Redox modulation of Rubisco conformation and activity through its cysteine residues

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Abstract

Treatment of purified Rubisco with agents that specifically oxidize cysteine-thiol groups causes catalytic inactivation and increased proteolytic sensitivity of the enzyme. It has been suggested that these redox properties may sustain a mechanism of regulating Rubisco activity and turnover during senescence or stress. Current research efforts are addressing the structural basis of the redox modulation of Rubisco and the identification of critical cysteines. Redox shifts result in Rubisco conformational changes as revealed by the alteration of its proteolytic fragmentation pattern upon oxidation. In particular, the augmented susceptibility of Rubisco to proteases is due to increased exposure of a small loop (between Ser61 and Thr68) when oxidized. Progressive oxidation of Rubisco cysteines using disulphide/thiol mixtures at different ratios have shown that inactivation occurs under milder oxidative conditions than proteolytic sensitization, suggesting the involvement of different critical cysteines. Site-directed mutagenesis of conserved cysteines in the Chlamydomonas reinhardtii Rubisco identified Cys449 and Cys459 among those involved in oxidative inactivation, and Cys172 and Cys192 as the specific target for arsenite. The physiological importance of Rubisco redox regulation is supported by the in vivo response of the cysteine mutants to stress conditions. Substitution of Cys172 caused a pronounced delay in stress-induced Rubisco degradation, while the replacement of the functionally redundant Cys449-Cys459 pair resulted in an enhanced catabolism with a faster high-molecular weight polymerization and translocation to membranes.

These results suggest that several cysteines contribute to a sequence of conformational changes that trigger the different stages of Rubisco catabolism under increasing oxidative conditions.

Key words: Chlamydomonas reinhardtii, chloroplast, critical cysteines, oxidative inactivation, proteolytic enhancement, redox control, Rubisco catabolism, senescence, site-directed mutants, thiol-disulphide exchange.

Redox regulation in the chloroplast

Regulation by redox balance is a recurrent feature that pervades many aspects of plant biology (Buchanan and Balmer, 2005). It seems particularly fit that redox regulation mechanisms should exist inside the chloroplast because redox shifts are strongly correlated with changes in the physiological state and photosynthetic activity of the organelle. The shining of light on the thylakoids produces an electron flow through the components of the photosynthetic chain up to ferredoxin (Fig. 1). From that point, reducing power is distributed between NADPH, the main cofactor transferring reducing equivalents to anaerobic pathways, and the chloroplast thioredoxins, signalling molecules which, in turn, activate target proteins through reduction of critical cysteine residues (Schürmann and Jacquot, 2000). Besides, the redox state of plastoquinone, one intermediate electron carrier of the photosynthetic chain, is also known to regulate the transcription of specific chloroplast genes (Pfannschmidt et al., 1999).

From NADPH, reducing power is also transferred to glutathione (GSH) and ascorbate by means of successive redox reactions catalysed by the NADPH–glutathione

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Abbreviations: AP/GR, ascorbate peroxidase/glutathione reductase; CSH, cysteamine; CSSC, cystamine; GSH, glutathione; GSSG, glutathione disulphide; ROS, reactive oxygen species; Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulphate; SOD, superoxide dismutase.

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reductase and the glutathione–ascorbate reductase, respectively (Fig. 1). GSH and (specially) ascorbate are very abundant molecules in the chloroplast stroma, their concentrations reaching the millimolar range (Law et al., 1983). Thereby, ascorbate and GSH constitute a pool of redox buffers, providing inertia that opposes fast redox fluctuations inside the chloroplast. At a certain point, the balance of reductive and oxidative activities taking place inside the chloroplast defines a stromal redox status, which may be estimated as the ratio of oxidant to reductant forms of any one of the main redox pairs that are mutually connected through redox exchange (e.g. NADP+/NADPH, GSSG/GSH, dehydroascorbate/ascorbate or oxidized/reduced thioredoxin).

As a side reaction of photosynthetic electron transfer, and under certain conditions (such as excess light), some electrons may divert from their path, react with ambient O₂ molecules, and produce incompletely reduced forms of oxygen, known as ‘reactive oxygen species’ or ROS (Fig. 1). Although ROS can also act as intracellular signals (Apel and Hirt, 2004), these species are very active as oxidants, recruiting the missing electrons from a variety of nearby molecules and, thereby, potentially damaging functional photosynthetic structures. ROS production is usually minimized under physiological conditions but it is altogether unavoidable in a changing environment. Moreover, generation of ROS is increased when the components of the photosynthetic electron transport chain are dismantled (during senescence) (McRae and Thompson, 1983) or damaged (as a result of a variety of environmental stresses). ROS bursts are usually counteracted by (i) the glutathione–ascorbate redox buffer pool (Noctor and Foyer, 1998); (ii) ROS-deactivating enzymes (converting the most damaging species to less reactive forms) such as superoxide dismutase (SOD) (Alschcer et al., 2002), peroxiredoxin (Dietz et al., 2006), or the ascorbate peroxidase/glutathione reductase (AP/GR) system (Shigeoka et al., 2002); and (iii) ROS-quenching (i.e. electron donor) substances such as tocopherol (Krieger-Liszkay and Trebst, 2006) and carotenoids (Davison et al., 2002) (Fig. 1). However, under prolonged acute stress or at later stages of senescence, the protective agents are eventually overridden, and the chloroplast milieu turns increasingly oxidative. Lipid peroxidation and protein oxidative modifications are common markers for monitoring the degree of oxidation achieved inside the chloroplast at a given stage.

**Redox regulation of Rubisco**

The CO₂ (and O₂) -fixing enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco), controls the crucial step of partitioning ribulose 1,5-bisphosphate between the assimilatory photosynthetic metabolism and its carbon-releasing counterpart, the photorespiratory pathway. Besides, it is a very abundant protein, densely filling the chloroplast stroma of photosynthetic eukaryotes and storing a remarkable percentage of the macronutrients (specially N) allocated inside the chloroplasts (Evans and Seeman, 1989). Several aspects of Rubisco synthesis and activity have been proposed to be regulated by redox poise under physiological conditions. For instance, the stability of the chloroplastic rbcL mRNA (encoding the large subunit of Rubisco) has been shown to decrease as a result of the daily dark-to-light transition in a redox-dependent manner (Salvador and Klein, 1999). This effect is thought to be mediated by redox-sensitive protein factors binding to mRNAs. Besides, it has been proposed
that the nascent Rubisco large subunit may itself block its own translation under oxidative conditions by exposing a domain that binds to RNA when oxidized (Yosef et al., 2004). The enzymatic activity of Rubisco may also be affected by redox balance in some species through the activity of the thioredoxin-dependent large isoform of the Rubisco activase, an auxiliary enzyme that releases tightly-binding natural inhibitors from the catalytic site of Rubisco (Zhang et al., 2002). The nuclear-encoded small subunit of Rubisco has also been identified as a direct target of thioredoxin in a comprehensive screening among chloroplast proteins (Motohashi et al., 2001). However, the potential regulatory effects derived from this interaction are yet to be described.

During senescence or under different types of stress, Rubisco is known to undergo several oxidative modifications, such as thiol oxidation (García-Ferris and Moreno, 1994), formation of carbonyl adducts at certain residues (Eckardt and Pell, 1995; Junqua et al., 2000), acetylation and multimerization mediated by an enzymatic oxidase system (Ferreira and Davies, 1989; Ferreira and Shaw, 1989) and non-enzymatic fragmentation caused by ROS (Ishida et al., 1998; Nakano et al., 2006). As a result of these modifications, an increasing fraction of Rubisco becomes cross-linked to high molecular weight polymers (Ferreira and Shaw, 1989, Marín-Navarro and Moreno, 2006), and associated to membranes (Mehta et al., 1992; García-Ferris and Moreno, 1994). All these processes are thought to underlie or facilitate the fast and selective degradation of the enzyme that takes place habitually under such conditions (Albuquerque et al., 2001; Ferreira et al., 2000). However, not all types of modifications are detected under different stresses, suggesting that Rubisco catabolism may follow different courses depending on the nature of the stress and the specific conditions. Indeed, the co-existence of different catabolic routes for Rubisco turnover seems to be a reasonable assumption in view of the divergent experimental evidence on Rubisco degradation found under different conditions (Hörtenstein and Feller, 2005). In this review, the focus is exclusively on cysteine thiol oxidation, and its consequences for Rubisco performance.

**Cysteine-dependent redox properties of Rubisco**

Cysteine-thiol oxidation is a widely encountered means of modifying the activity of functional proteins in redox changing environments. Besides, it is also frequent as a marking step for protein turnover (Stadtman, 1990). Thiol groups may undergo progressive degrees of oxidation, from the mild disulphide (a reversible S–S bond established with another thiol) to the sulphenic (−SOH), sulphinic (−SO₂H), and sulphonic (−SO₃H) acid derivatives. The lower oxidation states of disulphide and sulphinic acid may easily be reverted again to the sulphydryl state. Disulphides can be regenerated by disulphide exchange with free thiols, while sulphenic acid may be reduced by thioredoxins and glutaredoxins (Hancock et al., 2006). Some sulphenic acid derivatives can also be reduced back to sulphenic acid through the action of specific ATP-dependent sulphiredoxins, which are known to be present in the chloroplast (Rey et al., 2007), while sulphonic acid is habitually considered an irreversible final oxidation step.

Rubisco was first described as a ‘cysteine-sensitive’ enzyme on the basis of its inactivation by thiol-directed reagents such as p-chloromercuribenzoate (Sugiyama et al., 1968) or the affinity label N-bromoacetylthiophanilomine phosphate (Schloss et al., 1978). The latter labelling allowed the identification of Cys172 and Cys459 as the targets for the affinity reagent and, thus, as the residues that were critical for enzymatic activity. Therefore, when the structure of the enzyme was solved, it came as a surprise that no cysteine appeared to play a significant role in the catalytic mechanism nor to be directly implicated in the active site architecture (Andersson et al., 1989). Hence, inactivation by cysteine reagents appears to result from indirect structural effects caused by the covalent bonding of cysteines to relatively bulky moieties. Indeed, it had already been detected that some of these modifications led to holoenzyme disassembly (Sugiyama et al., 1968).

It was later found that the activity and conformation of Rubisco can be modified by mild oxidative treatments affecting cysteines (Tenaud and Jacquot, 1987; Peñarrubia and Moreno, 1990; García-Ferris and Moreno, 1993) that do not disrupt the quaternary structure of the enzyme (Marín-Navarro and Moreno, 2003). To achieve this transition mimicking different redox ambient in vivo, the purified Rubisco is incubated in the so-called redox buffers (Gilbert, 1982). These are (pH-buffered) aqueous solutions containing a mixture of a small disulphide (such as cystamine) and its corresponding thiol (cysteamine) at different ratios; the higher the disulphide/thiol ratio, the more oxidizing the environment. The use of these disulphide/thiol buffers under a nitrogen atmosphere (to avoid other spontaneous oxidation processes) ensures that only cysteine residues are affected and, furthermore, that cysteine oxidation progresses only to the disulphide state. Under those conditions, a typical redox-dependent behaviour is exemplified by the *Chlamydomonas reinhardtii* Rubisco in Fig. 2. The exposure to an increasingly oxidative environment triggers a sharp transition to a fully inactive form of Rubisco with a midpoint corresponding to a disulphide/thiol ratio of about 1.5 (Fig. 2A). Moreover, Rubisco oxidation also induces structural alterations, as revealed by a change of the fragmentation pattern produced by low-specificity proteases (such as subtilisin or proteinase K) acting on purified Rubisco upon oxidation.
An obvious goal to understand the nature of the redox modulation of Rubisco has been the identification of the critical residue (or residues) whose oxidation results in the observed transitions. The fact that redox modulation of Rubisco is extended among eukaryotic enzymes points to phylogenetically conserved residues. However, this consideration still leaves a number of candidates. For example, in *C. reinhardtii*, nine (out of a total of 16) Cys residues are highly conserved among green-type eukaryotic Rubiscos. These are cysteines 84, 172, 192, 247, 284, 427, and 459 of the large subunit, and 41 and 83 of the small subunit. As a first approach, the scanning of the functional roles of these cysteines was started through site-directed mutagenesis of each of the conserved residues, exchanging it by serine (a conservative substitution replacing a single sulphur atom by oxygen). Perhaps surprisingly, all single mutants of these highly conserved residues were recovered as photosynthetically competent strains (Moreno and Spreitzer, 1999; Marín-Navarro and Moreno, 2006), thereby reinforcing the idea that these cysteines are not crucial for catalytic activity and may be conserved for other reasons. The characteristic properties and phenotypes of some of these Rubisco mutants will be described below. It should be noted, however, that redox modulation of Rubisco can result from the additive, cooperative, or even redundant contribution of several cysteines and, thus, the role of a specific residue may not be readily appreciable in a single-cysteine mutant. A hint on the implication of several cysteine residues in the oxidative transitions of Rubisco comes from the fact that the midpoint of proteolytic sensitization of the enzyme does not match that of inactivation, but it is shifted to more oxidative conditions (corresponding to a cysteamine/cysteamine ratio between 4 and 5) (Fig. 3). This suggests that oxidation of further cysteines is needed for triggering the conformational changes that modify the proteolytic pattern. Moreover, it predicts that, when subjected to increasing oxidative conditions, Rubisco will first become inactive (with little structural change) and afterwards experience the conformational alterations that render it sensitive to proteases. It has been proposed that these features can be relevant *in vivo*, specially under

![Fig. 2](https://academic.oup.com/jxb/article-abstract/59/7/1605/639472)

**Fig. 2.** Redox-dependent inactivation (A) and modification of the proteolytic pattern (B) of Rubisco. (A) Redox buffers were prepared by combining cystamine (CSSC) and cysteamine (CSH) at different ratios while keeping the monomeric concentration (CSH+2CSSC) constant and equal to 40 mM, in a Rubisco activation buffer (100 mM TRIS–HCl, 10 mM NaHCO₃, 10 mM MgCl₂ pH 8.2). Purified Rubisco (0.13 mg ml⁻¹) was incubated with the redox buffers for 2 h at 30 °C under a nitrogen environment and the residual carboxylase activity was determined. The activity as a percentage of that of the fully reduced enzyme (CSSC/CSH=0) is represented. Error bars indicate standard deviation from triplicates. (B) Purified Rubisco (0.13 mg ml⁻¹) either reduced with 40 mM CSH or oxidized with 20 mM CSSC as above was subjected to a proteolysis with subtilisin (0.5 μg ml⁻¹) in Rubisco activation buffer. At the indicated times, the reaction was stopped adding 2 mM phenylmethylsulphonyl fluoride and keeping on ice for 10 min. The samples were subsequently analysed by SDS-PAGE. The 55 kDa Rubisco large subunit (LS) and its degradation products of 53 kDa (band 1) and 47 kDa (band 2) are indicated with arrows.

![Fig. 3](https://academic.oup.com/jxb/article-abstract/59/7/1605/639472)

**Fig. 3.** Inactivation (open circles) and proteolytic sensitization (closed circles) of Rubisco incubated in redox buffers at different disulphide/thiol ratios. Purified Rubisco was incubated with redox buffers at different CSSC/CSH ratios, as detailed in Fig. 2A. The residual carboxylase activity, as a percentage of that of the fully reduced enzyme, is shown on the left axis. A proteolytic assay with subtilisin was performed with the treated Rubisco samples as described in Fig. 2B. The amount of intact large subunit remaining after 20 min of proteolysis, determined by SDS-PAGE analysis, is indicated on the right axis as a percentage of that obtained from fully reduced Rubisco in the same conditions. Error bars indicate standard deviation from triplicates.
senescence or stress processes, which create oxidative conditions and lead to an enhanced catabolism of Rubisco (García-Ferris and Moreno, 1993; Moreno et al., 1995). In the following, current knowledge about each of these redox regulated transitions (inactivation and proteolytic sensitization) will be reviewed and the evidence of their possible physiological role will be examined.

**Oxidative inactivation of Rubisco**

As shown in Fig. 2A, oxidation of Rubisco with cysteamine/cystamine buffers leads to a complete inactivation of the enzyme. Under the experimental conditions used in this assay, the activity loss should result from modification of thiol groups to disulphides, either internal ones (i.e. those bonding two protein cysteines) or mixed disulphides with cysteamine. Because the transition to the inactive form takes place around a disulphide/thiol ratio of 1.5, the critical cysteines should have a standard redox potential similar to (or slightly more oxidizing than) the cysteamine/cysteamine pair. The spinach Cys172 and Cys459 residues have been identified as the target of the affinity labelling that causes also Rubisco inactivation (Schloss et al., 1978). However, the absence of Cys172 in the *C. reinhardtii* C172S mutant Rubisco does not shift, or otherwise alter, the shape of the transition to the inactive form in redox buffers (Moreno and Spreitzer, 1999) suggesting that this residue is not involved in disulphide inactivation. Nevertheless, Cys172 and the proximal Cys192 are thought to be responsible for inactivation by arsenite (a reagent for vicinal dithiols) because the C172S mutant is not sensitive to arsenite (Moreno and Spreitzer, 1999). This further supports the notion that disulphide inactivation operates through a mechanism which is different from those due to chemical modification by a variety of other reagents.

By contrast, mutation of Cys459 and Cys449 (a moderately conserved residue located within a disulphide bonding distance from Cys459) affects redox-dependent inactivation. These mutations do not prevent a substantial loss of activity upon oxidation, but the enzyme cannot be completely inactivated even at very high cystamine/cysteamine ratios (Marín-Navarro and Moreno, 2006). This indicates that these cysteines contribute (among other residues) to the changes leading to Rubisco oxidative inactivation. The fact that the percentage of activity remaining after oxidation is even more noticeable in the C449S-C459S double mutant (in which both Cys449 and 459 have been replaced by serines) (Marín-Navarro and Moreno, 2006) implies that the contribution of these cysteines to the inactivation process may be additive and/or redundant, but not co-operative (as would be expected if the effect would be derived from establishing a disulphide bridge between these residues). Indeed, a previous kinetic analysis had indicated that cysteines involved in inactivation do not appear to engage in internal disulphide bonding (García-Ferris and Moreno, 1993). The possible physiological advantages of this fact have been discussed elsewhere (Moreno et al., 1995). Besides, the case of Cys449 and Cys459 illustrates the feasibility of affecting the catalytic performance from a considerable distance (about 2 nm from the Cys449/Cys459 pair to the nearest active site). Thus, it seems that cysteine oxidation can induce subtle conformational changes that may propagate through the Rubisco structure reaching remote regions. Structural rearrangements that are known to occur as a result of cysteine oxidation will be described in the next section.

**Conformational changes induced by oxidation**

Oxidation of Rubisco has been shown to weaken its quaternary structure, as demonstrated by the thermal and solvent sensitivity of the oxidized enzyme (Marín-Navarro and Moreno, 2003). Nevertheless, the oxidized holoenzyme still remains assembled at physiological temperature in aqueous solutions. An analysis of the proteolysis of *C. reinhardtii* Rubisco with subtilisin revealed that, besides the clipping of the unstructured N-terminus of the large subunit (which does not affect the structural stability of the enzyme and occurs in a redox-independent manner), the oxidized enzyme becomes more sensitive to proteolysis due to an enhanced processing at a specific loop (Ser61–Thr68) located between the B α-helix and a neighbouring 3_{10}-helix within the N-terminal domain of the large subunit (Marín-Navarro and Moreno, 2003). Thus, oxidation of Rubisco involves conformational changes that facilitate the access of the protease to the Ser61–Thr68 loop. Moreover, it has been shown that clipping of this piece (up to Thr68), which remains transiently attached to the holoenzyme, favours the disassembly of the quaternary structure and the subsequent unrestricted proteolysis of the released subunits (Marín-Navarro and Moreno, 2003). A quantitative analysis of the kinetics of proteolysis suggests that holoenzyme disassembly follows the proteolytic processing at the Ser61–Thr68 loop of both of the large subunits integrating any of the four dimers that scaffold the holoenzyme core (Marín-Navarro and Moreno, 2003). Hence, the clipped N-terminal pieces apparently remain attached to the holoenzyme only until the structure is destabilized by a cut at the Ser61–Thr68 loop in one large subunit whose dimerizing neighbour has already been processed at the same site. Since the proteolytic cut at the Ser61–Thr68 loop is obstructed in the reduced enzyme, this mechanism offers a double-check redox-dependent path for full proteolytic access to the otherwise protease-resistant holoenzyme structure of Rubisco (Fig. 4).
The Cys172 residue of the large subunit was the first to be related to redox conformational changes of Rubisco because its substitution by serine produces a mutated enzyme which displays a protease-sensitivity transition shifted to more oxidative conditions (Moreno and Spreitzer, 1999). Nevertheless, the mutated enzyme eventually becomes as degradable as the wild type when incubated at high disulphide/thiol ratios (Marín-Navarro and Moreno, 2006). Therefore, it seems that the oxidation of Cys172 delays the structural changes rendering the enzyme sensitive to proteolysis until compensating conformational changes are brought about by the oxidation of other (less reducing) cysteines. The latter residues do apparently fulfill a somewhat redundant role for this transition in the wild-type enzyme. Similarly, the simultaneous mutation of both Cys449 and Cys459 produces a diminished sensitivity to processing at the Ser61–Thr68 loop, even at high disulphide/thiol ratios (Marín-Navarro and Moreno, 2006). Therefore, it seems that the oxidation of Cys449 and Cys459 contribute to the conformational changes that expose the Ser61–Thr68 loop to proteolytic attack. Because no alterations were found in the C449S and C459S single mutants (Marín-Navarro and Moreno, 2006), it might be assumed that Cys449 and Cys459 are also functionally redundant with regard to this structural transition.

The role of cysteine-mediated redox modulation of Rubisco in vivo

While the conditions needed for Rubisco inactivation or conformational transition may be too oxidizing to be reached during the daily light/dark redox oscillation, it has been repeatedly suggested that these oxidative processes may take place in vivo under senescence or stress scenarios, which are known to trigger a fast catabolism of Rubisco (Peñaarrubia and Moreno, 1990; Mehta et al., 1992; García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999; Marín-Navarro and Moreno, 2003). There is indeed ample evidence of the development of strong oxidative conditions inside the chloroplast during senescence or stress processes, as detected by increase of the GSSG/GSH ratio (Law et al., 1983), rise of ROS (McRae and Thompson, 1983), or lipid peroxidation (Mishra and Singhal, 1992). Besides, during stress processes the number of titrable cysteine–thiol groups of Rubisco has been shown to decrease (García-Ferris and Moreno, 1994) and oxidative cross-linking of Rubisco subunits can be demonstrated comparing reducing and non-reducing SDS–PAGE electrophoregrams (García-Ferris and Moreno, 1994; Marín-Navarro and Moreno, 2006). Moreover, inactivation of Rubisco has been shown to precede its degradation during senescence of leaves (Wittenbach, 1978; Hall et al., 1978) or isolated chloroplasts (Seftor and Jensen, 1986), as predicted by Fig. 3. Furthermore, a Rubisco proteolytic fragment, generated by incubation with lysates from senescing leaves, has been shown to have Val69 at its N-terminus (i.e. it has been processed at the Ser61–Thr68 loop) (Yoshida and Minamikawa, 1996). It cannot be ignored that other identified fragments produced by endogenous processing have displayed different N-terminal sequences (Desimone et al., 1996; Kokubun et al., 2002) or proved to be generated by a different (non-enzymatic) mechanisms (Ishida et al., 1999). As already stated above, it transpires from the accumulated evidence that many different paths of Rubisco degradation are likely to exist, one or several of them prevailing under specific conditions. Cysteine-dependent oxidative inactivation and proteolytic sensitization may be just one of these catabolic paths.

Even if consideration of the whole of the above circumstantial facts is strongly suggestive, the most compelling evidence for a physiological role of cysteine oxidation in Rubisco catabolism comes from the analysis of the phenotype of the C. reinhardtii cysteine mutants under stress conditions. For instance, Fig. 5 shows the

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**Fig. 4.** Steps for the proteolytic degradation of Rubisco. (A) Schematic representation of the N-terminal domain of Rubisco large subunit highlighting the two regions (site 1 and site 2) that are accessible to proteolytic attack in vitro. A conformational change between the reduced (RR) and oxidized (RO) forms can be detected through an enhanced access of broad specificity proteases to site 2, while proteolysis at site 1 is not affected by redox conditions. (B) Scheme for the mechanism of Rubisco degradation in vitro. Rubisco holoenzyme is composed by four dimers of large subunits (represented by complementary half ovals) and eight small subunits (represented by grey ovals). The N-terminal end (up to Lys 18) of each large subunit is depicted as a protruding stretch. The proteolytic cut at site 1, which releases this N-terminal fragment does not compromise the structural stability of the holoenzyme. Processing at site 2 of the two large subunits integrating a dimer promotes the disassembly of the holoenzyme and the subsequent unrestricted degradation to small peptides and amino acids of all the subunits. (C) Rubisco holoenzyme structure highlighting one dimer of large subunits in blue and green. The N terminal fragment arising from the cut at site 2, which remains attached to the holoenzyme core until a double cut occurs at any of the dimers of the same molecule, is shown in red.
response of the C172S and C192S mutants to salt stress compared with the wild-type strain. It can be appreciated that 10 h of stress with 0.3 M NaCl caused only a slight decrease of chlorophyll content (Fig. 5A) (indicating little dismantling of thylakoidal structures) but a noticeable loss of total protein per cell (Fig. 5C). The amount of Rubisco in the wild-type strain fell in the same period to an even lower percentage (Fig. 5B). Thus, the Rubisco-to-total-protein ratio experienced a steady decrease throughout the treatment (Fig. 5D) showing a preferential degradation of Rubisco compared with the bulk of other proteins. However, the C172S mutant, when subjected to the same stress intensity (as witnessed by the chlorophyll and total protein loss in Fig. 5), showed a distinctly slower Rubisco degradation rate (Fig. 5B). Monitoring of the Rubisco-to-total-protein ratio indicated that the substitution of Cys172 by serine suppressed the preferential degradation of Rubisco in the C172S mutant during the first 6 h of stress (Fig. 5D). A similar delay of Rubisco turnover has been reported for the same mutant under osmotic or oxidative stress (Moreno and Spreitzer, 1999). Interestingly, a C172A mutant (i.e. having Cys172 replaced by alanine) of a cyanobacterial Rubisco has also been shown to display retarded catabolism under nitrogen deprivation (Marcus et al., 2003). This suggests that the mechanism for timing of Rubisco degradation that involves Cys172 may be shared by prokaryotic and eukaryotic green-type Rubiscos. By contrast, the substitution of the vicinal Cys192 does not alter the Rubisco degradation rate in the C. reinhardtii C192S strain (Fig. 5), nor in the cyanobacterial mutant (Marcus et al., 2003).

The delayed degradation of Rubisco in mutants lacking Cys172 could be a consequence of the shift of the proteolytic sensitivity of the enzyme to more oxidative conditions as observed in vitro (Moreno and Spreitzer, 1999). However, in the case of Cys449 and Cys459, the single substitutions produced no apparent phenotype while the C449S-C459S double mutant displayed an accelerated catabolism with enhanced oxidative cross-linking and association to membranes (Marín-Navarro and Moreno, 2006). This agrees with the observation of an altered conformation of the double mutant (but not of the single mutants) upon oxidation, as detected in vitro by divergent kinetics of proteolysis using subtilisin as a structural probe (Marín-Navarro and Moreno, 2006). Nevertheless, there is an apparent paradox in that the elimination of cysteines (i.e. of potentially oxidizable thiol groups) results in an enhanced oxidative catabolism. In that instance, it has to

Fig. 5. Evolution of chlorophyll (A), Rubisco (B), total protein (C), and Rubisco to total protein ratio (D) in C172S (closed circles), C192S (closed squares), and wild-type (open circles) strains of C. reinhardtii subjected to salt stress. C. reinhardtii cultures of the different strains were grown up to a density of 2×10^6 cells ml^{-1} before the application of stress (0.3 M NaCl). At the indicated times of stress, an aliquot of the culture was taken to determine the chlorophyll content, and the amount of total protein and Rubisco as described elsewhere (Marín-Navarro and Moreno, 2006). In all cases, the amounts are given as a percentage of the initial value. Error bars represent standard deviation from 9 (C172S), 15 (C192S) or 21 (wild type) experiments.
be admitted that the oxidation of Cys449 and/or Cys459, and the derived structural changes, may act to shield or protect other cysteine residues from further oxidation (Fig. 6). Hence, the Cys449–Cys459 pair could serve to adjust the rate of Rubisco degradation by delaying further stages of catabolism.

Concluding remarks
Modulation of Rubisco activity and structure by the redox state of its conserved cysteine residues appears as a suggestive regulatory mechanism for adapting the enzyme to the changing functional status of the chloroplast. While the potential for regulation has always been clear from in vitro experiments, the actual implication of this mechanism as physiologically relevant in vivo has been difficult to prove. The definitive evidence has come from the verification that site-directed mutants on conserved cysteine residues display an abnormal Rubisco catabolism in vivo. Moreover, the fact that the mutants on highly conserved cysteines are all photosynthetically active and show an altered phenotype only under stress conditions

Fig. 6. A model for cysteine-mediated redox modulation of Rubisco catabolism. Under sustained stress, photosynthetic organisms experience increasing oxidative conditions within the chloroplast stroma (indicated by the gradient of grey shade at the top bar). (A) In wild-type Rubisco, each cysteine has a characteristic redox potential determined by its structural microenvironment and represented in the figure by a grey-shaded cube, with a lighter shade intensity correlating with a lower redox potential. As an arbitrarily simplified example, only five critical cysteines (numbered from 1 to 5) are shown. When the redox potential in the chloroplast milieu (r) matches that of a specific cysteine, the originally reduced residue (open circle) becomes oxidized (closed circles). The oxidation of different cysteines results in specific conformational changes that progressively direct Rubisco to the catabolic pathway, including inactivation, polymerization, mobilization to a membrane fraction, and degradation. The rate at which each conformational variant is guided to these routes is represented by an increasing number of vertical arrows. A higher versatility of the stress response is achieved by a higher number of structural variants, and this gradual modification may be tuned by cysteine networking. In the figure, the oxidation of both Cys 1 and 2 causes a conformational change that converts Cys 3 into a less reducing thiol. Besides, Cys4 and Cys 5 are assumed to be functionally redundant because the oxidation of Cys 5 alone is supposed to reproduce (among other changes) the conformational distortions caused by the oxidation of Cys 4. Replacement of cysteines by serines in (B) and (C) is represented by switching from circles to triangles. (B) Double mutation of Cys 1 and 2, which have a redundant role (these would be exemplified by Cys449 and Cys459 from Rubisco large subunit), causes an acceleration of the catabolic processes because the critical Cys 3 is now oxidized at a lower redox potential. (C) Mutation of Cys 4 retards catabolism (this would be the case of Cys172 from Rubisco large subunit) because completion of the conformational transition now has to wait for the oxidation of the least reducing Cys 5.
further reinforces the idea that these residues are conserved because of their redox properties which are important for regulating Rubisco degradation. However, redox modulation of Rubisco through its cysteines appears as an intricate process where several residues seem to contribute in an additive, co-operative or redundant manner. The set of critical cysteines seems to act as a network of redox sensors that drives the progression of Rubisco through the different stages of catabolism by means of a sequence of structural changes taking place under increasingly oxidative conditions (Fig. 6). Each of these conformational changes is induced by the oxidation of specific cysteine residues and seems to act as a checkpoint to ensure an adequate timing for the different steps of Rubisco catabolism within the general framework of chloroplast dismantling following acute stress or senescence conditions.

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