Abbreviations: Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 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 ; Ω , CO_2/O_2 specificity factor; 2CABP, 2-carboxyarabinitol 1,5-bisphosphate; NCS, non-crystallographic symmetry; r.m.s.d., root-mean-square deviation.

Key words: *Chlamydomonas reinhardtii*; chloroplast mutants; Rubisco; specificity factor; proximal cysteines; X-ray crystallography.

SYNOPSIS: Proximal cysteines 172 and 192 in the large subunit of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) are evolutionarily conserved among cyanobacteria, algae, and higher plants. Mutation of Cys-172 has been shown to affect the redox properties of Rubisco *in vitro* and to delay the degradation of the enzyme *in vivo* under stress conditions. Here we report the effect of the substitution of Cys-172 and Cys-192 by serine on the catalytic properties, thermostability, and three-dimensional structure of *Chlamydomonas reinhardtii* Rubisco. The most striking effect of the C172S substitution was an 11% increase in the specificity factor compared to the wild-type enzyme. The specificity factor of C192S Rubisco was not altered. The V_{\max} for carboxylation was similar to that of wild type Rubisco in the case of the C172S enzyme, but about 30% lower for the C192S Rubisco. In contrast, the K_m for CO_2 and O_2 were similar for C192S and wild-type enzymes, but distinctly higher (about double) for the C172S enzyme. C172S Rubisco showed a critical denaturation temperature about 2 °C lower than wild-type Rubisco and a distinctly higher denaturation rate at 55 °C, whereas C192S Rubisco was only slightly more sensitive to temperature denaturation than the wild-type enzyme. X-ray crystal structures reveal that the C172S mutation causes a shift of the main chain backbone atoms of β -strand 1 of the α/β -barrel affecting a number of amino acid side-chains. This may cause the exceptional catalytic features of C172S. In contrast, the C192S mutation does not produce similar structural perturbations.

INTRODUCTION

The bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco¹, EC 4.1.1.39) operates at a metabolic branch point channelling carbon either to the photosynthetic carbon fixation (Calvin-Benson) cycle or to the photorespiratory pathway [1]. The enzyme converts ribulose 1,5-bisphosphate (RuBP) to a reactive enediolate form, which is prone to attack by either CO₂ or O₂. The resulting carboxylated or oxygenated intermediate is hydrolyzed in two further steps (hydration and C₂-C₃ bond cleavage) producing two molecules of 3-phosphoglycerate or one each of 3-phosphoglycerate and 2-phosphoglycolate, respectively, as final products [2]. Because the carboxylation and oxygenation reactions share the same enediol substrate, the oxygenation reaction may be an unavoidable consequence of the catalytic mechanism of the enzyme [3,4], which evolved under anoxic conditions. The intrinsic preference of Rubisco for one or the other reaction is measured by the so called specificity factor (Ω), which is defined as the ratio of the V_{\max} for carboxylation (V_c) to the K_m for CO₂ (K_c), divided by the ratio of the V_{\max} for oxygenation (V_o) to the K_m for O₂ (K_o) [5]. The value of Ω ($V_c K_o / V_o K_c$) is known to vary among Rubisco enzymes from different species [6]. Because of the relevance to plant growth of the photosynthesis/photorespiration ratio, the beneficial manipulation of crop-plant Ω has been an unfulfilled biotechnological goal.

The crystal structures of the active enzyme has been solved for a number of species including prokaryotes, algae, and land plants [7]. The most common form of Rubisco (type I present in cyanobacteria, green algae, and higher plants) is composed of eight chloroplast-encoded large subunits (ca 55 kDa) and eight nuclear-encoded small subunits (ca 15 kDa). Two large subunits assemble into functional dimers with two active sites at the dimer interface. Catalytic competence requires carbamylation by CO₂ of the ϵ -amino group of a specific lysine residue in the active site, and subsequent binding of a Mg²⁺ ion to the carbamyl group [8]. Four small subunits cap the top and bottom of four pairs of the large subunits to form the hexadecameric holoenzyme. The knowledge of Rubisco structure has fostered an understanding of central features of the catalytic mechanism and other properties of Rubisco, but the structural determinants of the variations in Ω have remained elusive.

Analysis of Rubisco mutants has been helpful in evaluating the functional role of the substituted residues or deleted sequences [1,9-12]. Many of these studies have been carried out in *Chlamydomonas reinhardtii*, a unicellular green alga that allows for straightforward genetic manipulation of both the chloroplastic and nuclear genomes. Because of their singular redox properties, Cys residues are potential candidates for harboring regulatory effects and, therefore, may be considered as primary targets for site-directed mutagenesis. In fact, it is known that the specific oxidative modification of Cys residues by disulfide exchange causes inactivation and increases proteolytic susceptibility of the purified Rubisco *in vitro* [13-15]. These findings suggest a physiological role of Cys oxidation in Rubisco catabolism. Whereas the critical Cys residues have not been unambiguously identified, the conserved Cys-172 residue from the Rubisco large subunit has attracted some attention [16,17]. Furthermore, specific modification of this Cys residue by an affinity label has been shown to abolish enzymatic activity [18], suggesting a yet undefined role for this residue in catalytic performance. Substitution of Cys-172 by Ser in *C. reinhardtii* yields an active enzyme but eliminates arsenite sensitivity, shifts the critical redox potential that controls proteolytic susceptibility *in vitro*, and increases resistance to stress-induced degradation *in vivo* [16]. A slower catabolism of Rubisco under stress has been also reported after substitution of the same residue in a prokaryotic Rubisco [17], whereas the replacement of the adjacent Cys-192 or other conserved Cys residues (Cys-247) did not delay the stress-induced degradation [17].

Analysis of the crystal structures of Rubisco enzymes from different sources reveals that Cys-172 and Cys-192 are close to each other, but, in most cases, not close enough to form a disulfide bond [7]. In one of the structures of the non-carbamylated enzyme from tobacco [19], a disulfide bond is observed, whereas a second structure, obtained with similar conditions, does not feature the 172-192 disulfide bond [20]. Structures of activated (carbamylated) Rubisco [21-23] show residues 172 and 192 as free Cys residues. In any case, the proximity of the Cys residues is likely to support redox interactions that may have structural, catalytic, and/or regulatory relevance. In an attempt to define the structural and functional roles of these proximal Cys residues, they have been replaced by site-directed mutagenesis and chloroplast transformation of *C. reinhardtii*. The C172S and C192S mutant

Rubisco enzymes have been analyzed in detail with respect to catalytic function and their X-ray crystal structures have been solved.

EXPERIMENTAL

Strains and cell cultures

Chlamydomonas reinhardtii strain C172S, with a Cys-172 to Ser substitution in the Rubisco large subunit, has been already described [16]. Strain C192S was created by site-directed mutagenesis and chloroplast transformation as described below. Strain 2137 mt+ is the wild type [24]. Non-photosynthetic strain 18-7G, which has a nonsense mutation in the *rbcL* large-subunit gene, was used as the host for transformation [25]. All strains were kept on standard solid medium supplemented with acetate [24] at 25 °C in the dark. For Rubisco purification, 1.5 liter flasks of standard liquid culture medium supplemented with acetate were inoculated with the appropriate strains and the cells were grown at 28 °C in the light (3 x 30W white light tubes at 30 cm, supplying about $10 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) with moderate (120 rpm) shaking up to a density of 10^7 cells/ml. The cells were collected by centrifugation ($3000 \times g$ for 5 min) and the pellet was frozen in liquid nitrogen and stored at -80 °C.

Directed mutagenesis and chloroplast transformation

Plasmid *prbcLP* containing a HpaI fragment (bases -743 to +1927), which encompasses the entire *rbcL* coding region (bases +1 to +1428), cloned into the SmaI-site of a pBluescript SK- (Stratagene) vector was used as a source of the *C. reinhardtii* Rubisco large subunit chloroplast gene. Site-directed mutagenesis of Cys-192 to Ser was achieved by changing TGT (Cys192) to AGC, using the QuickChange kit of Stratagene. This substitution created a new HindIII restriction site (between bases +573 and +574) that expedited the screening of transformants. The mutated plasmid (called pLS-C192S) was used for chloroplast transformation via projectile bombardment of *C. reinhardtii* strain 18-7G according to published procedures [16,26]. Transformation of the non-photosynthetic 18-7G mutant with pLS-C192S yielded photosynthetically competent colonies on minimal medium. The transformants became homoplasmic (as checked by HindIII restriction analysis of

polymerase chain reaction amplified *rbcl*) after successive rounds of single-colony isolation, replating in selective minimal medium, and growth under light. Finally, the entire coding region of *rbcl* was sequenced to confirm that only the intended mutation was present. The strain thus created was named C192S.

Purification of Rubisco

Rubisco was extracted and purified by 35-60% (w/v) ammonium sulfate precipitation, sucrose gradient centrifugation, and anion exchange chromatography according to a published procedure [14]. Alternatively, for the determination of kinetic constants, Rubisco was purified following a somewhat different procedure described elsewhere [27].

Rubisco activity assays

Rubisco carboxylase activity was routinely determined using a ^{14}C -fixation method previously described [14]. Kinetic constants were measured according to a reported procedure [28], and Ω was independently determined using the dual-labelling method [29].

Crystallization and Structure Determination

Crystals of *C. reinhardtii* Rubisco-mutant enzymes were grown using hanging drop vapor diffusion at 20 °C. The drops were prepared by mixing a solution containing 10 mg/ml Rubisco (in 50 mM HEPES pH 7.5) and 1 mM 2-CABP with an equal volume of a well solution containing 50 mM HEPES (pH 7.5), 0.05 - 0.2 M NaCl, 7-12% (w/v) PEG 4000, 10 mM NaHCO_3 , 5 mM MgCl_2 . Crystals grew within one week. Prior to data collection, crystals were transferred to a solution containing the well solution with 30% (v/v) ethylene glycol as a cryoprotectant and frozen in liquid nitrogen. Data were collected from single crystals at 100 K on beam line ID14-3 at ESRF, Grenoble, France (C172S), and on beam line I711 at Max-lab, Lund, Sweden (C192S). The data were processed using DENZO and SCALEPACK [30] to a resolution of 2.30 Å for C172S, and 2.65 Å for C192S (Table S1, see online supplementary data).

The mutant crystal structures were solved by molecular replacement using the program AMORE [31,32]. The search model consisted of one set of large and small subunits of wild-type *C. reinhardtii* Rubisco (PDB code 1GK8). In each case, eight solutions corresponding to eight different orientations of the search unit within one hexadecamer in the asymmetric unit were found.

Refinement was performed using REFMAC v. 5 [33]. For cross-validation, 5% of the data was excluded from the refinement for R_{free} calculations. Initial electron density maps calculated after one round of rigid body refinement showed clear density for the substituted residues 172 and 192, respectively. After building the corresponding residues into density, the mutant enzyme structures were further refined using a maximum likelihood target function and overall B-factor refinement. Tight non-crystallographic symmetry (NCS) restraints were imposed for the eight copies of large and small subunits in the asymmetric unit at the beginning of the refinement. Towards the end of refinement, NCS restraints were relaxed or removed for regions of the structure that clearly deviated from NCS. Solvent molecules were added using ARP/wARP [34]. Throughout the refinement, $2mF_o-DF_c$ and mF_o-DF_c sigma A weighted maps [35] were inspected and the models manually adjusted using O (32). The structures of C172S and C192S were refined to final $R_{\text{cryst}}/R_{\text{free}}$ values of 0.171/0.204 and 0.205/0.234 respectively. Coordinates and structure factors have been deposited at the Protein Data Bank with accession codes 2vdh (for C172S) and 2vdi (for C192S).

RESULTS

Isolation and growth of the C192S mutant

Chloroplast transformation of the *C. reinhardtii* 18-7G mutant lacking Rubisco [25] with the pLS-C192S plasmid, containing the *rbcL* gene with a C192S substitution, yielded transformant colonies that grew on minimal medium in the light. Amplification and sequencing of the *rbcL* gene confirmed that the transformants carried the single C192S mutation. This showed that the C192S mutant Rubisco was catalytically active. The C192S strain grew at a similar rate as the wild type, both in liquid culture and on solid media, under photoautotrophic, photoheterotrophic, or strictly heterotrophic conditions (data not shown). In general, the growth of the

C192S strain presented no particular features when compared to the wild type, except for a somewhat reduced survival of cells in old cultures.

Thermal stability of the mutant enzymes

The C172S Rubisco has been already shown to be thermosensitive [16]. In order to compare the thermal stability of the C192S mutant protein with the C172S and the wild-type enzymes, purified Rubisco from all three strains was incubated at different temperatures (in the 48 to 62 °C range) for 30 min (Figure 1A), and at 55 °C for different times (Figure 1B), and the carboxylase activity was subsequently determined. The C172S enzyme showed a critical denaturation temperature (54 °C) about 2 °C lower than the wild-type (Figure 1A) and a distinctly higher denaturation rate at 55°C (Figure 1B). In contrast, the denaturation temperature of the C192S Rubisco (near 55.5 °C, Figure 1A) was only slightly (about 0.5 °C) lower than the wild-type, and C192S denatured at a similar rate as the wild type at 55 °C (Figure 1B). In a previous report (16), it was suggested that the lower stability of the C172S mutant protein could result from the inability of the mutant to form a Cys-172 to Cys-192 disulfide bond, which might be present in wild-type Rubisco. Because the C192S enzyme is relatively stable, it can now be concluded that the thermosensitivity of the C172S mutant Rubisco is not caused by the absence of a putative Cys-172 to Cys-192 disulfide bridge, but reveals instead a significant structural role played by the Cys-172 residue, which cannot be fully complemented by Ser at the same position. In contrast, the replacement of Cys-192 by Ser seems to have a lesser effect on structural stability.

Catalytic performance of the mutant enzymes

Table 1 displays the mean and standard deviation values of the kinetic constants and Ω values determined for wild-type and mutant Rubisco enzymes. V_c was similar to that of the wild-type enzyme in the case of C172S, but somewhat lower (about 30%) for C192S Rubisco. In contrast, K_c and K_o were similar for C192S and wild-type Rubisco, but distinctly higher (about double) for the C172S mutant enzyme. This indicates that the lower carboxylation specific activity reported for C172S Rubisco previously [16] was likely due to the increased K_c value (Table 1).

Whereas the Ω value of C192S Rubisco was identical to that of wild-type Rubisco ($\Omega = 61$), the C172S mutant enzyme showed a significantly ($P > 0.95$) higher value of 68 (Table 1). With respect to the C192S enzyme, a lower V_c was compensated by a lower K_c resulting in an unchanged Ω value. However, in the case of C172S Rubisco, an even greater increase in K_o relative to an increased K_c is apparently responsible for an 11% increase in Ω (Table 1).

X-ray Crystal Structures of the mutant enzymes

To determine the molecular basis for their altered properties, the purified mutant enzymes were crystallized and their structures were determined. The crystallographic data collection and refinement statistics for the mutant-enzyme crystal structures are summarised in Table S1 (see online supplementary data). The final electron density maps showed density for all 140 residues of the small subunit. The amino-terminus of the large subunit displayed disorder that varied in extent from subunit to subunit. Density was observed for residues 11-475 (from a total of 475 residues) of the large subunit. In several of the subunits, an additional 2-4 amino-terminal residues could be built into density. Clear density was observed for the substituted residues in each case. However, because the substituted residue is isosteric, it cannot be verified by inspection of difference Fourier maps in this case.

In general, no dramatic differences in large-subunit $C\alpha$ backbone atoms were observed among the wild-type, C172S, and C192S structures. A superposition of the wild-type large subunit with the large subunits of C172S and C192S using the algorithms encoded in O [36] gave root-mean-square deviations of 0.198 and 0.200 Å for all $C\alpha$ atoms, respectively. This is close to the coordinate errors of the respective structures. Inspection of the region around residue 172 in C172S Rubisco reveals that the main chain atoms of residues 170-174 are displaced significantly (ca. 0.4 Å) compared to wild-type Rubisco (Figure 2A, B) resulting in a shift of β -strand 1 of the α/β -barrel. This is a larger displacement than the average for this β -strand and also for the average of all strands of the α/β -barrel (mean displacement 0.2 Å). Cys-192 is located in α -helix 6 of the α/β -barrel facing Cys-172. Substitution of Cys-192 with Ser did not cause a similar displacement of the backbone atoms of α -helix 6 or β -strand 6. In the wild-type enzyme, the distance between the sulfur atoms

of residues 172 and 192 is 3.9 Å, which is too long for establishing a disulfide bond. In the C172S and C192S mutant enzymes, the oxygen-to-sulfur distances are even somewhat longer, 4.1 Å. In contrast, the distance between the sulfur atoms of Cys-449 and Cys-459 (2.0 Å) and their conformations in the wild-type enzyme are ideal for disulfide formation [23]. In C172S, the sulfur to sulfur distance between Cys-449 and Cys-459 was similar to that of wild-type Rubisco (2.1 Å). In C192S this distance was lengthened to 2.9 Å (Figure 3), resulting in a shift of the α -helix harboring Cys-449 (residues 438-452), breaking the disulfide bond.

A superposition of the wild-type small subunit with the small subunits of C172S and C192S gave root-mean-square deviations of 0.235 and 0.224 Å for C α atoms 1-126, respectively. This is close to the coordinate errors of the respective structures, indicating that the C172S and C192S substitutions in the large subunit had no noticeable effect on the small-subunit structure.

Alterations in the Active Site

Residue 172 is located in β -strand 1 of the α/β barrel and is close to the active site. The most immediate effect of the C172S substitution is to shift the main-chain atoms of β -strand 1 by 0.4 Å relative to wild-type. This shifted residue 172 by 0.4 Å and resulted in an increase (by 0.2 Å) in the distance between the side chains of residues 172 and 192. It also resulted in a significant increase in the distance (by more than 0.3 Å) between Thr-173 and CABP relative to wild type Rubisco (Figure 2). All other shifts of active-site residues were close to coordinate error.

DISCUSSION

Cys residues 172 and 192 of the large subunit are highly conserved among both eukaryotic and cyanobacterial Rubiscos. Their structural vicinity, potentially allowing for a disulfide bond to form between them, could be a possible source of redox regulatory effects on the enzyme. Analysis of the high-resolution (1.4 Å) structure of the wild-type *C. reinhardtii* enzyme [23] clearly shows the presence of free Cys-172 and Cys-192 residues in the electron density maps. The distance and geometries of the two residues in activated Rubisco are also not compatible with the

geometry required for a disulfide bond. This implies that, in order for a disulfide to form under oxidative conditions, the main chain backbone around the residues would have to move considerably. Nevertheless, disulfide bonding between Cys-172 and Cys-192 appears to be possible if Rubisco becomes decarbamylated, as observed in the unactivated tobacco enzyme [19]. On the other hand, conformational changes are known to be induced in Rubisco by oxidative modification of Cys residues [15]. Therefore, Cys-172 and Cys-192 offer, in principle, a potential for redox modulation of Rubisco through structural changes induced by oxidation.

We have addressed the structural and functional role of the conserved Cys residues by substitution with Ser via site-directed mutagenesis and chloroplast transformation of *C. reinhardtii*. As a result of this single-atom substitution (O for S) the redox sensitivity of the residue is abolished. We have shown that the substitution of Cys-172 with Ser increases Rubisco thermosensitivity (Figure 1), perturbs the structure near the substitution site (Figure 2), and alters the kinetic constants of the enzyme (Table 1). Because the symmetric substitution of Cys-192 does not induce the same changes, it appears that the described effects are not directly related to the presence/absence of a disulfide bond between the proximal Cys residues. Thus, it seems that Cys-172 plays by itself a remarkable role in the structure and catalytic properties of Rubisco.

The substitution of Cys-172 by Ser increases the K_m for both gaseous substrates (Table 1). Because CO_2 and O_2 do not bind to the enzyme before reacting with the enediolate form of RuBP, the increased K_m values might result from a structural change that causes a restricted accessibility of the bound enediolate RuBP to CO_2 and O_2 . However, because the Ω value of the C172S enzyme is also altered (Table 1), it is more likely that conformational changes occur that affect the stabilities of the carboxylation and oxygenation transition states differently [3]. Cys-172 is situated in β -strand 1 of the α/β -barrel, which forms the scaffold for the active site [37]. Thus, replacement of this residue in the barrel may affect active-site residues. Such perturbations, however subtle, may have dramatic consequences for the kinetics of the reaction. Indeed, substitution of either of the flanking residues, Gly-171 or Thr-173, in *C. reinhardtii* Rubisco eliminates carboxylase activity [9]. Our X-ray diffraction analysis of the C172S mutant Rubisco indicates that the substitution to Ser at position 172 causes a significant shift of the main chain

backbone atoms of β -strand 1 of the α/β -barrel. This shift could disturb the geometry of the active site as well as the position of individual residues in the active site. In particular, the interactions of the proximal Thr-173, hydrogen bonding to the C2-hydroxyl of the CABP transition-state analogue [38], and perhaps Lys-175, which is thought to participate as a secondary proton acceptor in generating the reactive enediolate [2], are likely to be strained by the displacement of the β -strand. In contrast, the C192S mutation, which produces relatively minor alterations of enzymatic properties (Table 1), does not involve structural changes around the catalytic site.

Because the effect of C172S on enzyme kinetics appears to be structural and indirect rather than resulting from the elimination of the redox active sulfhydryl group, the particular impairment imposed on the catalytic site by the substitution might be expected to depend on the volume and nature of the substituting residue. Accordingly, the replacement of Cys-172 by Ala in a prokaryotic Rubisco produced only a limited increase of K_c but a significant reduction of V_c [17]. In this regard, the modification of Cys-172 with the bulky N-bromoacetyethanolamine phosphate affinity label is likely to cause a stronger structural distortion resulting in activity loss [18].

Another pair of proximal Cys residues, Cys-449 and Cys-459, are located close to the surface and about 20 Å away from the 172/192 pair. A survey of all available *C. reinhardtii* Rubisco structures to date [23,39-41] revealed that whereas the sulfur to sulfur distance of the 172/192 pair remains fairly constant, the distance of the 449/459 pair varies considerably. This variation is also illustrated by the present structures. In C172S, the sulfur to sulfur distance of the 449/459 pair was similar to that of wild-type, but in C192S this distance was considerably lengthened and there was no sign of a covalent bond in the electron density maps. This variation at a location close to the molecular surface may suggest a role for the 449/459 pair as a redox sensor. It is not clear at this stage if a long-distance structural connection exists between both redox centers or if some of the redox effects associated with the C172S substitution [16] might in fact result from the perturbation of the redox properties of Cys-449 and Cys-459. However, the *in vitro* and *in vivo* redox properties of mutant enzymes in which Cys-449 and Cys-459 have been replaced by Ser are very different from those of C172S [42]. Therefore, a physiologically

relevant effect resulting from the cooperation between the two Cys pairs seems unlikely.

It is noteworthy that the C172S substitution produces a modest but significant increase in Ω (Table 1). Changes in Ω have been proposed to result from differences in the differential stabilization of the carboxylation and oxygenation transition states [3,43]. It has been recently hypothesized by Tcherkez and co-authors [4] that an increase in Ω should result in a decreased V_c , and that a negative correlation between Ω and K_c may also exist [4]. These predictions are, however, not fulfilled in the case of the C172S mutant enzyme, which displays an increased Ω value without alteration of V_c but with a significant increase of K_c (Table 1).

Considering that Cys-172 is a critical residue that renders Rubisco inactive when oxidatively modified by a number of agents [16-18], one may wonder why it is so highly conserved and why it has not been substituted by Ser during evolution for the gain of substrate specificity and oxidative resistance. Moreover, the only apparent disadvantages of the C172S substitution would be a slight structural destabilization that does not seem to be relevant at physiological temperatures (Figure 1) and increases in K_c and K_o (Table 1). Despite the increased Ω value, the increase in K_c would likely cause a decrease in net CO_2 fixation, which is determined by the difference in the velocities of carboxylation and oxygenation at physiological concentrations of CO_2 and O_2 [5]. Nevertheless, the *C. reinhardtii* C172S mutant has been shown to grow at the same rate as the wild type under photoautotrophic conditions [16]. There may be, however, additional reasons for the conservation of Cys-172. Perhaps it is precisely its ability to act as an oxidation-activated switch, as a part of a wider design of redox control of CO_2 fixation and Rubisco turnover, what has prevented the substitution of this residue during evolution.

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Table 1 *Kinetic properties of Rubisco from wild-type C. reinhardtii, C172S and C192S mutants.*

Enzymes	Ω^* ($V_c K_o / V_o K_c$)	V_c^* $\mu\text{mol}\cdot\text{h}^{-1}\cdot\text{mg}^{-1}$	K_c^* μM	K_o^* μM	K_o/K_c
wild type	61 ± 4	130 ± 20	24 ± 3	620 ± 50	26
C172S	68 ± 2	120 ± 30	47 ± 3	1360 ± 130	29
C192S	61 ± 2	92 ± 16	18 ± 2	550 ± 60	31

* Values are means \pm SD (n-1) of three or more separate enzyme preparations.

FIGURE LEGENDS

Figure 1. Thermal inactivation of C172S (●), C192S (□), and wild type (○) Rubiscos. Purified Rubisco (0.2 mg/ml) was incubated at the given temperatures for 30 min (A), or at 55 °C for the indicated times (B). Afterwards, the Rubisco samples were chilled on ice for 5 min, and assayed for RuBP carboxylase activity at 30 °C. Activity values are given as a percentage of the activity at 30 °C (1.29, 1.27 and 1.97 μmol of CO_2 fixed per minute and mg of enzyme, respectively for C172S, C192S and wild type Rubiscos), which was taken as the initial value in B. Error bars indicate \pm standard deviation of independent triplicates. Spline interpolated curves have been included in A.

Figure 2. Displacement of β -strand 1 in the C172S mutant enzyme relative to wild-type Rubisco. (A) $\text{C}\alpha$ trace showing β -strands in the α/β -barrel. (B) Structural differences around residues 172 and 192 and part of the active site in the C172S mutant enzyme relative to wild type. In the wild-type structure, carbon atoms are colored blue, and in C172S carbon atoms are colored yellow. In 2CABP, in the lower right of (B), phosphorus atoms are colored magenta.

Figure 3. Region around residues Cys449 and Cys459 in the C172S and the C192S mutant enzymes. In C172S, carbon atoms are colored yellow. In C192S, carbon atoms are coloured gray.

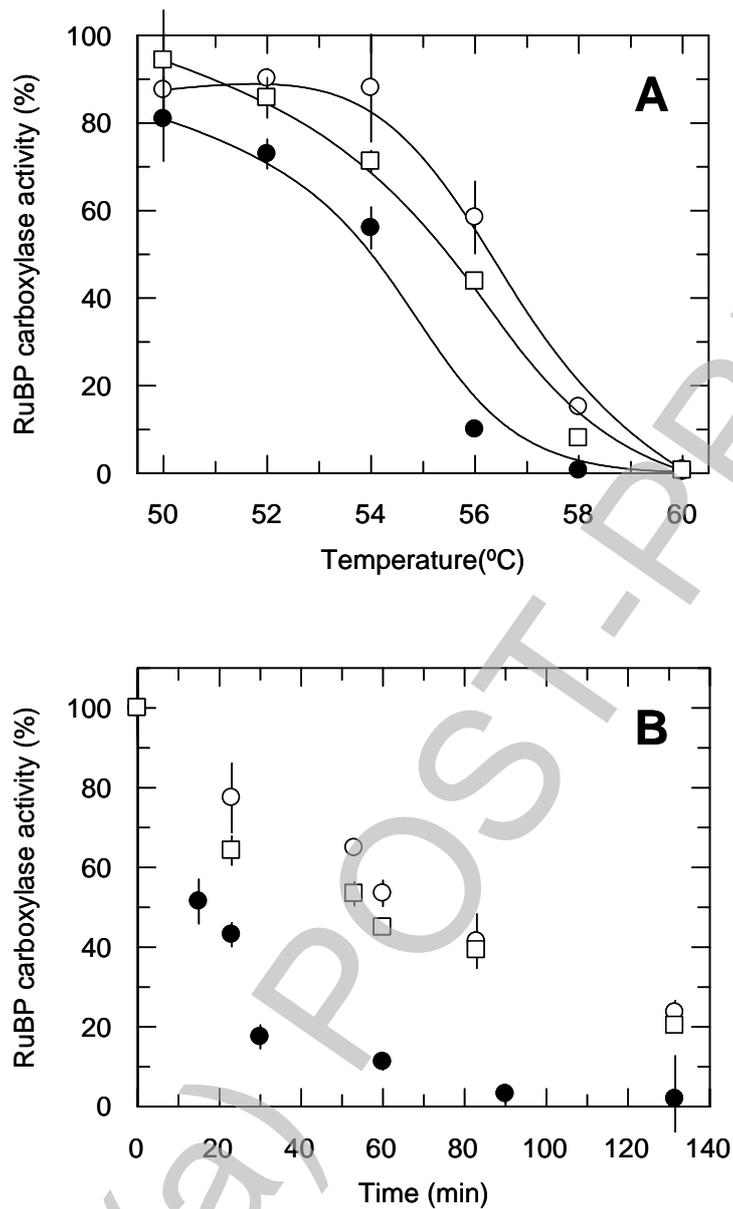


Figure 1

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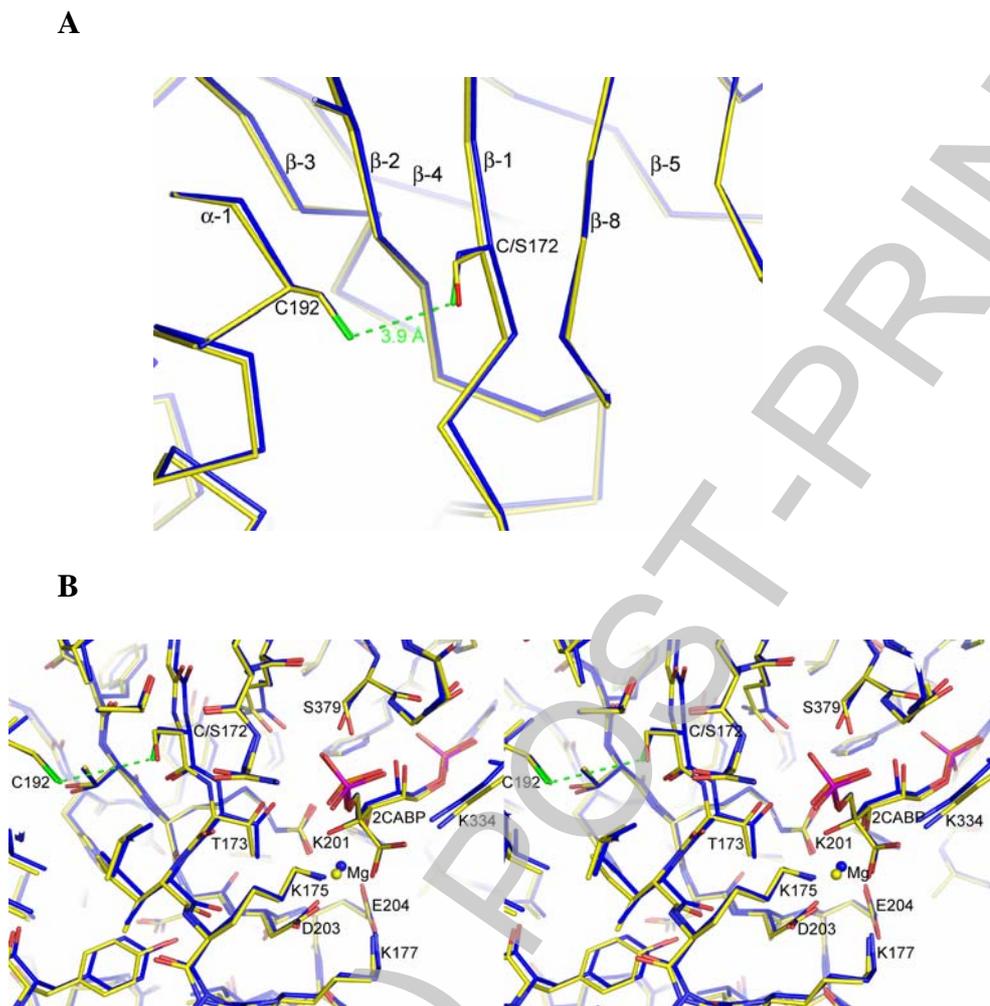


Figure 2

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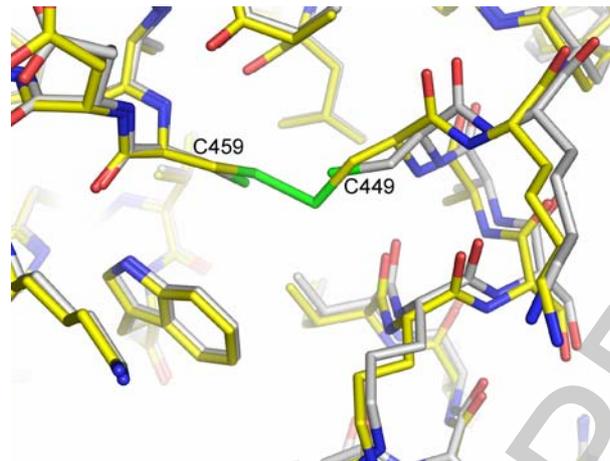


Figure 3