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CONTROL OF THE ACTIVITY OF RUBISCO FROM CHLAMYDOMONAS REINHARDTII THROUGH THE REDOX STATE OF ITS CYSTEINE RESIDUES

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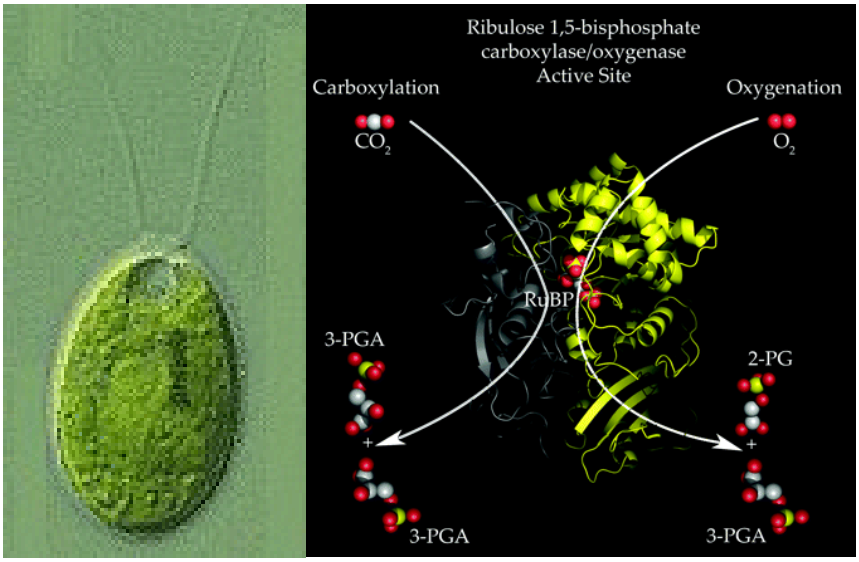

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CERTIFICA que el trabajo titulado "CONTROL OF THE ACTIVITY OF RUBISCO FROM *CHLAMYDOMONAS REINHARDTII* THROUGH THE REDOX STATE OF ITS CYSTEINE RESIDUES" ha sido realizado bajo su dirección por **Hemanth Phani Kumar SUDHANI** en el laboratorio de Biología Molecular de Plantas del citado departamento, como tesis doctoral para optar al título de Doctor dentro del Programa Oficial de Posgrado de BIOTECNOLOGÍA..

Para que así conste, firma el presente certificado en Burjassot, a 24 de julio de 2012

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Abbreviations

Ac-CoA	Acetyl coenzyme A
AS	Arsenite
Asc	Ascorbate
AP/GR	Ascorbate peroxidase/glutathione reductase
^{14}C	Carbon-14
CA1P	2-carboxy-D-arabinitol-1-phosphate
CABP	Carboxy-arabinitol-1,5-bisphosphate
CBB	Calvin-Benson-Bassham
Cys	Cysteine
CSH	Cysteamine (2-mercaptoethylamine)
CSSC	Cystamine (2-mercaptoethylamine disulfide)
C.reinhardtii	*Chlamydomonas reinhardtii*
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
DTT	Dithiothreitol
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
E. coli	*Escherichia coli*
EtSH	2- mercaptoethanol
GSNO	S-nitrosoglutathione

Abbreviations

GSH	Reduced glutathione
GSSG	Oxidized glutathione
Grx	Glutaredoxin
G3P	Glyceraldehyde 3 Phosphate
IAM	2-Iodoacetamide
IAA	Iodoacetic acid
V_c	Maximum Velocity for Carboxylation
K_C	Michaelis-Menten constant for carboxylation
kDa	Kilodalton
V_o	Maximum Velocity for Oxygenation
K_O	Michaelis-Menten constant for oxygenation
MW	Molecular weight
NAC	N-acetylcysteine
NADPH	β-nicotinamide adenine dinucleotide 2′-phosphate
NEM	N-Ethylmaleimide
PDB	Protein data bank
3-PGA	3-Phosphoglycerate
2PG	2-phosphoglycolate
PMSF	Phenylmethanesulfonylfluoride/Phenylmethylsulfonyl fluoride

POPOP	1,4-bis(5-phenyloxazol-2-yl) benzene
PPO	2,5-Diphenyloxazole
r	Disulfide/thiol ratio
ROS	Reactive oxygen species
RubisCO	Ribulose 1,5- biphosphate Carboxylase/Oxygenase
RuBP	Ribulose 1,5- biphosphate
SDS	Sodium dodecyl Sulphate
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TCEP	Tris (2-carboxyethyl) phosphine
Tris	Tris(hydroxymethyl)aminomethane
Trx	Thioredoxin
wt	Wildtype
XuBP	Xylulose 1,5- biphosphate
Ω	Relative Specificity Factor

INTRODUCTION

FUNCTIONS OF RUBISCO

The Calvin (or Calvin-Benson-Bassham) cycle was the first autotrophic carbon fixation pathway to be recognized. It was worked-out in the late 1940s and the 1950s by Melvin Calvin, Andrew Benson, James Bassham, and others (Wildman and Bonner, 1947; Calvin and Benson, 1948, 1949; Benson *et al.*, 1951). This metabolic route is found in most autotrophic organisms, ranging from prokaryotes (cyanobacteria and other phototrophic and chemoautotrophic bacteria) to eukaryotes (various algae and higher plants). The incorporation of carbon (as CO_2) into the cycle is catalyzed by the Ribulose-1,5-bisphosphate carboxylase oxygenase (EC.4.1.1.39, RubisCO). RubisCO catalyzes the fixation of one molecule of CO_2 to the pentose-bisphosphate sugar ribulose-1,5-bisphosphate (RuBP), yielding two molecules of the 3-carbon 3-phosphoglyceric acid (PGA). PGA undergoes reduction to glyceraldehyde 3-phosphate (thereby consuming NADPH and ATP, which have been generated by the light reactions of photosynthesis) and then, it is reshuffled in a series of reactions regenerating RuBP but saving some carbon for the net synthesis of carbohydrates (fig. 1). This metabolic scheme constitutes the Calvin Cycle.

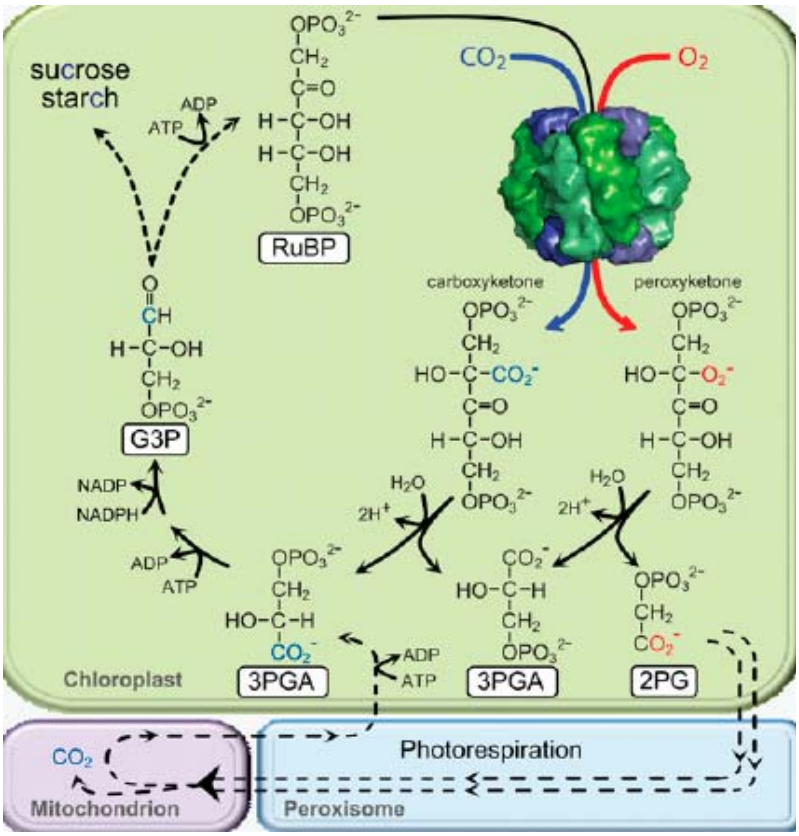


Fig.1. RuBP metabolism: RubisCO catalyzes both the carboxylation reaction and the oxygenation of RuBP, being the starting point of two opposing processes: photosynthesis and photorespiration [Taken from Maurino and Peterhansel (2010)].

In addition, RubisCO catalyzes also the oxygenation of RuBP to form one molecule each of 2-phosphoglycolate and PGA (fig.1) (Bowes *et al*., 1971). The latter is streamed into the Calvin cycle. Phosphoglycolate, however, is more difficult to recycle and has to circulate from chloroplast to the peroxisomes and then to the mitochondria, undergoing a number of reactions that consume ATP and reducing power, while losing CO_2, before it is converted to PGA and returns to the Calvin Cycle (fig.1). This sequence of reactions, started by the oxygenase activity of RubisCO, is the photorespiratory pathway. The net balance of photorespiration is opposed to photosynthetic carbon assimilation. Therefore, in contrast to RuBP carboxylation, the oxygenation of RuBP is regarded as a wasteful reaction of the RubisCO enzyme (Andrews and Lorimer, 1987). It would appear that eliminating or reducing the RubisCO oxygenase activity would potentially increase carbon assimilation, thereby rising photosynthetic efficiency significantly (Long *et al.,* 2006).

Besides its involvement in the Calvin cycle and photorespiration, RubisCO is also active in some non-photosynthetic tissues (such as the oil seeds of *Brassica napus*) contributing to a metabolic pathway that increases the efficiency of carbon use during the synthesis of fatty acids (Schwender *et al.,* 2004a). In these tissues, where the Calvin cycle is not functional, CO_2 fixation by RubisCO contributes to the metabolic flux that converts carbohydrates into fatty acids (Schwender *et al.,* 2004b) (Fig. 2). This is achieved in three stages: first, hexose phosphates are converted to ribulose-1,5-bisphosphate by the enzymes of the non-oxidative reactions of the pentose phosphate pathway together with phosphoribulokinase; second, ribulose-1,5- bisphosphate and CO_2 (most of which is produced by pyruvate dehydrogenase) are converted to PGA by RubisCO; and third, PGA is metabolized to pyruvate and acetyl-CoA through the terminal reactions of glycolysis thereby connecting with the fatty acids synthesis (FAS) pathway (fig. 2). Thus, RubisCO, together with phosphoribulokinase and the non-oxidative enzymes of the pentose phosphate pathway, allows the conversion of carbohydrate into 20% more acetyl-CoA (and oil) than does the direct glycolytic path, with 40% less carbon lost as CO_2.

Other cellular functions of RubisCO are related to its remarkable abundance, being usually present inside the cell at a much higher concentration than any other protein. This is especially true in higher plants, where it amounts up to 50% of the total soluble protein content of the leaves and other photosynthetic tissues, reaching a millimolar concentration inside the chloroplast stroma (Pickersgill, 1986). Because of its high concentration and its widespread distribution in the biosphere, it was arguably considered a plausible candidate to

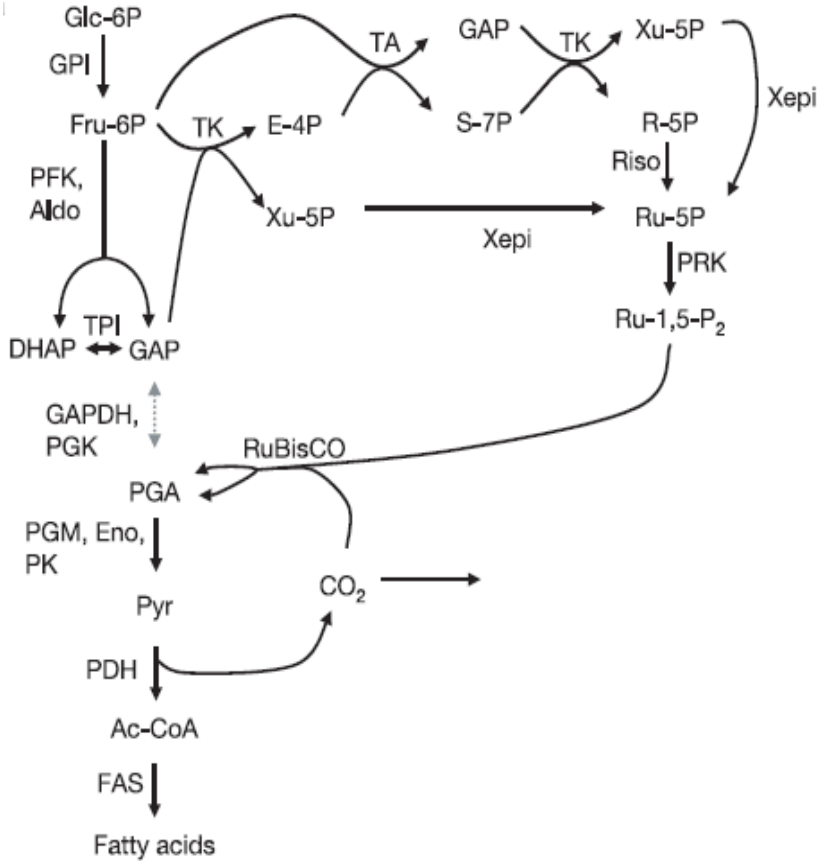


Fig.2. Metabolic Transformation of sugar into fatty acids: Conversion of hexoses phosphate to pentose phosphate through the non-oxidative steps of the pentose phosphates pathway and subsequent formation of PGA by RubisCO bypasses the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase while recycling half of the CO_2 released by pyruvate dehydrogenase (PDH). PGA is then further processed to pyruvate (Pyr), acetyl-coenzyme A (Ac-CoA) and fatty acids [Taken from Schwender *et al.*, (2004a)].

"the most abundant protein in the world" (Ellis, 1979). It is usually assumed that the abundance of RubisCO compensates for the low efficiency of the enzyme and its slow catalytic activity (Ellis, 1979; Spreitzer and Salvucci, 2002). In any case, photosynthetic organisms are forced to invest a significant portion of its nitrogen and sulfur content to synthesize large amounts of RubisCO. Thus, for example, leaves of higher plants typically allocate in RubisCO about one fourth of the total nitrogen found in these tissues (Mae *et al.*, 1983; Evans and Seeman, 1989). It has been repeatedly observed that, when photosynthetic organisms are faced with adverse conditions, the specific degradation of RubisCO acts as a source of nitrogen, sulfur and carbon for the rapid synthesis of an enzyme complement and other components that are essential to overcome stress. Moreover, during natural senescence of higher plants, proteolysis of RubisCO in mature organs contributes a significant fraction of the nutrients allocated in the growing new tissues (Friedrich and Huffaker, 1980; Kang and Titus, 1980; Mae *et al.*, 1983). Therefore, RubisCO acts as a nutrient-storage protein in many species of higher

plants (Huffaker and Peterson, 1974; Millard, 1988). Because most photosynthetic organs undergo senescence and abscission as a final step of development, the specific degradation of RubisCO and the subsequent reuse of its nutrients through the mobilization of its amino acids to growing tissues, play a crucial role in the nutritional balance of plants (Ferreira *et al.*, 2000).

Besides this nutrient storage functions, it has been proposed that RubisCO could act also as a metabolic buffer in the chloroplast stroma of algae and higher plants (Ashton, 1982). Because of the high concentration of RubisCO active sites and their ability to bind sugar phosphates, adenosine phosphates and NADPH, it has been suggested that sequestration of these compounds by RubisCO may have a physiological function modulating the availability of these metabolites in free form (Ashton, 1982).

FORMS AND PHYLOGENY OF RUBISCO

All RubisCO enzymes are multimeric. Two different types of subunits may occur: a large subunit (L) of 50-55 kDa and a small subunit (S) of 12-16 kDa. On the basis of the number of subunits and their structural arrangement, as well as the corresponding sequence divergence, RubisCOs found in nature can be classified into four different forms, which integrate the RubisCO super family. These are called forms I to IV. Table 1 summarizes the characteristics of the different forms of RubisCO.

Table.1. Main features of the different forms of RubisCO

	Form I	Form II	Form III	Form IV
Holoenzyme composition	L_8S_8	$(L_2)_n$	L_2 and $(L_2)_5$	L_2
RubisCO activity	+	+	+	--
Function	Calvin Cycle	Calvin Cycle	RuPP pathway (?)	Methionine salvage (a.o.)

These four different RubisCO holoenzyme forms have often unique features, yet the fundamental structural unit, common to all forms, is the catalytic large subunit dimer (Tabita *et al.*, 2008).

FORM I AND II RUBISCO

Form I RubisCO is the most abundant. It is found in higher plants, algae, and many autotrophic bacteria (including all cyanobacteria) (Tabita, 1999). The holoenzyme is a hexadecamer, denoted as $RbcL_8S_8$, containing eight large subunits and eight small subunits. The ca 550 kDa oligomer has a cylindrical shape with a diameter of ~110 Å and a height of 100 Å, letting an axial channel accessible to the solvent. The molecule exhibits local 4:2:2 symmetry and consists of a core of four L_2 dimers arranged around a four-fold axis, capped at each end by four small subunits (Knight *et al.*, 1990) (fig. 3).While RubisCOs classed as forms II to IV lack S-subunits, containing only L-subunits arranged into L_2 dimers or $(L_2)_n$ complexes, form I enzymes assemble four S-subunits, placed in between the four L_2 dimers that constitute the central core, at each of the two poles of the molecule (fig. 3). Albeit not strictly required for CO_2 fixation, the S-subunits are thought to be essential, providing the structural stability needed to maximize carboxylation efficiency within this structural framework (Andersson and Backlund, 2008).

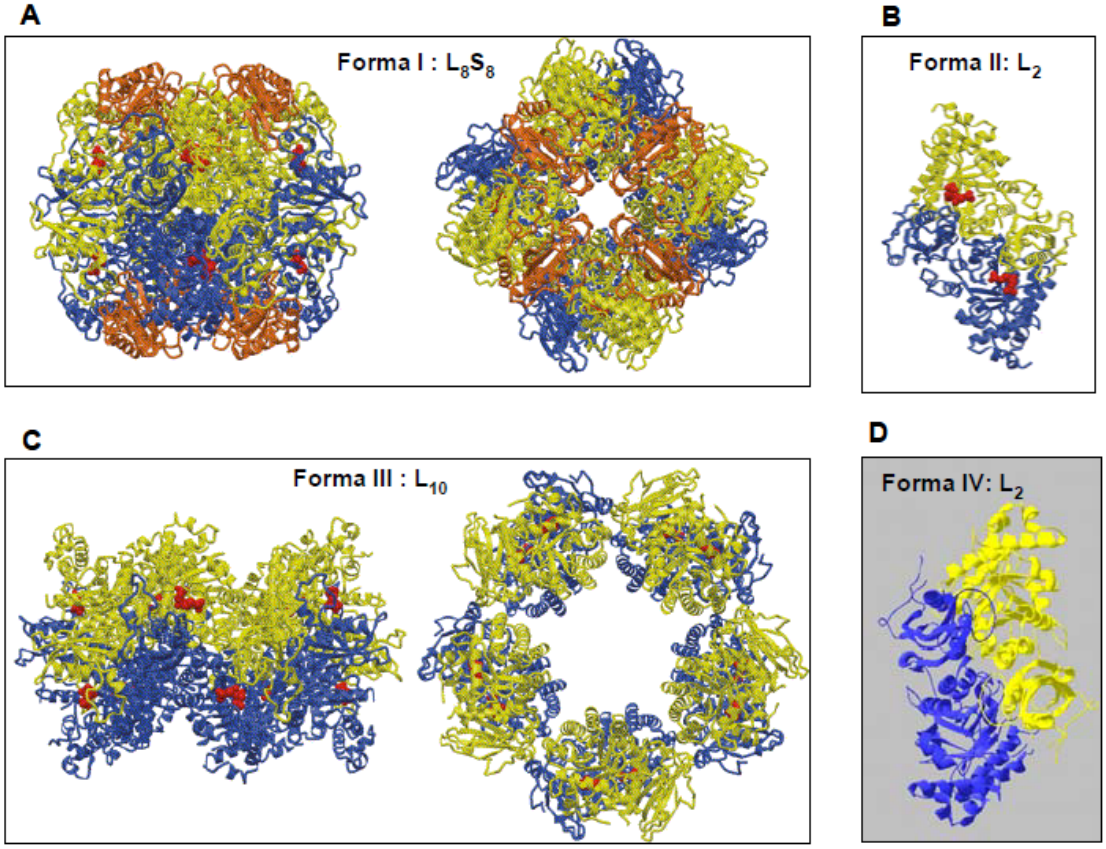


Fig.3. Representative structures of different forms of RubisCO: All forms are comprised of dimers of catalytic large subunits. Form I is comprised of four dimers with small subunits decorating the top and bottom of the L_8 octomeric core. Only form I has small subunits. Form II is comprised of dimers of L, ranging from L_2–L_8 depending on the source. Form III is found only in some archaea and is comprised of dimers of L in either an L_2 or $(L_2)_5$ arrangement. Form IV (the RubisCO-like Protein or RLP) appears thus far always to have an L_2 structure [from Li *et al.*(2005); Tabita *et al.*(2007)].

Analysis of amino acid sequence divergence has revealed two distinct phylogenetic branches of the form I RubisCOs (fig. 4). Accordingly, a distinction has been made between green-type enzymes (forms I A and B from cyanobacteria, green algae and higher plants) and red-type enzymes (forms I C and D from non-green algae and phototrophic bacteria) (Delwiche *et al.*, 1996 and Tabita *et al.*, 1999). Form II RubisCO is a dimer of large subunits (fig. 3) which is structurally very similar to any of the dimers integrating the core large subunit octamer of form I. Indeed, the main structural differences with form I are related to the absence of small subunits. Form II RubisCOs are primarily present in chemoautotrophs, phototrophic proteobacteria, and dinoflagellates (Tabita 1999). An interesting feature of form II RubisCO is that it is often found in organisms that also contain form I. It has been suggested that form II enzymes are adapted to low-O_2 and high-CO_2 environments (Badger and Bek, 2008).

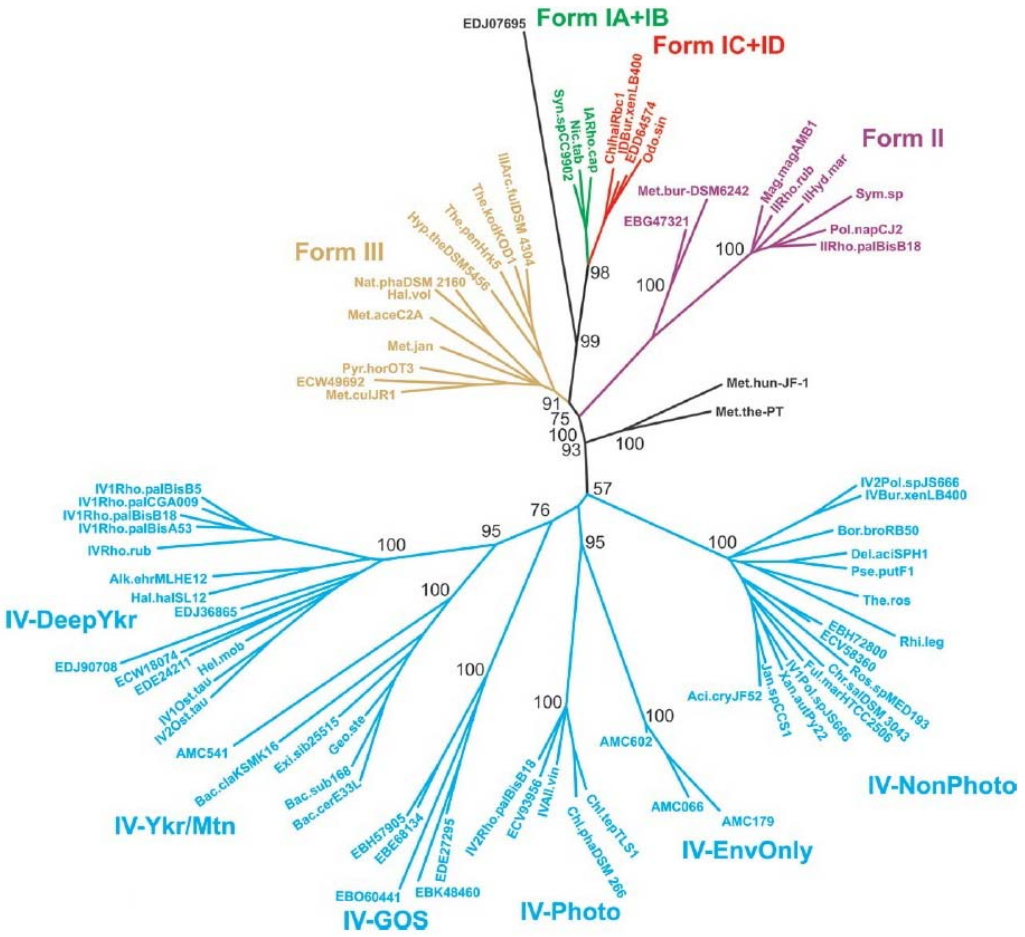


Fig.4. Phylogenetic tree illustrating the three classes of RubisCO and the six clades of RLP: The tree was constructed from a CLUSTALW alignment of a non-redundant set of RubisCO and RLP sequences taken from publicly accessible sequence databases, primarily GenBank. The nonredundant set was selected such that each sequence was no more than 76% identical at the amino acid level to any other sequence in the alignment. The tree was produced by Neighbor–Joining in the MEGA 4.0 software suite using the p-distance model and pair wise gap deletion. Bootstrap values are the percentage of 1000 trials in which a given node was present [Taken from Tabita *et al.*(2008)].

FORM III RUBISCO

Studies carried out during the last two decades have described form III RubisCO in anaerobic archaea growing at high temperatures, such as *Methanococcus jannaschii*, and some mesophilic heterotrophic methanogens (Bult *et al.*, 1996; Klentk *et al.*, 1997; Mueller-Cajar and Badger, 2007; Tabita *et al.*, 2008). Sequences of these proteins are phylogenetically well separated from known RubisCO forms I and II (fig. 4). In all these cases RubisCO is found to assemble in L_2 homodimers. Moreover, in the Form III RubisCO purified from the hyperthermophilic archaebacterium *Thermococcus* (formerly *Pyrococcus*) *kodakarensis* (Ezaki *et al.*, 1999) large subunit dimers are arranged in a pentameric structure $(L_2)_5$ (fig. 3). It has been shown that these form III enzymes are capable of catalyzing RuBP-dependent CO_2 fixation *in vitro* (Watson *et al.*, 1999) and are active *in vivo* (Finn and Tabita, 2003) under anaerobic conditions, displaying an extremely high affinity for O_2 (Kreel and Tabita 2007). However, form III RubisCOs probably do not contribute to net carbon fixation since archaebacteria lack phosphoribulokinase (which catalyzes the formation of RuBP from ribulose-5-phosphate) (Watson and Tabita 1999 and Sato *et al.*, 2007) and therefore, cannot regenerate RuBP through the Calvin cycle. It is assumed that archaeal form III RubisCOs probably fulfil a catabolic role removing RuBP produced during purine/pyrimidine metabolism. Thus, RuBP is produced from 5-phospho-D-ribose-1-pyrophosphate (RuPP), as shown in the methanogenic archaeon *Methanoccocus jannaschii* (Finn and Tabita 2004), and further metabolized by form III RubisCO as a catabolic step in the RuPP pathway.

Form III RubisCOs differ among themselves in their optimal temperature for activity, as well as their degree of sensitivity to O_2 (Finn and Tabita, 2003). The study of these special features of form III presents several additional points of interest. For example, a comparative study with RubisCOs from aerobic species may illuminate the evolutionary changes associated with the increased presence of O_2. Furthermore, the ability to catalyze RuBP carboxylation at extreme temperatures is a structural adaptation that could provide relevant information about the structure-function relationship in the catalytic site of RubisCO (Finn and Tabita, 2003). Although the classification of RubisCO enzymes into forms I, II, and III is generally supported by sequence phylogenies, quaternary structures, and functional properties together, there may be exceptions as in the structural and biochemical properties of the RubisCO enzyme from the archaeon *Methanococcoides burtonii*, which correlate to form III despite close sequence identity to form II RubisCOs (Alonso *et al.*, 2009).

FORM IV RUBISCO

Form IV is also called the RubisCO-like protein (RLP) because this protein does not catalyze RuBP carboxylation or oxygenation (Hanson and Tabita 2001). Yet, there are similarities in the primary (Hanson and Tabita 2001) and tertiary (Li *et al.,* 2005; Imker *et al.,* 2007) structures of these proteins that clearly indicate that RLPs are homologues of carboxylating RubisCOs and are derived from some common ancestor (Tabita *et al.,* 2007). Sequence analysis suggests six different clades of RLPs (fig 4). Structural information is scarce but indicates that this form may be a L_2 dimer. RLPs do not possess RubisCO activity because they have nonidentical residues at positions where key conserved residues of the active site of RubisCO are normally found.

It is known that the *Bacillus subtilis* (Ashida *et al.,* 2003) and *Geobacillus kaustophilus* (Imker *et al.,* 2007) RLPs, the cyanobacterial RLP from *Microcystis aeruginosa* (Carre-Mlouka *et al.,* 2006) and RLPs from the photosynthetic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas palustris* participate in a methionine salvage metabolic pathway by catalyzing the enolization of the 2,3-diketo-5-methylthiopentyl-1-phosphate. This substrate is a RuBP analogue, and the enolization reaction is very similar to one of the steps of the RubisCO carboxylation/oxygenation reaction mechanism (see below). On the other hand, physiological results indicate that RLP from the green sulphur bacterium *Chlorobium tepidum* is involved in thiosulphate oxidation (Hanson and Tabita 2001, 2003); even if the precise reaction catalyzed by the RLP has not been identified. Other clades of RLP molecules from different organisms have not yet been assigned a function. Interestingly many of these latter RLP genes do not complement RLP-knockout strains from organisms with defined functions, suggesting different physiological roles (Singh and Tabita 2009).

Only one archaeon, *A. fulgidus*, and one eukaryote, the alga *Ostreococcus tauri*, have thus far been shown to contain an RLP gene (Tabita *et al.,* 2007). Additionally, each of these organisms contains a functional CO_2-fixing RubisCO, a form III enzyme in *A. fulgidus* (Watson *et al.,* 1999) and a form I enzyme in *O. tauri* (Robbens *et al.,* 2007). It has been suggested that photosynthetic RubisCOs may have evolved from an ancestral RLP that was functional in the methionine salvage pathway (Ashida *et al.,* 2005). Diversification of RuBP-carboxylating RubisCO forms may have occurred later, driven by changes in atmospheric composition and adaptation to particular habitats. A plausible outline of this process is presented in fig. 5.

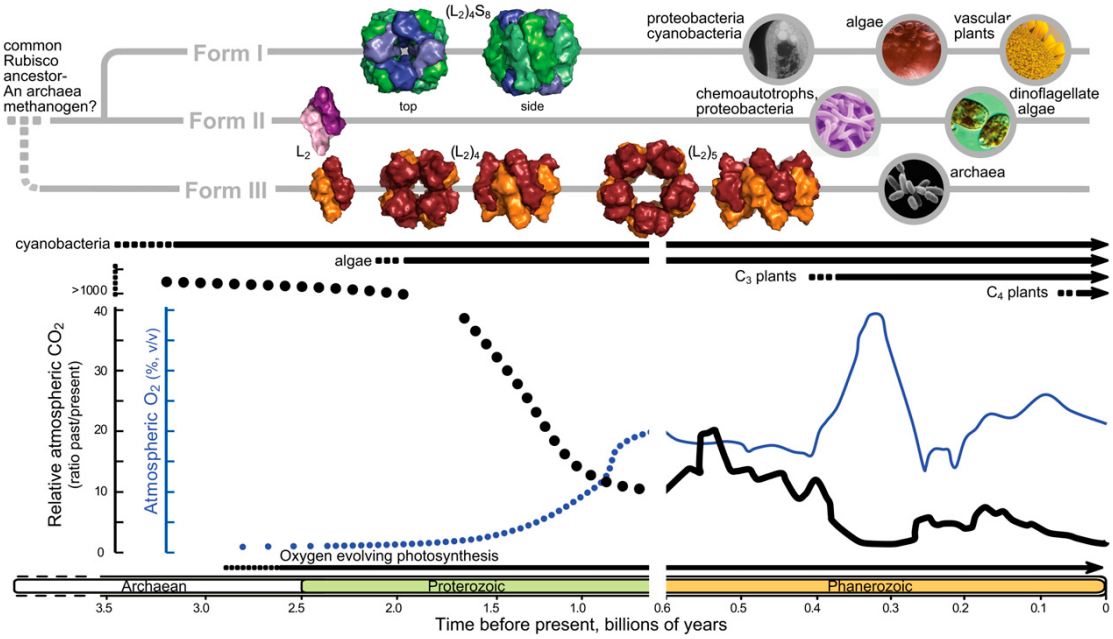


Fig.5. Hypothetical profiles of RubisCO phylogeny: the evolutionary timelines of different photosynthetic organisms, and the variation in atmospheric CO_2 (thicker line) and O_2 levels during earth's history. Hypothetical atmospheric CO_2 and O_2 levels prior to 0.6 billion years ago are represented by dotted lines. Quaternary structures of each RubisCO were drawn with Pymol using Protein Data Bank coordinates for the spinach (Spinacia oleracea) (L2)4S8 (8RUC), R. rubrum L2 (5RUB), Pyrococcus horikoshii (L2)4 (2CWX), and Thermococcus kodakaraensis (L2)5 (1GEH) enzymes. Structures for larger form II (L2)n RubisCOs are unavailable. Encircled images depict types of organisms where the different RubisCO forms are found [Adapted from Tabita *et al.*, (2008) and Badger *et al*., (2002)].

SYNTHESIS AND ASSEMBLY OF RUBISCO

GENE ORGANIZATION

Genetic studies of RubisCO began in earnest after Wildman and coworkers investigated large and small subunit tryptic peptide differences in interspecific crosses of tobacco (Chan and Wildman 1972; Kawashima and Wildman 1972; Kung *et al.*, 1974). The large subunits were transmitted uniparentally and small subunits were transmitted biparentally. The uniparental inheritance of the large subunit implied that its gene (called *rbc*L) would reside in the chloroplast DNA. Gene cloning and sequencing confirmed this assumption for the maize chloroplast (McIntosh *et al.*, 1980). The *rbc*L gene in higher plants and green algae is present as a single copy per chloroplast genome, but because many copies of the genome are present in each plastid, the actual *rbc*L copy number per chloroplast can be high. With rare exceptions, *rbc*L does not contain introns and encodes ~ 475 amino acids (Gutteridge and Gatenby, 1995). In higher plants and green algae, the small subunit is encoded by a multigene family (*rbc*S genes) in the nuclear genome (Dean *et al.*, 1989; Clegg *et al.*, 1997). This gene family may consist of 2 to 22 members (or even more in wheat), depending on the species (Sasanuma, 2001; Spreitzer, 2003). The model green alga *C. reinhardtii* contains only two

*rbc*S genes (Goldschmidt-Clermont and Rahire, 1986). Almost all *rbc*S genes have introns in highly conserved positions (Spreitzer, 2003) and encode a polypeptide of some 123 residues. Members of an *rbc*S gene family are generally more similar to each other than to members of a family in a different species, resulting in only a few amino acid differences in the small subunits within a family (Spreitzer, 2003). Some photosynthetic eukaryotes, other than higher plants and green algae, have a different location for the RubisCO genes. The chloroplast genome of brown (Chromophyte) and red (Rhodophyte) algae holds the genes encoding both the large and the small RubisCO subunits. On the other hand, dinoflagellates, which lack *rbcS* genes because they only have the form II of the enzyme in their chloroplasts, encode *rbcL* in the nuclear genome. When RubisCO subunit genes are encoded together in the same genome, as in bacteria and non-green algae (where both subunits are encoded in the chloroplast), they are often part of a much larger operon regulated by a single promoter (Tabita, 1999) (fig. 6). The proteobacterial operon encoding RubisCO genes contains also structural genes for other enzymes of the Calvin-Benson-Bassham cycle (hence termed *cbb* operon).

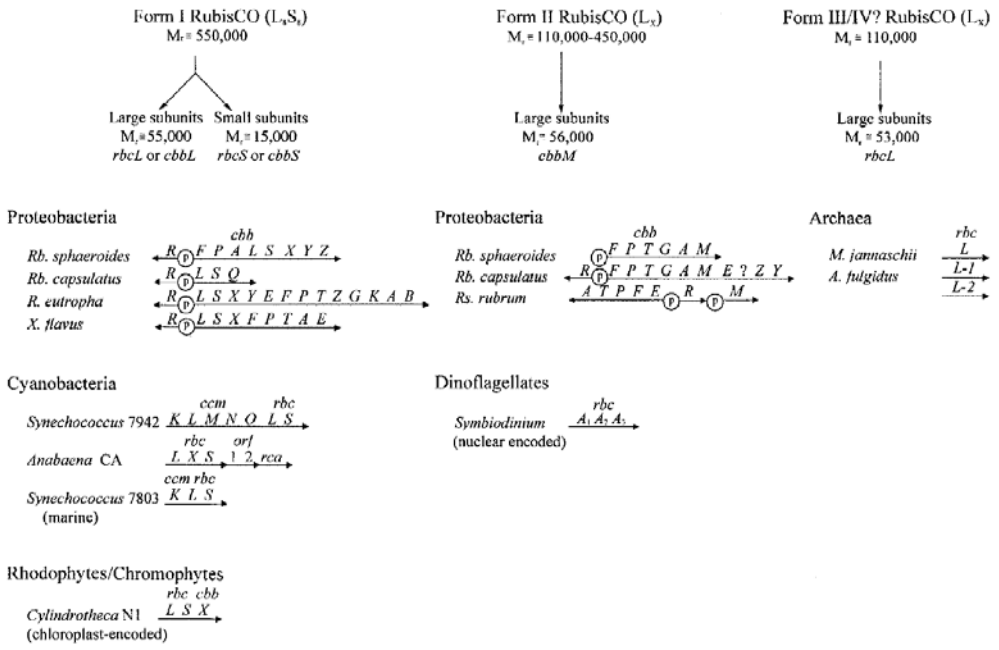


Fig.6. Organization of microbial RubisCO genes from proteobacteria, cyanobacteria, archaea, and eukaryotic rhodophytes, chromophytes, and dinoflagellates. Arrows refer to the direction of transcription, with the arrowhead delimiting the various gene clusters comprising individual operons; encircled P's indicate promoter sequences controlling transcription. In the proteobacteria, the form I RubisCO genes (*cbbLS*) are located in an operon with other Calvin cycle structural genes, as is the form II RubisCO gene (*cbbM*). These include fructose 1,6/sedoheptulose 1,7 bisphosphatase (*cbbF*), phosphoribulokinase (*cbbP*), aldolase (*cbbA*), phosphoglycolate phosphatase (*cbbZ*), transketolase (*cbbT*), glyceraldehyde phosphate dehydrogenase (*cbbG*), pentose 5-phosphate 3-epimerase (*cbbE*), phosphoglycerate kinase (*cbbK*) and genes of unknown function (*cbbX*, *cbbY*, *cbbA*, and *cbbB*). In all cases, transcription of the *cbb* operons is controlled by the product of the divergently transcribed *cbbR* gene. In *Synechococcus* 7942, the *rbc* genes are not cotranscribed with the *ccm* genes, but *ccmK* is cotranscribed with *rbcLS* in marine cyanobacterial WH strains. A *cbbZ* sequence is invariably found downstream from the form I *rbcLS* genes of eukaryotic nongreen algae [Taken from Tabita (1999)].

BIOSYNTHESIS OF RUBISCOS SUBUNITS

Under natural conditions, the regulation of the expression of RubisCO genes in higher plants and red algae is carried out at both at the transcriptional and post-transcriptional level. The transcription of the chloroplastic *rbc*L gene, driven by a strong promoter, is significantly increased during light periods although part of this effect may not be under the direct control of light but of the circadian clock (Misquitta and Herrin, 2005). In addition, control is also exerted at the level of transcript stability. The half-life of *rbc*L transcripts is known to be about 15 times lower in the light than in the dark (Salvador *et al.,* 1993). Hence, light promotes the degradation of the *rbc*L transcript and this effect is mediated by a redox signal since inhibition of photosynthetic chain increases the stability of the transcripts in illuminated cultures (Salvador and Klein, 1999). It appears that some sequences in the 5' UTR may be crucial for stabilizing the *rbcL* transcript in the dark or under non-reducing conditions (Salvador *et al.,* 2004; Suay *et al.,* 2005). The expression of *rbcS* is also regulated by light at the level of transcription (Thompson and White 1991). In gymnosperms and green algae, however, the *rbcS* mRNA and its translational product have been reported to be accumulated in the dark (Yamamoto et al. 1991, Goldschmidt-Clermont and Rahire 1986, Malnoe *et al.,* 1988).

Translation of the *rbcS* transcript on cytosolic ribosomes of higher plants and green algae produces a precursor polypeptide. Translocation across the chloroplast envelope removes the N-terminal plastid-targeting peptide, and releases the mature form in the stroma of chloroplasts. On the other hand, the synthesis of the large subunit polypeptide occurs entirely on chloroplast ribosomes of higher plants and green algae. The biosynthesis of large and small subunits is carried out in a regulated manner. It was previously reported that silencing of *rbc*S expression by introducing antisense RNA (Rodermel *et al.,* 1996) or by a gene knockout (Khrebtukova and Spreitzer, 1996) prevented the accumulation of the L-subunit. Similarly, when the translation of the *rbc*L transcript was inhibited by a nonsense mutation or by chloramphenicol, the S-subunit was rapidly degraded, indicating that it cannot accumulate in its free and unassembled form (Schmidt and Mishkind, 1983; Spreitzer *et al.,* 1985). In higher plants and in green algae, expression of subunits that are part of large protein complexes is highly coordinated by chloroplast encoded subunits (Choquet *et al.,* 1998, 2001). The basis for this control system is that elimination of one subunit reduces the expression of the other subunits in the complex by their

translational arrest, or by degradation of unassembled subunits (Cohen *et al.*, 2006).

In bacteria and non-green algae, RubisCO genes are usually expressed as components of an operon, In proteobacteria, which encode RubisCO together with other Calvin cycle components in the same operon, it is noteworthy that, even if all operon genes are co-transcribed, the intracellular level of RubisCO far exceeds that of the other Calvin cycle enzymes (Gibson *et al.*, 1991; Meijer *et al.*, 1991; Schaferjohann *et al.*, 1995) indicating a different post-transcriptional processing of the different cistrons.

HOLOENZYME ASSEMBLY

In photosynthetic eukaryotes, the assembly of RubisCO from its constituent subunits takes place in the chloroplast. Early experiments revealed that a binding protein (Cpn60) was associated with newly synthesized RubisCO in chloroplasts (Barraclough and Ellis 1980; Bloom *et al.*, 1983; Roy *et al.*, 1988). Although at that time the exact role of this protein was not known, it was hypothesized that it could aid RubisCO folding by avoiding unwanted non-covalent interactions of the unstructured polypeptides, somehow acting as a scaffold to bring about the right conformation of the enzyme.

Indeed, the term "chaperone", which was first coined for this function (Gatenby and Ellis, 1990), was rapidly extended to a growing family of proteins that help the folding of other proteins in different ways. It was later demonstrated that a certain subset of chaperones, the chaperonins, were required to obtain assembled RubisCO folding in a bacterial host system (Goloubinoff *et al.*, 1989b) and for *in vitro* refolding of form II RubisCO (Goloubinoff *et al.*, 1989a). These are special chaperones that isolate polypeptides restricting them to closed environment and assist their folding in a process that consumes ATP.

Chloroplast chaperonins Cpn60 and Cpn21, which are homologues of the components of the bacterial GroEL/GroES system (Hemmingsen *et al.*, 1988), facilitate the correct folding of the L-subunits which assemble in an octamer core. Afterwards, the S-subunits join the L-subunit core to form the final L_8S_8 holoenzyme in an ATP-independent manner (Gatenby and Ellis 1990; Hartman and Harpel 1994). It is likely that the assembly of the whole holoenzyme requires several types of chaperones, as suggested by the fact that the bacterial DnaK/DnaJ/GrpE chaperone system aids in folding of recombinantly expressed RubisCO (Checa and Viale 1997), but the chaperonins are still found to be essential for the initial steps of folding and assembly of RubisCO large subunits under all circumstances.

Form II RubisCO and prokaryotic form I RubisCO can be recombinantly expressed and assembled in *E.coli* (Gatenby *et al.,* 1985; Somerville and Somerville 1984). However, it has not been possible to assemble eukaryotic form I RubisCO outside of the chloroplast hitherto, even in the cases in which chloroplast chaperonins were also coexpressed in *E. coli* (Cloney *et al.,* 1993). It appears that further specific folding or assembly factors that occur in the chloroplast (and are missing in *E. coli*) are necessary for building the correct holoenzyme structure.

POST-TRANSLATIONAL MODIFICATIONS

Both the large subunit and the small subunit of RubisCO are extensively modified either during or after translation (Houtz and Portis, 2003). Evidence for these amino acid modifications comes primarily from structural analyses of the mature forms of the polypeptides and subsequent comparison with the known DNA sequences. Most of these post-translational modifications have unknown function, but they are supposed to be involved in the regulation of enzyme interactions with other proteins or in maintaining the stability of the enzyme.

The large subunit of RubisCO of higher plants, algae and cyanobacteria undergoes post-translational modifications such as acetylation, N-methylation, phosphorylation and possibly transglutamination (Hartman and Harpell, 1994, Houtz and Portis, 2003). The tri-methylation of the Lys 14 residue is found in RubisCOs of some higher plants (tobacco, tomato, pea, soybean, potato) but not in others (spinach, wheat) (Houtz *et al.,* 1992). In the particular case of *C. reinhardtii*, X-ray crystallography revealed also the methylation of cysteines 256 and 369, and the hydroxylation of prolines 104 and 151 (Taylor *et al.,* 2001; Mizohata *et al.,* 2002).

Before small subunits are imported into the chloroplast, the N-terminal transit sequence is phosphorylated (probably at Ser-21) as part of a process that regulates import (Su *et al.,* 2001). During or immediately after import, the transit sequence is removed by a stromal processing peptidase in what appears to be a two-step proteolytic process (Oblong and Lamppa 1992; Su *et al.,* 1993, 1999; Robinson and Ellis 1984a,b). The final processing exposes a conserved Met residue, which is methylated to form an $^{\alpha}$N-methylmethionine (Grimm *et al.,* 1997; Whitney and Andrews 2001). This is a widespread modification found in the small subunit of RubisCOs from a number of species (Grimm *et al.,* 1997; Mizohata *et al.,* 2002; Taylor *et al.,* 2001; Ying *et al.,* 1999). The enzyme responsible for this modification, the RubisCO S-subunit $^{\alpha}$N-methyltransferase (SSMT) (Ying *et al.,* 1999), is related to the L-subunit $^{\varepsilon}$N-methyltransferase

(LSMT) which catalyzes the methylation of Lys-14 in the L-subunit (Houtz *et al.*, 1991).

The carbamylation of Lys 201, which is crucial for conforming the catalytically competent active site, as well as the oxidative modifications of certain amino acid residues, which occur usually under stress or senescence conditions and are marking steps of the enzyme catabolism, will be specifically discussed in later sections.

RUBP CARBOXYLATION/OXYGENATION ACTIVITY OF RUBISCO

This section and subsequent ones will concentrate on the activity and characteristics of the most studied type of RubisCO: the green-like form I from cyanobacteria, green algae and higher plants.

RUBISCO ACTIVATION

RubisCO must be activated to carry out carboxylation or oxygenation. The catalytically competent form of RubisCO is a complex of enzyme-CO_2-Mg^{2+}, in which the molecule of CO_2 is different from the substrate. The first step in the activation process is carbamylation by CO_2 at an uncharged amine of a lysine residue (Lys201, in the spinach enzyme numbering) located near the active site of RubisCO. The carbamylated Lys201 is stabilized by coordinating a Mg^{2+} ion at the active site in concert with residues Asp203 and Glu204 (Cleland *et al.*, 1998; Kellogg and Juliano 1997).

Formation of the carbamate at Lys201 with CO_2 is spontaneous *in vitro* (Houtz and Portis 2003). However, RuBP can bind to RubisCO before carbamylation creating a dead-end enzymatically-inactive complex (fig.7). Additionally, other naturally occurring sugar phosphate analogs, such as 2-carboxy-arabinitol-1-phosphate (CA1P), D-xylulose-1,5-bisphosphate (XuBP), and D-glycero-2,3-pentodiulose-1,5-bisphopsphate (PDBP), can also bind tightly to the active site thereby inhibiting RubisCO catalytic activity (Lorimer *et al.*, 1977; Parry *et al.*, 2003; Parry *et al.*, 2008). The removal of tightly bound inhibitors from the catalytic site of the carbamylated and decarbamylated forms of RubisCO requires an ancillary enzyme, RubisCO activase, and the hydrolysis of ATP. (fig.7). RubisCO activase is a AAA+ protein that releases inhibitor substrates from the active site of RubisCO in an ATP-dependent manner and allows the reactivation of RubisCO (Kellogg and Juliano 1997).

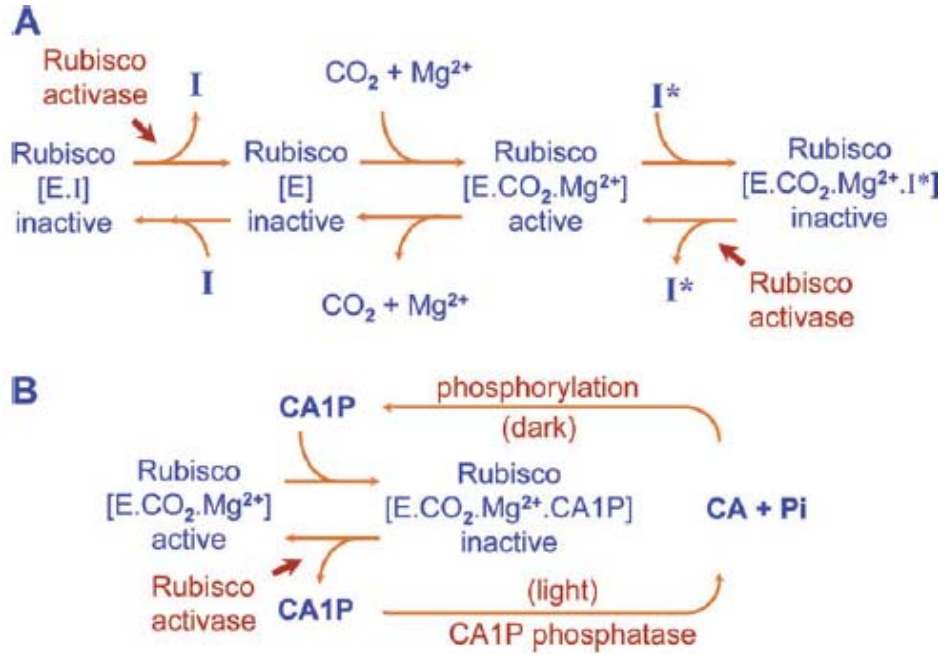


Fig.7. (A) Decarbamylated RubisCO, E, can bind substrate, EI, and become inhibited. Carbamylated RubisCO, $E.CO_2 .Mg^{2+}$, can also become inhibited when bound with substrate analogues, $E.CO_2 .Mg^{2+}.I^*$. RubisCO activase is needed in both of these conditions to remove the inhibitory sugar-phosphate compound. (B) Carbamylated RubisCO can be inhibited by 2-carboxy-arabinitol-1- phosphate (CA1P) or D-glycero-2,3-pentodiulose-1,5-bisphosphate (PDBP) in the dark and reversibly activated by RubisCO activase in the light [Taken from Parry *et al.*, (2008)].

RubisCO activase is regulated by the ratio of ATP to ADP (being inhibited by high concentrations of ADP), therefore RubisCO is activated by RubisCO activase in response to light due to the increase in ATP concentrations from the light-dependent reactions during the day (Graciet *et al.*, 2004; Parry *et al.*, 2003). In addition, the pumping of protons into the thylakoids driven by the photoelectronic transport chain promotes alkalization and release of Mg^{2+} ions in the stroma, thereby favouring RubisCO carbamylation. In this way, these regulatory effects ensure that, immediately after the onset of light, the RubisCO active site is freed from inhibitors, becomes carbamylated, and is ready to participate in catalysis (Parry *et al.*, 2008).

CATALYTIC MECHANISM

Carboxylation of ribulose-1,5-bisphosphate yields an unstable phosphorylated six-carbon intermediate (2-carboxy-3-keto-arabinitol-1,5-bisphosphate), which decays while still bound to the enzyme into two molecules of glycerate-3-phosphate. As a first step of the reaction mechanism, a proton is abstracted from C-3 of the ribulose 1,5-*bis*phosphate bound to the enzyme to create a 2,3-enediolate intermediate which is highly reactive. The nucleophilic enediolate attacks CO_2 producing the 2-carboxy-3-ketoarabinitol 1,5-bisphosphate, which is subsequently hydrated to an unstable gem diol

intermediate. The C-2-C-3 bond of this intermediate is immediately cleaved, generating a carbanion and one molecule of 3-phosphoglycerate. Stereospecific protonation of the carbanion yields a second molecule of 3-phosphoglycerate, thereby concluding the catalytic cycle (fig.8).

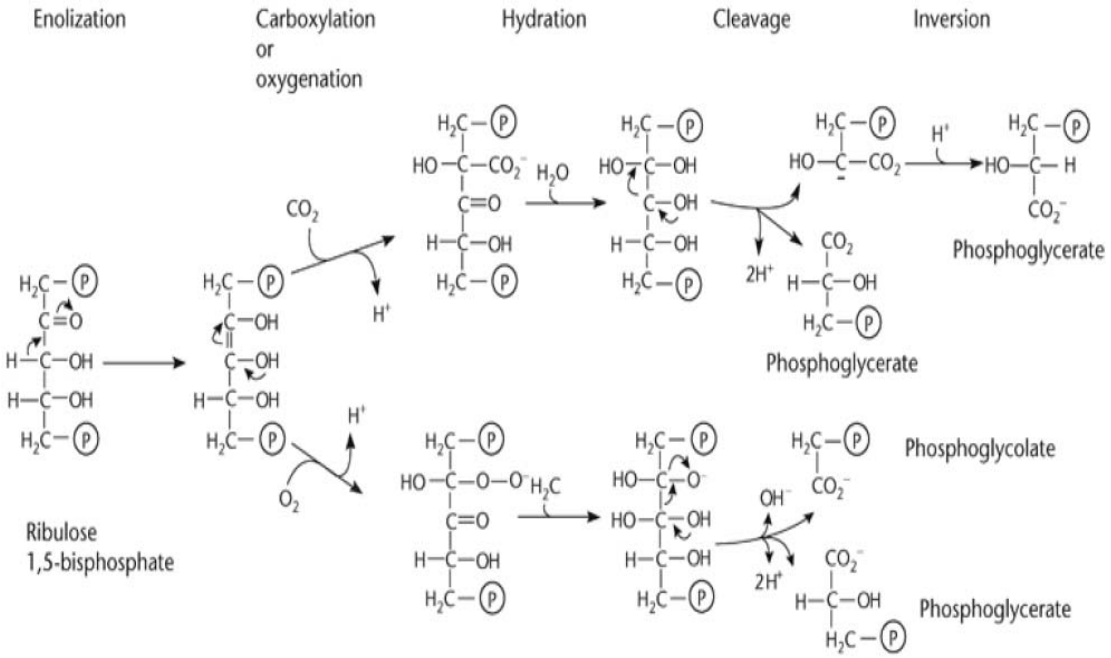


Fig.8. Catalytic cycle of carboxylation and oxygenation of RuBP by RubisCO [Adapted from Hartman and Harpel (1994)].

In the competing reaction of oxygenation, the RubisCO-RuBP enediolate complex captures O_2 and through similar steps of hydration and cleavage yields only one PGA molecule and one molecule of 2-phosphoglycolate (fig. 8). After elucidation of the carboxylation mechanism of RubisCO, it became clear that there was no specific mechanism for oxygenation. The latter reaction takes place as an unavoidable side-reaction of carboxylation due to the fact that the enediolate intermediate is reactive to O_2 as well as to CO_2 (Andrews and Lorimer 1987). Because neither of the alternative substrates (O_2 or CO_2) binds to the enzyme before reacting, the enzyme cannot control the access of O_2 and the subsequent production of the useless 2-phosphoglycolate. To retrieve the carbons in 2-phosphoglycolate, a complicated process of photorespiration takes place, incurring a net loss of CO_2 (about one CO_2 molecule per two captured O_2 molecules) and, therefore, reducing the net photosynthetic carboxylation rate. Thus, the opposing oxygenase activity is an intrinsic characteristic of RubisCO due to a catalytic mechanism failure (probably reflecting the fact that the CO_2-fixing activity of RubisCO was evolutively established when there was no substantial amount of oxygen in the atmosphere to compete), and the photorespiratory

pathway is likely to have evolved subsequently to avoid 2-phosphoglycolate accumulation and to recycle three-fourths of its carbon at the price of losing the other fourth.

STRUCTURAL CHARACTERISTICS OF THE CATALYTIC SITE

The characteristics of the catalytic mechanism are directly dependent on the structural details of the active site. X-ray crystallography has been used to elucidate the folding of a number of RubisCO enzymes. The three-dimensional structure of the form II RubisCO from *Rhodospirillum rubrum* was the first to be solved (at 2.9Å resolution) (Somerville and Somerville, 1984, Schneider *et al.*, 1986), followed by the structures of the form I enzymes from spinach (Andersson *et al.*, 1989; Knight *et al.*, 1989; Knight *et al.*, 1990), tobacco (Curmi *et al.*, 1992; Schreuder *et al.*, 1993a; Schreuder *et al.*, 1993b). *Synechococcus* (Newman *et al.*, 1993, Newman and Gutteridge, 1993; Newman and Gutteridge, 1994) and, later, many others including representatives of forms III and IV. Despite apparent differences in amino acid sequence and function (in the case of the RLPs) of the various forms, the tertiary structure of the large (catalytic) subunit is extremely well conserved throughout the different forms of RubisCO (reviewed in Andersson and Taylor, 2003; Andersson and Backlund, 2008).

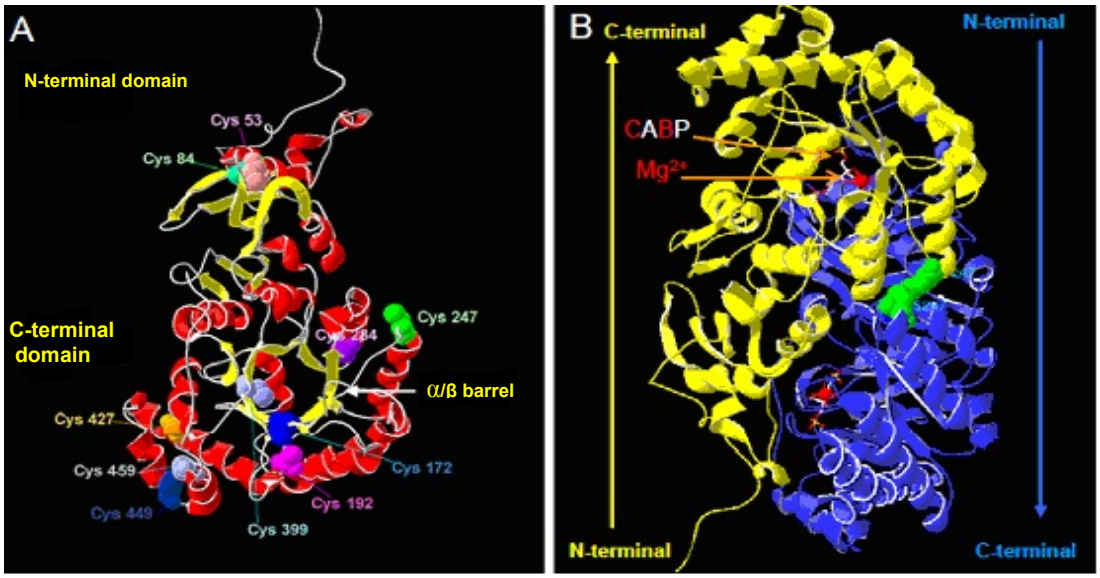


Fig.9. Folding of the large subunit of RubisCO of *C. reinhardtii*: (A). β strands are shown in yellow and α helices in red. Each subunit consists of a small N-terminal domain and large C-terminal domain, containing a central α/β barrel structure. The position of some cysteine residues is indicated. (B). Structural unit of the enzyme, comprising a dimer of antiparallel large subunits. The active site is conformed with residues of the α/β barrel located in the C-terminal domain of one subunit, and with residues of the N-terminal domain of the other subunit. Each subunit is in one color (yellow and blue). CABP and Mg^{2+} bound to the one active site are indicated. Cys247 residues maintaining the dimer linked by a disulfide bridge are highlighted in green. Taken from García-Murria, (2006).

The overall fold of the large (catalytic) subunit is composed of a smaller amino-terminal domain consisting of a four-to-five-stranded mixed β sheet with helices on one side of the sheet, and a larger carboxy-terminal domain (Andersson and Taylor 2003). The carboxy-terminal domain consists of eight consecutive βα-units linked by loops of varying length and arranged as an eight-stranded parallel α/β barrel structure. The active site is located at the carboxy-terminal end of the β-strands, with the loops connecting the βα-units contributing several residues involved in catalysis and substrate binding. Residues from the amino-terminal domain of the adjacent large subunit in the dimer complete the active site. Thus, the functional unit structure of RubisCO is an L2 dimer of large subunits harbouring two active sites (fig.9).

The determination of the structure of RubisCO with the RuBP-analogue inhibitor CABP (2-carboxy-D-arabinitol-1,5-bisphosphate) bound to the enzyme facilitated the identification of the specific residues involved in catalysis. The substrate binds in an extended conformation across the opening of the α/β barrel and is anchored by two distinct phosphate-binding sites at opposite locations of the α/β barrel, and in the middle by the magnesium-binding site (fig. 10).

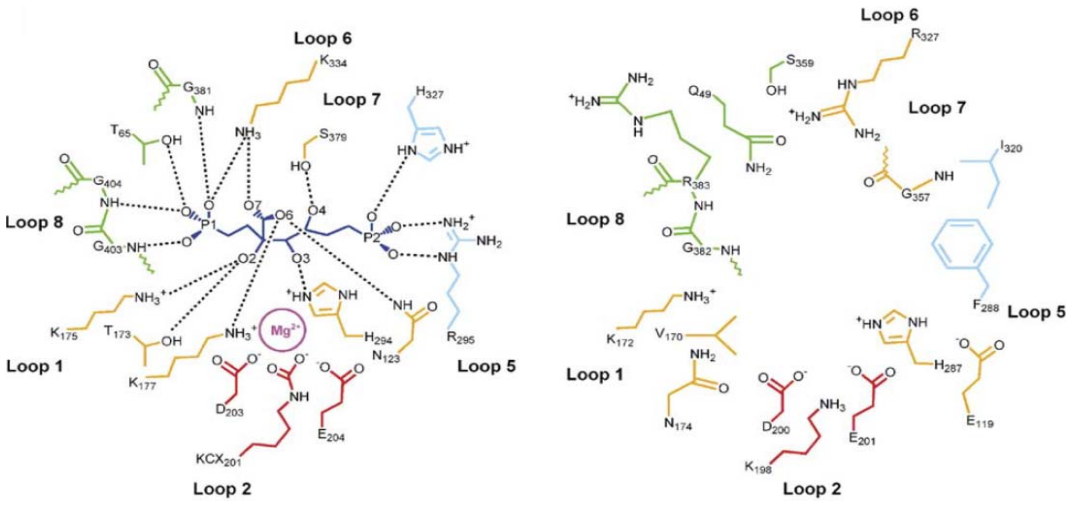


Fig.10. Schematic representation of the active site of spinach RubisCO in an empty state (right) or occupied by the substrate-analogue CABP (left): The left picture shows the hydrogen bonds between the active site residues and CABP. CABP is shown in dark blue. Procedence of each residue is indicated by the loop where it resides (loops connecting the βα-units of the α/β barrel are numbered from the N-terminal end). Residues are numbered relative to spinach RubisCO (Adapted from Li *et al.*, (2005)).

Residues involved in CABP binding can be divided into four groups: those forming hydrogen bonds with (1) P1 phosphate, (2) P2 phosphate, and (3) the carboxyarabinitol backbone; and those (4) coordinating the metal ion (Knight *et al.*, 1990). P1 binding residues are located at loops 7 (Gly381) and 8 (Gly403,

Gly404) of the C-terminus and at the N-terminus (Thr65) of the adjacent subunit. Binding of phosphate P2 is accomplished by residues of the loops 5 (Arg295) and 6 (His327) of the C-terminal domain (Knight *et al.,* 1990).

The Mg^{+2} ion is coordinated by the carboxyl group of the carbamylated Lys201 and the hydroxyl groups of Asp203 and Glu204 (from loop 2). The remaining coordination sites are occupied by water molecules and the hydroxyl groups at C2 and C3 from the sugar phosphate (Knight *et al.,* 1990). Crystallographic data together with mutagenesis and computational studies over the last three decades have contributed to dissect the catalytic mechanism, assigning precise functions to specific residues. Thus, the carbamylated Lys201 is crucial for starting the reaction by acting as a base that abstracts the proton in the enolization step, while Lys 334 (from loop 6) stabilizes the carboxylated intermediate and His294 (from loop 5) seems to have a wider role, being involved in hydration and C2-C3 bond scission (Kannappan and Gready, 2008).

The loop 6 (connecting β-strand 6 with α-helix 6 in the large subunit carboxy-terminal α/β –barrel) is loosely bound to the rest of the structure acting as a mobile flap that alternates between an open and a closed active site conformation. The open conformation lets the sugar substrate and products diffuse in and out of the active site while the closed conformation appears to be necessary for catalytic competence. Thus, in every catalytic cycle, loop 6 should change conformation, switching from open (to let RuBP in) to closed (in order the reaction to proceed) and back to open (to let the products out) again. Two strictly conserved glycine residues, Gly333 and Gly337, maintain flexibility in the hinge of loop 6. The other strictly conserved residue is Lys334 which, in the closed conformation, extends its side chain into the active site interacting with one of the two oxygen atoms of the six-carbon intermediate (Knight *et al.,* 1990; Andersson, 1996) (fig. 10).

CARBOXYLATION vs OXYGENATION: THE RELATIVE SPECIFICITY FACTOR

Due to the competitive cross-inhibition between CO_2 and O_2, the rates of carboxylation (v_c) and oxygenation (v_o) by RubisCO are defined by the product of an intrinsic factor of the enzyme (Ω) and the concentration ratio of CO_2 to O_2 at the active site:

$$(v_c/v_o) = \Omega \cdot ([CO_2]/[O_2]$$

Hence, carboxylation may be favoured over oxygenation by increasing the CO_2 (or decreasing the O_2) concentration in the environment surrounding RubisCO. This has been accomplished by many algae by pumping water-dissolved bicarbonate inside compartments containing RubisCO and carbonic anhydrase (to

release CO_2 from excess bicarbonate). While this procedure is not amenable to non-aquatic organisms, two special pathways have evolved to the same end in some land plants: the C4 and the crassulacean acid (CAM) metabolisms. Both pathways are additional to the Calvin cycle and increase the supply of CO_2 to RubisCO, thereby reducing the oxygenation reaction and the flux to the photorespiratory pathway. In C4 plants such as maize (*Zea mays*) and sugarcane (*Saccharum officinarum*), CO_2 is taken up in mesophyll cells by an oxygen-insensitive carboxylase and released by specific decarboxylases in bundle sheath cells (which are internal cells around veins) where RubisCO is located (reviewed by Drincovich *et al.,* 2010). Similarly, crassulaceans capture CO_2 at night through a transient organic acid acceptor, and release it near RubisCO during the day with closed stomata to avoid CO_2 and water escape.

On the other hand, the intrinsic preference of the RubisCO enzyme for carboxylation over oxygenation is measured by Ω, known as the relative specificity factor, and defined as:

$$\Omega = (V_C \cdot K_O / V_O \cdot K_C)$$

Where V_C and V_O are, respectively, the maximum rates of carboxylation and oxygenation, while K_C and K_O are the respective Michaelis constants for CO_2 and O_2 (Laing *et al.,* 1974). Thus Ω equals the ratio of the catalytic to Michaelis constants relation for carboxylation and oxygenation. The relative specificity values of RubisCO enzymes from different origins were found to differ substantially (Jordan and Ogren, 1981). Higher values of Ω reflect a biased propensity of the enzyme for carboxylation. Indeed, comparison of the kinetic parameters of RubiCOs from different species (fig. 11) shows adaptive trends suggesting that selection pressure must play a role in shaping RubisCO catalytic performance. For example, the specificity factor of the RubisCO from *R. rubrum* (a bacterium living in O_2 deprived media) is much lower (Ω = 12.3) than that of the spinach enzyme (Ω = 80). Yet the structural features of the catalytic sites are highly similar in both species offering no straightforward explanation for the sequence or conformation changes that may support the different specificity (Schneider *et al.,* 1990).

Changes in Ω have been proposed to result from the differential stabilization of the carboxylation and oxygenation transition states (Chen and Spreitzer, 1991). By assuming this hypothesis, it can be calculated that the seven-fold factor between the Ω of the *R. rubrum* and the spinach enzymes corresponds to an increase of about 1 kcal/mol in the differential activation energies of the two transition states (Lorimer *et al.,* 1993). This is less than the energy usually involved in a single hydrogen bond. Thus, the key to the efficiency

of any particular RubisCO enzyme lies hidden in the fine details of its three-dimensional structure, and this has motivated intense research with the ultimate aim of boosting carboxylation and improve crop yields through genetic engineering (reviewed in Spreitzer and Salvucci, 2002; Parry *et al.*, 2003). Phylogenetic and structural comparisons of variant RubisCOs have identified candidate L- and S-subunit residues that could benefit catalytic specificity. Several of these sites and regions have been explored experimentally in *C. reinhardtii*, identifying important roles for loop 6 and Asp-473 in the L-subunit and for the loop between β-strands A and B of the S-subunit (Chen and Spreitzer, 1989; Satagopan and Spreitzer, 2004; Karkehabadi *et al.*, 2005; Karkehabadi *et al.*, 2007).

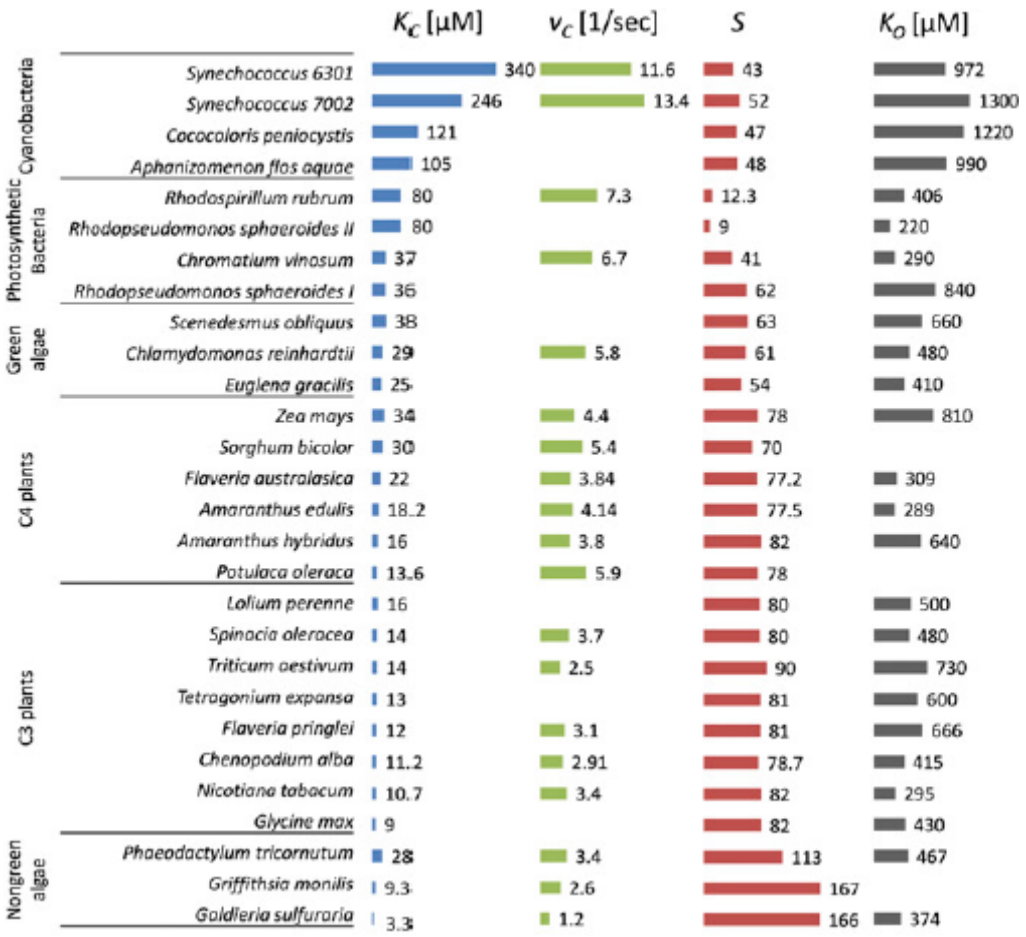


Fig.11. Four kinetic parameters of 28 RubisCOs from 27 species: the effective Michaelis–Menten (MM) constant for CO_2 (K_C); the carboxylation turnover rate (v_C); the relative specificity factor (S); and the effective MM constant for O_2 (K_O). K_C and S are known for all 28 RubisCOs, K_O has been measured for 25 of the enzymes, while all of the kinetic parameters are available for 16 RubisCOs. All enzymes in the dataset belong to the more widespread form I, except for the form II RubisCOs from *R. rubrum* and *R. sphaeroides* [Taken from Savir *et al.*(2009].

There might be, however, some limitations to the catalytic improvements that can be engineered in RubisCO. Correlations among the kinetic parameters of enzymes from various organisms, in particular, the negative Ω versus Vc correlation (Jordan and Ogren 1981, 1983; Zhu *et al.*, 2004) provide evidence for an interplay between constraints and selection, and support an underlying structural mechanism (Tcherkez *et al.*, 2006). For example, improvements in CO_2 fixation rate for forms I and II RubisCOs generally come at the expense of a lower affinity for CO_2. Therefore, photosynthetic organisms that live under high CO_2 and low O_2 (e.g. proteobacteria such as *R. rubrum*) or that have evolved energy-expensive CO_2-concentrating mechanisms around RubisCO possess enzymes with lower CO_2 affinities (i.e. higher K_c) but higher carboxylation rates (v_c) (fig.12 A, B). This is usually accompanied by lower specificity factors (fig. 12 C). In contrast, non-C4 land plants have higher CO_2 affinities, better specificity factors, but slower carboxylation rates (Whitney *et al.*, 2011).

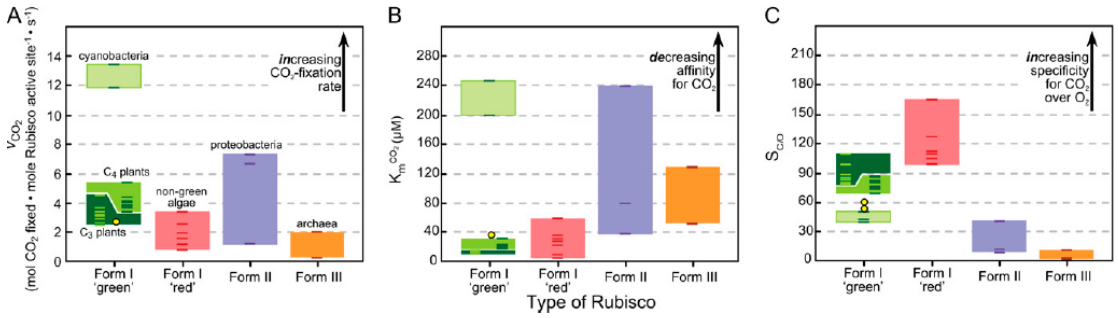


Fig.12. Comparative catalytic features of different RubisCO forms measured at 25^{0}C: Individual dashes in each column represent separate catalytic measurements for each RubisCO. Yellow circles indicate the catalytic measurements for green algal RubisCO. Specificity factor ($S_{C/O}$) values are calculated as $(V_C/K_O)/(V_O/K_C)$, where V_C and V_O are the maximum rates of RuBP carboxylation and oxygenation and K_O and K_C are the apparent K_m values for O_2 and CO_2, respectively [Taken from Whitney *et al.* (2011)].

Nevertheless, the high specificity factor displayed by form I RubisCOs of the red-like type (present in non-green algae) (fig. 11) and, furthermore, the extraordinarily high activity and specificity measured in some form III enzymes (Ezaki *et al.*, 1999) suggest that the catalytic features of the green-like form I RubisCOs (which include those of crop plants) could have been superior if they had evolved from a different sequence and/or enzyme architecture.

REDOX REGULATION AND CATABOLISM OF RUBISCO

REDOX REGULATION IN THE CHLOROPLAST

Redox states of atoms or molecules may act as signals to regulate a wide variety of biological phenomena (Pfannschmidt *et al.*, 2001; Buchanan and Balmer 2005; Foyer and Noctor 2009). In eukaryotes, redox reactions derived from photosynthesis and respiration are compartmentalized in chloroplasts and mitochondria, respectively. In chloroplasts, light-driven electrons flow through the intermediaries of the photosynthetic transport chain (which includes plastoquinone) reaching ferredoxin (Fd) which transfers electrons to $NADP^+$ and thioredoxins (Trx) (fig. 13). NADPH is partly consumed at the reductive steps of assimilatory metabolism, but also regenerates glutathione (GSH) and ascorbate, which constitute the main redox buffers in the chloroplast. Thioredoxins are small (approximately 12 kD), ubiquitous oxidoreductases that mediate the dithiol-disulfide exchange of cysteine residues, thereby modulating the function and stability of their target proteins (Schürmann and Buchanan, 2008).

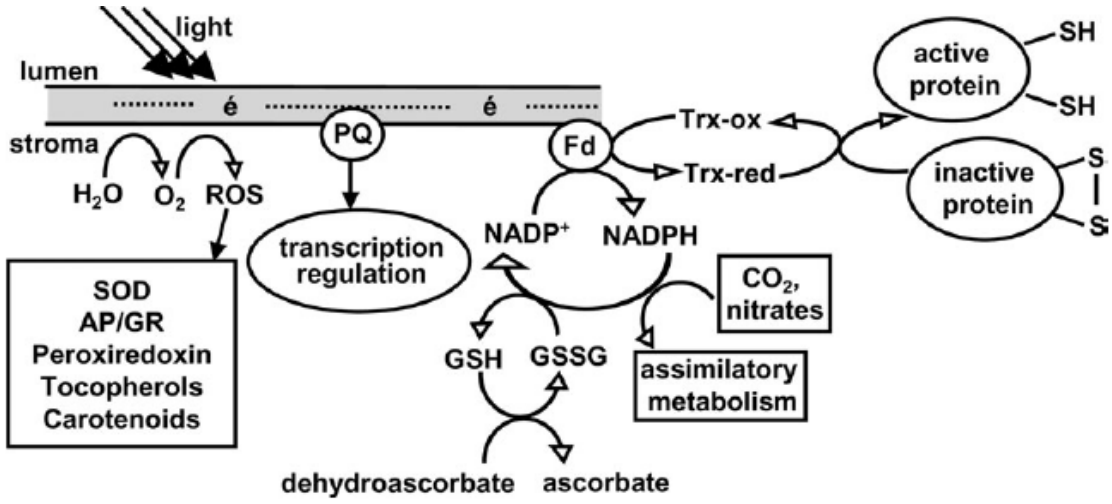


Fig.13. Distribution of light-generated reducing power inside the chloroplast: Photosynthetic electron flow driven by light reduces the plastoquinone (PQ) pool and reaches ferredoxin (Fd). From ferredoxin reducing power is distributed to NAPH and thioredoxins (Trx). In its turn, NADPH powers the reductive anabolic reactions and sustains the glutathione (GSH) and ascorbate pools, which are the main redox buffers of the chloroplast. The electron transport chain and photochemical reaction centers may also generate reactive oxygen species (ROS), which are neutralized by the redox buffers and specialized quenchers such as superoxide dismutase (SOD), ascorbate peroxidase/glutathione reductase (AP/GR), peroxiredoxin, tocopherols and carotenoids [Adapted from Moreno *et al.* (2008)].

Redox regulation inside the chloroplast may be directly exerted by the redox buffers (the NADPH, glutathione and ascorbate pools) or through a series of signalling effectors. Glutathione can directly combine with proteins through S-thiolation. The glutathionylated proteins may be reduced back through the activity

of glutaredoxins, specialized oxidoreductases of the thioredoxin superfamily (Zaffagnini *et al.*, 2012) that get their reducing power either from ferredoxin or directly from glutathione. The main signalling effectors inside the chloroplast are thioredoxins, plastoquinone and ROS. Several chloroplast proteins (including Calvin cycle enzymes) are regulated by thioredoxins through disulfide exchange. Target proteins usually contain vicinal dithiols whose redox state (either free sulfhydryls or an internal disulfide) functions as an activity switch (Schürmann and Buchanan, 2008).

The redox state of the plastoquinone pool has also been reported to control the expression of some chloroplast genes (Pfannschmidt *et al.*, 1999; Pfannschmidt, 2003). Reactive oxygen species (ROS) are usually generated by the escape of electrons from the photosynthetic redox chain when damaged or overloaded. ROS can be quenched or converted to less harmful species by several detoxifying mechanisms, including superoxide dismutase, the ascorbate peroxidase/glutathion reductase system, peroxiredoxins, tocopherol, and carotenoids (fig.13) (Halliwell and Gutteridge, 2007) . Nevertheless, ROS may act also as intracellular signals inducing the expression of genes through redox-sensing factors which are not yet well characterized in plants (Mittler *et al.*, 2011).

REDOX REGULATION OF RUBISCO

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 *rbc*L 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 which detect the rise of reducing potential at the start of the photosynthetic electron flow, subsequently binding to mRNAs. Besides, it has been proposed that the nascent RubisCO large subunit polypeptide may itself block its own translation under oxidative conditions by exposing a domain that binds to RNA when oxidized (Yosef *et al.*, 2004). In this case, the response seems to be directly triggered by a rise of the oxidixed/reduced ratio of the glutathione pool (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 (Zhang *et al.*, 2002). Here, the auxiliary enzyme that releases tightly-binding natural inhibitors from the catalytic site of RubisCO would be activated by the light signal (through the ferredoxin/thioredoxin system),

thereby allowing the carbon fixation activity to be resumed. Besides, both subunits of RubisCO have been identified as targets of thioredoxins in comprehensive screenings among chloroplast proteins (Motohashi *et al.*, 2001; Balmer *et al.*, 2004). Proteomic studies have detected RubisCO also as a target of glutaredoxins (Rouhier *et al.*, 2005). These reports suggest that RubisCO is subjected *in vivo* to physiological control focused on the redox state of cysteine residues. Nevertheless, this evidence might not be taken as conclusive because these global proteomic studies rely always on a step of selective "fishing" of the targets in which RubisCO (due to its notorious abundance) could be an unwanted contaminant. In any case, the potential regulatory effects derived from these modifications under habitual physiological conditions are yet to be described.

OXIDATIVE MODIFICATIONS RELATED TO RUBISCO CATABOLISM

While usually a very stable protein, RubisCO is rapidly degraded at the onset of natural senescence or imposed stress. As mentioned above, due to its extraordinary abundance RubisCO acts habitually as a store of nitrogen and sulfur in higher plants and their mobilization from senescing or declining organs is crucial for nutrient economy (Feller *et al.*, 2008). The enhanced turnover of RubisCO takes place after experiencing a number of modifications of oxidative nature which are thought to be part of a catabolic program leading to a controlled degradation of the enzyme (Ferreira *et al.*, 2000). The oxidative modifications described in different species include thiol oxidation (Garcia-Ferris and Moreno, 1994), formation of carbonyl adducts at certain residues (Eckardt and Pell, 1995; Junqua *et al.*, 2000), acidification and multimerization mediated by an enzymatic oxidase system (Ferreira and Davies, 1989; Ferreira and Shaw, 1989), S-nitrosylation (i.e. the reaction of cysteine sulfhydryls with nitric oxide) (Abat and Deswal, 2009) and non-enzymatic fragmentation caused by ROS (Ishida *et al.*, 1998; Nakano *et al.*, 2006). Moreover, as a result of these modifications, a temporally increasing fraction of RubisCO becomes cross-linked to high molecular weight polymers (Ferreira and Shaw, 1989, Marin-Navarro and Moreno, 2006), and associated to membranes (Mehta *et al.*, 1992; Garcia-Ferris and Moreno, 1994).

All these modification processes are thought to underlie the fast and selective degradation of RubisCO that takes place habitually under senescence or stress conditions (Albuquerque *et al.*, 2001; Ferreira *et al.*, 2000). For example, the oxidation of cysteine residues is known to alter the conformation of the L-subunits as to expose a loop (between Ser61 and Thr68 in *C. reinhardtii*) to proteases. Proteolytic cuts at both L-subunits integrating one of the four core

dimers forces holoenzyme disassembly and subsequent full degradation of the entire protein (Marín-Navarro and Moreno, 2003). RubisCO degradation may take place in the chloroplast and some candidate proteases have been advanced (Feller *et al.*, 2008; Prins *et al.*, 2008). On the other hand, proteolysis of RubisCO in some species has been shown to take place in the vacuolar compartment after transport of the enzyme in small vesicles budding from the chloroplast and finally fusing with vacuoles (Ishida *et al.*, 2007). It is likely that the oxidative cross-linking of RubisCO to membranes is a previous step to vesicle engulfment. However, because not all types of modifications are detected under different stresses or in different species, 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 (Hörtensteiner and Feller, 2005).

ROLE OF SINGULAR CYSTEINE RESIDUES IN RUBISCO CATABOLISM

Cysteine-thiol oxidation is a widely encountered mechanism for modifying the activity of enzymes 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 disulfide (a reversible S–S bond established with another thiol) to the sulfenic (–SOH), sulfinic ($–SO_2H$), and sulfonic ($–SO_3H$) acid derivatives. The lower oxidation states of disulfide and sulfenic acid can be reverted again to the sulfhydryl state. Disulfides can be regenerated by disulfide exchange with free thiols, while sulfenic acid can be reduced by thioredoxins and glutaredoxins (Hancock *et al.*, 2006).

RubisCO activity was early described as sensitive to thiol-directed reagents such as p-chloromercuribenzoate (Sugiyama *et al.*, 1968) or the affinity label N-bromoacetylethanolamine phosphate (Schloss *et al.*, 1978). It was later found that the activity of RubisCO can be modified by mild oxidative treatments affecting cysteines (Tenaud and Jacquot, 1987; Penarrubia and Moreno, 1990) including those restricted to disulfide exchange (Garcia-Ferris and Moreno, 1993). Spontaneous exchange with a small disulfide such as cystamine (2-mercaptoethylamine disulfide) leads to complete inactivation of RubisCO and also to conformational alterations that render the holoenzyme more prone to proteolytic attack and disassembly (Marín-Navarro and Moreno, 2003). It is noteworthy that the cysteine-dependent inactivation and proteolytic sensitization

of the enzyme is widely extended, having been described in RubisCOs from cyanobacteria (Marcus *et al.,* 2003), photosynthetic protists (Garcia-Ferris and Moreno, 1993; 1994), green algae (Mehta *et al.,* 1992; Moreno and Spreitzer, 1999), diatoms (i.e. non-green algae of the red-like type) (Marín-Navarro *et al.*, 2010), and higher plants (Peñarrubia and Moreno, 1990; Marín-Navarro and Moreno, 2003). It appears that this is a highly conserved trend which is aimed to trigger the first stages of the catabolism of the enzyme under natural senescence, environmental stress or other circumstances generating oxidative conditions (Moreno *et al.*, 2008).

Changes brought forth by disulfide exchange rely in the oxidation of critical cysteines that, acting as redox sensors, operate the switch that turn the enzyme to the inactive and protease-sensitive conformation. The identity of these critical residues has not been clearly established to date. It is however known that more than one cysteine must be implied because the inactivation and the proteolytic sensitization of the enzyme occur at a different redox potential, the latter requiring a more oxidizing environment (García-Ferris and Moreno, 1993; Moreno *et al.,* 2008). Because critical redox-sensor residues are likely to be conserved, the physiological effect of substituting some of the conserved cysteines of RubisCO have been studied in organisms (such as *C. reinhardtii* and *Synechocystis*) that allow the transformation with a site-directed mutagenized RubisCO gene.

The first substitution to be studied was the replacement of Cys172 by serine in *C. reinhardtii* (Moreno and Spreitzer, 1999). Exchange of this residue did not affect the oxidative inactivation of RubisCO, but the conversion to a protease-susceptible form *in vitro* required a more oxidizing environment than the wild type enzyme, and the stress-induced degradation of RubisCO was delayed *in vivo* (Moreno and Spreitzer, 1999; Moreno *et al.,* 2008). Furthermore, these effects were later confirmed substituting the same residue by alanine in the *Synechocystis* enzyme (Marcus *et al.,* 2003). In contrast, the substitution of the vicinal Cys192 apparently did not alter the proteolysis of RubisCO but sensitized the enzyme to inactivation under milder oxidative conditions (García-Murria, 2006).

On the other hand, the substitution of both of the Cys449-Cys459 vicinal pair of residues in *C. reinhardtii* produced an enzyme which could not be totally inactivated *in vitro*, retaining about 20% of the activity under extreme oxidizing conditions. The same mutant experienced enhanced aggregation and association to membranes *in vivo* during stress-induced catabolism (Marín-Navarro and Moreno, 2006). The substitution of only one of the two vicinal residues (i.e. either

Cys449 or Cys459) produced a less intense phenotype of the same characteristics indicating that, in this case, the contribution the residues appears to be additive (Marín-Navarro and Moreno, 2006). The only other cysteine residue potentially involved in internal disulfide bonding, Cys247, has been also mutagenized to alanine in the *Synechocystis* RubisCO with no apparent effect on its catabolism (Marcus *et al.*, 2003). The evidence accumulated hitherto suggests that different cysteine residues from RubisCO are likely contribute to the catabolism of the enzyme to variable extent, and they might do so in an additive, cooperative or redundant way (Moreno *et al.*, 2008). Therefore, this group of critical residues would be able to encode a complex but plastic response. It appears that a systematic screening of all conserved cysteines through site-directed mutagenesis could be a first step to analyze this redox signalling network.

OBJECTIVES

OBJECTIVES

The present works aims to continue the research on the modulation of RubisCO activity by disulfides and thiols. In particular, efforts will be directed to solve the following open questions:

A. The role of glutathione in the redox regulation of RubisCO activity.

As a preliminary step, experiments will be devised to determine which common disulfides and thiols are able to modify RubisCO activity, and to confirm that inactivation/reactivation of RubisCO occurs via disulfide exchange, as has been long assumed to be. In addition, because glutathione constitutes the major thiol/disulfide pool in the chloroplast stroma, it seems worth to investigate if Rubisco activity can be specifically controlled by glutathione, either by a direct disulfide exchange or through intermediaries such as other thiols, ascorbate or glutaredoxins.

B. The identity of the critical cysteine residues of RubisCO.

Due to the sensitivity of RubisCO to disulfides and cysteine-directed reagents, it has been postulated that the catalytic activity of RubisCO is affected by the redox state of some of its cysteines (the so called “critical” residues). It is assumed that, upon oxidation or chemical modification, these cysteines may induce long range conformational effects reaching the catalytic site. The identity of these cysteines has not been established yet. To that end, a site-directed mutagenesis scanning of the conserved cysteines of the *C. reinhardtii* RubisCO has already been started and will be completed with the present work. Besides, the sequential order of cysteine modification as the environment turns progressively more oxidizing will also be determined by differential labelling of the reduced and oxidized residues and mass spectrometry analysis.

C. The mechanism by which thiols increase the inhibitory effect of arsenite on RubisCO activity.

It has been described that arsenite produces a partial inactivation of Rubisco and that the inhibitory effect of arsenite can be notably enhanced by the presence of thiols. Although the reduction of a previously existing disulfide (releasing a dithiol that could subsequently react with arsenite) has been advanced as a plausible explanation, the actual mechanism, as well as its reversibility and effectiveness *in vivo*, will be investigated in the present work.

Thereafter, results will be organized in chapters according to the three objectives.

CHAPTER A

REDOX MODULATION OF RUBISCO ACTIVITY BY THIOL/DISULFIDE EXCHANGE: THE ROLE OF GLUTATHIONE

CHAPTER A

INTRODUCTION

Chloroplasts of plants and algae contain a highly organized thylakoid membrane system, harboring the light-harvesting complexes, the photosynthetic reaction center and the water splitting apparatus, which furnishes with electrons a chain of acceptors/donors (Blankenship, 2002). The light-driven electron transfer process involves a complex sequence of redox reactions leading to the reduction of final acceptors (like ferredoxin and NADPH) with highly negative standard redox potentials. Reducing power is subsequently distributed between driving assimilatory metabolic reactions (basically the reduction of oxidized carbon, nitrogen and sulfur compounds), anti-oxidant protection and redox signaling. While, under most circumstances, the major consumer of reducing equivalents is the metabolism (in particular, the Calvin cycle), antioxidant protection is crucial to shield the photosynthetic machinery from reactive radicals generated by uncontrolled escapes from the electron transfer activity itself (Noctor and Foyer, 1998). The chloroplast stroma contains a large pool (1-5 mM) of glutathione (GSH), and even larger (10-30 mM) of ascorbate (As), which buffer oxidant activity by giving away electrons, thereby switching to glutathione disulfide (GSSG) and dehydroascorbate (DHA), respectively (Foyer and Noctor, 2011). DHA is reduced back to ascorbate by GSH in a process catalyzed by the DHA reductase, while GSSG is reduced to GSH by NADPH through the activity of the glutathione reductase. Therefore, the redox state of the As/DHA and GSH/GSSG pairs is directly linked to that of NADPH/$NADP^+$. During light hours, most of the chloroplastic glutathione pool is maintained in the reduced state (GSH) by NADPH, which in its turn is reduced by ferredoxin, the final acceptor of the photosynthetic electron chain. In the dark (and also during stress conditions impairing photosynthestic electron flow) the GSH/GSSG and As/DHA ratios may decrease moderately, but the redox status is usually prevented to turn very oxidative by arresting the processes that act as electron sinks (such as the Calvin cycle) and generating reducing equivalents through the metabolization of starch (Baier and Dietz, 2005).

Many environmental changes affect, directly or indirectly, the efficiency of the photosynthetic transport, thereby shifting the redox potential (i.e. the oxidized/reduced ratio) of components of the electron transport chain as well as of the pools of photosynthesis-coupled redox pairs. The cell may use these shifts as

regulatory cues. For example, the expression of many chloroplastic genes is known to be modulated by the redox state of an electron chain carrier, the plastoquinone (Pfannschmidt *et al.*, 1999). In other instances, the redox signals are distributed by specialized intermediary proteins, such as thioredoxins and glutaredoxins. Thioredoxins catalyze reversible thiol-disulfide exchange reactions between their own pair of vicinal cysteine thiol groups and a disulfide from a target protein (Schürmann and Jacquot, 2000). When oxidized, chloroplastic thioredoxins are reduced back by ferredoxin through the activity of the ferredoxin-dependent thioredoxin reductase. Light-dependent modulation of some Calvin cycle enzymes by disulfide/thiol switching of critical cysteine residues is a well established mechanism of redox regulation that involves thioredoxins (Crawford *et al.* 1989, Scheibe 1991). On the other hand, glutaredoxins catalyze specific deglutathionylation of protein cysteine residues that have established a mixed disulfide bond with glutathione (Rouhier *et al.*, 2008). Glutathyonylation (i.e. the disulfide bonding of a protein cysteine to glutathione) may happen spontaneously as a result of local oxidative conditions habitually promoted by oxygen radicals (ROS), but in some instances it can also be catalyzed by specific glutaredoxins (Starke *et al*., 2003; Beer *et al.*, 2004).

The mechanism of glutaredoxins involves also thiol-disulfide exchange and their oxidized forms are reduced back inside the chloroplast either by GSH or by ferredoxin (Rouhier *et al*., 2008). Glutathionylation of chloroplastic proteins may have protective, regulatory or signaling functions (Zaffagnini *et al.,* 2012). Besides, reversible S-nitrosylation of sulfhydryl groups is but another mechanism of regulation of protein function based on the modification of critical cysteine residues. Nitric oxide production and signalling are well established in plants (Wilson *et al*., 2008). The modification of target proteins takes place usually through transnitrosation by S-nitrosothiols, which act as nitric oxide stores inside the cell. Inside the chloroplast, the main S-nitrosothiol is expected to be S-nitrosoglutathione (GSNO), a reactant that alternatively may also produce glutathionylation of protein sulfhydryls (Giustarini *et al.*, 2005).

Ribulose-1,5-bisphosphate carboxylase/oxygenase, (RubisCO) is the key enzyme of the Calvin cycle, the metabolic pathway that incorporates inorganic carbon to phosphorylated sugars. The three-dimensional structure of RubisCO from the unicellular green alga *Chlamydomonas reinhardtii* (Taylor *et al*., 2001; Mizohata *et*

al., 2002), and several other species has been solved and the conformation of the catalytic site is well known. Even if RubisCO cysteine residues do not appear to play a direct role on the catalytic mechanism, it has been shown that the enzymatic activity of RubisCO is lost when the purified protein is treated with some thiol-directed reagents (Sugiyama *et al.*, 1968, Schloss *et al.*, 1978). Moreover, RubisCO inactivation takes place also when exposed to mild oxidative conditions that may affect specifically sulfhydryl groups from cysteine residues (Tenaud and Jacquot 1987, Peñarrubia and Moreno 1990). Cysteine sensitivity of RubisCO may be exemplified by its full inactivation by cystamine (i.e. 2-mercaptoethylamine disulfide) *in vitro* (García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999, Moreno *et al.*, 2008), which is thought to result from disulfide exchange with critical cysteines. Accordingly, this effect can be reversed, recovering full activity through a treatment with the cysteamine (2-mercaptoethylamine) thiol (García Ferris and Moreno, 1993). It has been hypothesized that this feature may constitute *in vivo* a physiologically relevant redox-controlled switch (Peñarrubia and Moreno, 1990; Moreno *et al.*, 2008). In the last decade, several proteomic studies have identified RubisCO as a potential target of thioredoxins (Balmer *et al.*, 2003; Lemaire *et al*., 2004), glutaredoxins (Rouhier *et al*., 2005) and S-nitrosoglutathione (Abat et al., 2008; Abat and Deswal, 2009), reinforcing the notion of a physiological regulation of RubisCO activity through the redox state of critical cysteine residues.

In order to be physiologically meaningful, RubisCO inactivation should be connected somehow to the redox conditions of the chloroplast stroma, perhaps through its major thiol/disulfide buffer, the GSH/GSSG pool. The detection of glutathionylated forms of RubisCO (Rouhier et al., 2005) appears to support this view. However, it has been reported in some instances that GSSG was unable to inactivate RubisCO *in vitro* (García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999). In this work, the effect of disulfides and thiols on RubisCO has been reexamined. The modification of RubisCO activity by several disulfide and thiol compounds has been tested in order to gain insight on the chemical nature and peculiarities of this enzymatic switch. Besides, the feasibility of RubisCO activity being regulated by the GSH/GGSG pool, either directly or indirectly through intermediary redox-active metabolites, has been investigated.

MATERIALS and METHODS

Chemicals. Analytical grade sodium ascorbate ($C_6H_7NaO_6$), cysteamine (2-mercaptoethylamine) (CSH), cystamine (cysteamine disulfide) (CSSC), reduced (GSH) and oxidized (GSSG) glutathione, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), tris (2-carboxyethyl) phosphine (TCEP), and S-nitrosoglutathione (GSNO) were purchased from Sigma. Cysteine and dithiothreitol (DTT) were obtained from Merck. Oxidized DTT was from Calbiochem. $NaH^{14}CO_3$ (51.7Ci/ mol) was supplied by Perkin-Elmer. Solutions used in oxidative inactivation experiments were made with highly purified water (Milli Q from Millipore).

C. reinhardtii strain and growth conditions. The culture medium used for growth and maintenance of the *C. reinhardtii* strain was based on the TAP medium (Harris, 2009) with modified concentrations of K_2HPO_4 and K_2HPO_4, which were 0.1434 g/l and 0.0725 g/l respectively, and with sodium acetate (0.95 g/l) as the source of carbon. This medium is referred to as (liquid) acetate medium. Solid acetate medium was made adding 15 g of Bacto-agar (Difco) per liter of liquid medium and plating in Petri dishes after sterilization in an autoclave.
The wild type *Chlamydomonas reinhardtii* strain used was 2137 mt + (Spreitzer and Mets, 1981), maintained in solid acetate medium at 25 º C in the dark. For RubisCO purification, this strain was grown in liquid acetate medium at 28 °C under continuous light (provided by 3 white-light tubes of 30W at 30 cm of the cells) and orbital stirring (100 rpm) to a cell density of about 10^7 cells / ml. Approximately 3-4 g of wet cells were harvested from each liter of culture.

Purification of the RubisCO of C. reinhardtii. *C. reinhardtii* cells grown in liquid acetate medium were sedimented by centrifugation (3000*g* x 5min), washed with distilled water and frozen in liquid nitrogen and stored at -80 ° C until use. Except where noted, all steps in the purification of RubisCO were carried out at 4 °C. About 6-10g of frozen cells were suspended in homogenization buffer (100 mM Tris-sulfate, 10 mM $MgSO_4$, 20 mM β-mercaptoethanol, pH 8.0) containing a protease inhibitor cocktail (Complete, Roche) using a ratio of 5 ml of buffer per g of cells. The cell suspension was sonicated (Sonics Vibracell) through 30s-pulses of 75W, separated 30s, (until extensive rupture of cells as monitored by optical microscopy) in an ice bath. Then, 2% (w/v) insoluble polyvinyl-polypyrrolidone was added to the crude

extract and the mixture was stirred for 5 min. After a centrifugation step (35000g x 10min), the supernatant was subjected to fractional precipitation with $(NH4)_2SO_4$ between 35 and 60% saturation. The final precipitate (15000g x 15min) was dissolved in 2.5 ml of buffer A (10 mM Tris-H_2SO_4, 10 mM $MgSO_4$, 10 mM $NaHCO_3$, 1mM β-mercaptoethanol, pH 8) and was applied to a desalting column of Sephadex G - 25 (GE Healthcare, PD-10), previously equilibrated with buffer A, and eluted with 3.5ml of the same buffer. The eluate was divided between two polycarbonate tubes, each of them containing 16ml of a linear gradient of sucrose (0.2-0.8 M) in buffer A. Then, the tubes were centrifuged at 132000g x 4h in a fixed angle rotor (Ti 55.2, Beckman). Afterwards, the gradient was fractionated while monitoring absorbance at 280nm (UV-1 model, Amersham Pharmacia). The peak containing RubisCO was collected between 1 and 5 ml from the bottom of the tube and stored frozen at -20 ºC. The partially purified extract (diluted to an approximate volume of 25 ml) was further resolved by FPLC on a 6ml anion-exchange column Resource Q (Amersham Pharmacia), previously equilibrated with 20mM Tris-HCl pH 7.5 and eluted with 120 ml of a linear NaCl gradient (0 to 0.42 M in 20mM Tris-HCl pH 7.5) at a flow rate of 6ml/min at room temperature. RubisCO was collected directly as an intense 280 nm absorbance peak eluted around 0.15 M NaCl. The final protein preparation contained >95% RubisCO (ascertained by densitometry of Coomassie Blue-stained gels after SDS-PAGE) and was essentially free of nucleic acids (A_{280}/A_{260nm} > 1.7). Purified RubisCO was activated by gel filtration through a Sephadex G-25 column (GE Healthcare PD-10) equilibrated with 10 mM $MgCl_2$, 10 mM NaHCO3, 100 mM Tris-HCl, pH 8.2 (activation buffer), and further diluted with the same buffer as needed.

RubisCO carboxylase activity assay. Carboxylase activity was determined following essentially the procedure of Lorimer et al. (1977). The enzyme (about 4 µg) in 200 µl of activation buffer was kept in plastic vials (Biovial, Beckman) at 30°C for 10min. Then, the reaction was started by the addition of 50 µl of radioactive mixture [55 mM [^{l4}C]-$NaHCO_3$ (about $1{\cdot}10^9$ dpm/mmol), 2.3 mM RuBP, 10 mM $MgC1_2$, 100mM Tris-HCl pH 8.2]. The reaction was stopped after 5 min with 50 µl of 2 M HCl and the excess $NaH^{l4}CO_3$ was eliminated drying the samples in a vacuum oven at 80 °C (twice, after redissolving the dry material with 200 µl of water). The final residue was dissolved in 200 µl of water, suspended in 3 ml of scintillation mixture (Cocktail 22 Normascint, Scharlau), and counted for radioactivity. To determine precisely the specific radioactivity of the CO_2/bicarbonate used as substrate, in each experiment 50 µl of the radioactive mixture were directly counted

by adding 3 ml of an alkaline scintillation cocktail [57:50:5:3 (v/v) of toluene/ phenylethylamine/water/methanol containing 8.52 g/l of PPO and 0.174 g/l of POPOP] which retained the bicarbonate.

Inactivation / reactivation of RubisCO

All inactivation /reactivation treatments were carried out with the RubisCO dissolved in 10 mM $MgCl_2$, 10 mM NaHCO3, 100 mM Tris-HCl, pH 8.2 (activation buffer). The presence of bicarbonate and Mg^{2+} ions in the medium ensured that the enzyme adopted the catalytically competent conformation. Both inactivating and reactivating reagents were prepared immediately before use as fourfold-concentrated solutions in activation buffer readjusting the pH (8.2) after dissolving them. Inactivating reagents were mixed with purified RubisCO preparations (at a final enzyme concentration about 0.2 mg/ml) in open Eppendorf tubes and placed at 30°C in a vacuum oven which was twice evacuated and refilled with nitrogen to prevent uncontrolled spontaneous oxidation. 20µl samples of the incubated solutions were periodically taken, diluted to 200µl with activation buffer and assayed for carboxylase activity.

For reactivation assays, RubisCO (0.45 mg/ml) was previously inactivated with 20 mM CSSC at 30 ºC for 2h in under nitrogen atmosphere. Afterwards, CSSC was eliminated by desalting in a Sephadex G-25 column (GE Healthcare, PD-10). Aliquots (0.48 ml) of the desalted inactive RubisCO preparation were mixed with 0.16 ml of (4x) reactivating reagents and kept at 30 ºC in a vacuum oven under nitrogen. Aliquots of 20µl were taken periodically for carboxylase activity assay. All inactivation/reactivation assays were carried out as independent triplicate runs.

Spectrophotometric assay of disulfide reduction by ascorbate.

Disulfide to thiol reduction was monitored determining the appearance of sulfhydryl groups using the Ellman's reagent (DTNB). Reducing activity was determined from the slope of the time course of the optical density at 412 nm assuming an extinction coefficient of $1.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ for the TNB monomer (Russo and Bump, 1988).

RESULTS

Inactivation of RubisCO by various disulfides

Exposure of purified RubisCO from *C. reinhardtii* to different disulfides resulted in enzyme inactivation to a variable extent (fig. A1).

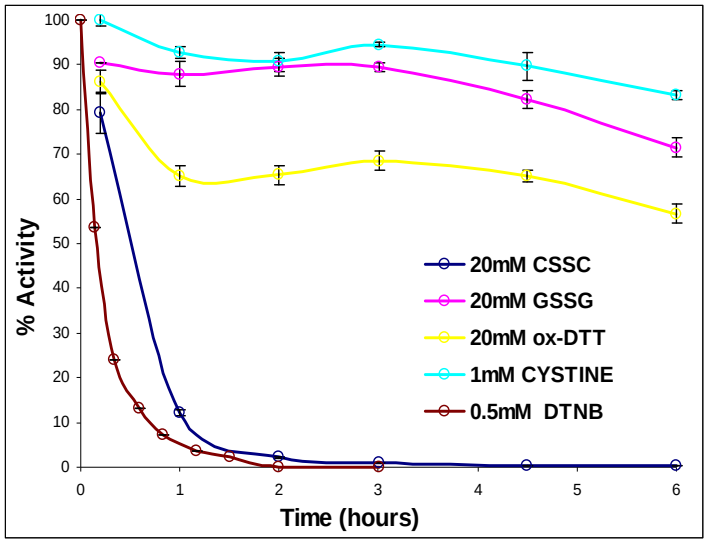


Fig. A1. Time course of RubisCO inactivation by disulfides. RubisCO (0.2 mg/ml) from *C. reinhardtii* was incubated with 20 mM GSSG, 20 mM CSSC, 20 mM oxidized DTT, 1 mM cystine or 0.5 mM DTNB in activation buffer at 30 ºC under a nitrogen atmosphere. At given times, samples (0.02 ml) from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the activity of a non-treated (control) sample incubated in parallel. Bars represent ± SEM from triplicates.

RubisCO was completely inactivated by some disulfides (CSSC and DTNB) and partially inactivated by others (oxidized DTT and GSSG). Cystine at 1 mM (close to its solubility limit) was ineffective contrasting with DTNB, which produced a fast inactivation at 0.5 mM concentration (fig. A1).

The rate of RubisCO inactivation was also dependent on the disulfide concentration. Treatment with CSSC led to extensive inactivation within 90 minutes at all tested concentrations (in the range of 5 to 20mM) (fig. A2, panel A). In contrast, increasing concentration of GSSG (from 10 to 40 mM), while slightly enhancing the rate of activity loss, still resulted in a very restricted inactivation within 6 hours (fig. A2, pa nel B).

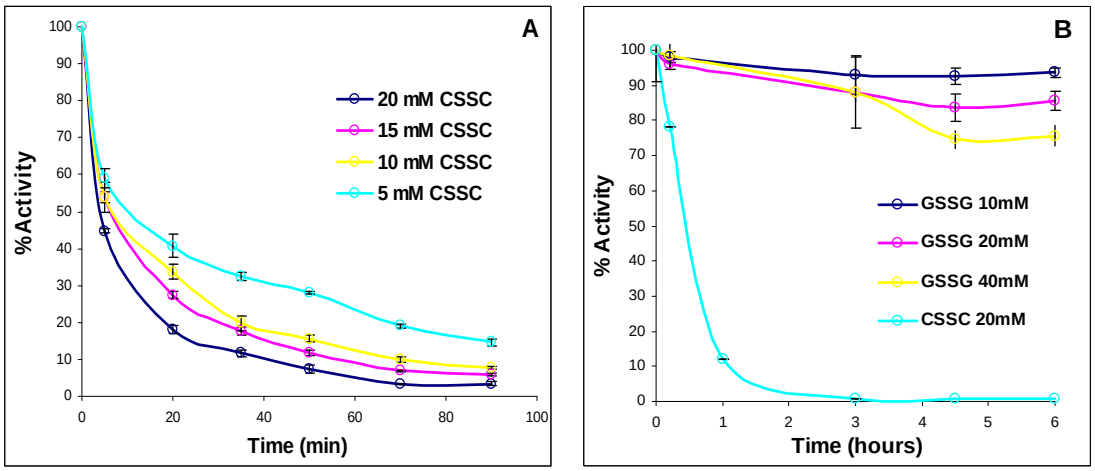


Fig. A2. Time course of RubisCO activity in the presence of CSSC (A) or GSSG (B) at various concentrations. The 20 mM CSSC time course is included also in B for comparison. RubisCO (0.2 mg/ml) in activation buffer was incubated with CSSC or GSSG at 30 ºC in a nitrogen atmosphere. At given times, samples from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the activity of a non-treated (control) sample incubated in parallel. Error bars represent ± SEM.

Because of the specificity and limited chemical reactivity of the disulfide reagents, the most likely hypothesis is that inactivation occurred through oxidation of critical Cys residues of RubisCO establishing mixed disulfides with the reactants:

-Cys-SH + XSSX <=========> -Cys-SSX + XSH
active inactive

Accordingly, inactivation with CSSC took place faster at pH 8.2 than at pH 7.0 (figure A3), suggesting that the modification of the critical cysteines was mediated by the thiolate ion, which is known to be an obligatory intermediate for disulfide exchange (Torchinsky, 1981). Alternatively, these substances could inactivate RubisCO by non-covalent binding to the enzyme, thereby interfering somehow with the structural conformation required by the catalytic mechanism.

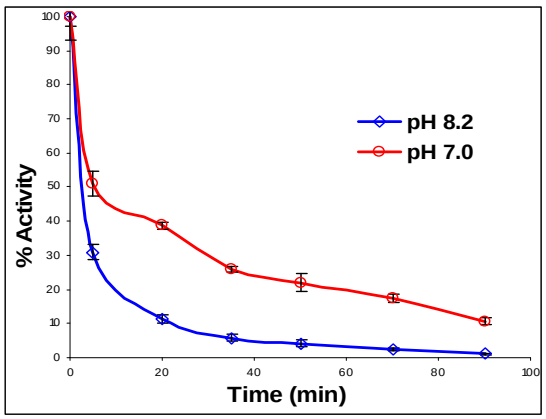


Fig. A3. Time course of RubisCO inactivation by 20 mM CSSC at pH 7.0 and pH 8.2. RubisCO (0.2 mg/ml) in activation buffer (either at pH 8.2 or pH 7.0) was incubated with 20 mM CSSC at 30 ºC. At given times, samples (0.02 ml) from independent triplicates were taken and assayed for carboxylase activity. The assay took place at pH 8.2 for all samples. Values are expressed as a percentage of the initial activity. Bars represent ± SEM from triplicates.

If inactivation takes place through the disulfide exchange mechanism, it might be assumed that disulfides failing to inactivate RubisCO (GSSG, cystine, oxidized DTT) do not exchange with the critical cysteine residues. Limited inactivation of RubisCO by some disulfides may be due to a premature approach to equilibrium because of a remarkable stability of the reagent in the disulfide form, as is known to be the case of oxidized DTT (Torchinsky, 1981). In other cases there might be kinetic barriers that hinder disulfide exchange, as may happen with GSSG, which seems to produce a steady inactivation at a very slow rate (figs. A1 and A2 panel B). It is also conceivable that these disulfides could indeed react with the critical Cys residues but the resulting mixed disulfides do not (or not fully) inactivate RubisCO. In the case of glutathione, this latter possibility deserves to be considered further because, if true, the glutathionylation of RubisCO cysteines could play a physiological role *in vivo* protecting against oxidative inactivation of the enzyme.

Reactivation of CSSC-oxidized RubisCO

After full inactivation of RubisCO with 20 mM CSSC and removal of the disulfide in a desalting column, the inactive enzyme preparation was treated with different sulfhydryl compounds to investigate a possible recovery of activity through disulfide exchange. Reactivation of CSSC-treated RubisCO was indeed achieved by a variety of thiols (fig. A4).

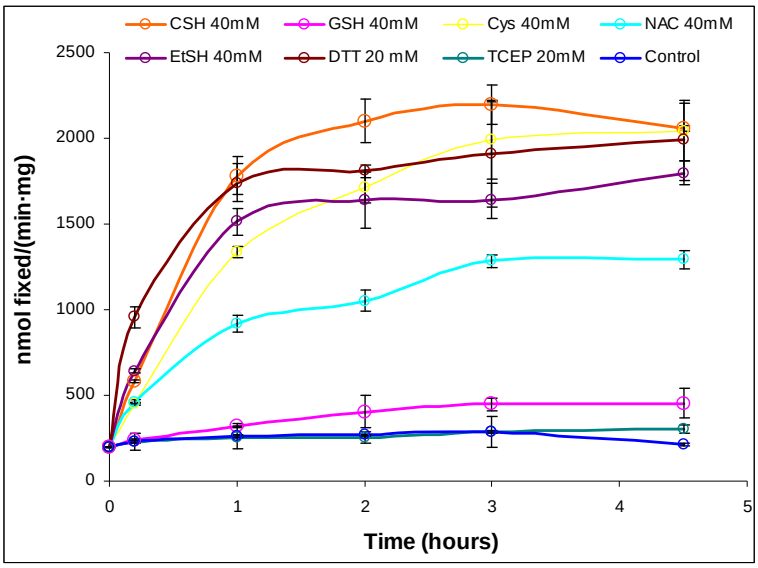


Fig. A4. Time course of the reactivation of CSSC-oxidized RubisCO by different reductants. A time course of unaided recovery (without reductant) is also included (Control). RubisCO. which had been inactivated with 20 mM CSSC for 2h at 30 ºC under a nitrogen atmosphere, was desalted afterwards to eliminate CSSC. Reductants at a final concentration of 40 mM (CSH, GSH, Cys, NAC, EtSH) or 20 mM (DTT, TCEP) were added to the desalted RubisCO aliquots (0.3 mg/ml) in activation buffer and kept at 30 ºC under nitrogen atmosphere. Samples were taken at the given times for activity assay. Activity is given as [nmol CO_2 fixed/(min · mg of RubisCO)]. Bars represent ± SEM from triplicates.

DTT and CSH produced a fast and complete recovery. Cys and EtSH were somewhat slower but also led to full (or almost full) recovery. NAC (N-acetyl cysteine) and GSH achieved only partial reactivation. NAC restored about half of the activity whereas GSH achieved a very limited recovery (about 10 % of the initial activity). No reactivation of the CSSC-oxidized RubisCO was observed in the absence of reductants (control in fig. A4) or in the presence of 20 mM tris (2-carboxyethyl) phosphine (TCEP), a non-thiol reagent that potentially can reduce disulfides according to the following scheme:

$$\text{-CysSSC} + \text{P}(\text{CH}_2\text{CH}_2\text{COO}^-)_3 + \text{H}_2\text{O} \;\; <===> \;\; \text{-CysSH} + \text{CSH} + \text{O=P}(\text{CH}_2\text{CH}_2\text{COO}^-)_3$$

Disulfide TCEP Thiols TCEP oxide

To investigate the possibility (considered above) of a spontaneous glutathionylation of the critical Cys residues of RubisCO that would not affect the catalytic activity of the enzyme, the inactive RubisCO was also incubated with 20 mM GSSG in the

presence of a low concentration (1 mM) of GSH (which was introduced to ensure the presence of thiolate ions to promote disulfide exchange). Under these conditions, disulfide exchange with GSSG would be expected to recover RubisCO activity if the mixed disulfides of the critical cysteines with glutathione do not compromise the catalytic competence of the enzyme. However, treatment of the oxidized enzyme with GSSG did not reactivate RubisCO (fig. A5). Thus, the hypothesis of a spontaneous protective glutathionylation of the critical residues by a direct disulfide exchange with GSSG is not supported by *in vitro* observations.

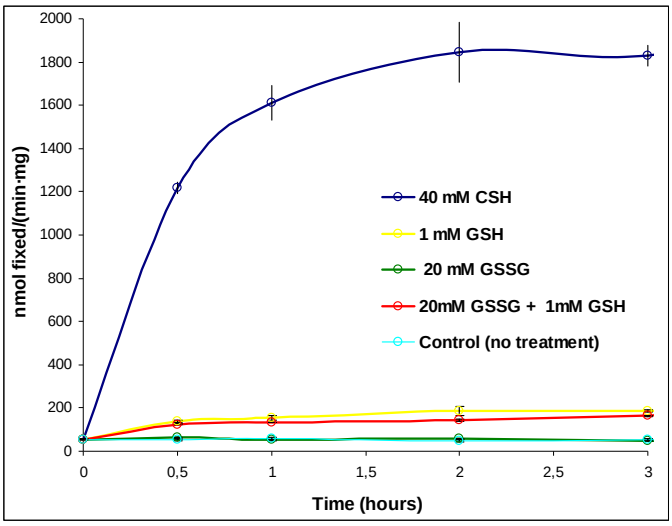


Fig. A5. Time course of the reactivation of CSSC-oxidized RubisCO by GSSG. RubisCO. which had been inactivated with 20 mM CSSC for 2h at 30 ºC under a nitrogen atmosphere, was desalted afterwards to eliminate CSSC. Aliquots of the oxidized RubisCO (0.3 mg/ml) were incubated at 30 ºC under nitrogen atmosphere with 20 mM GSSG + 1mM GSH to attempt reactivation. The time course of activity was compared to that in the absence of redox agents (control). In parallel, other aliquots were incubated with either 20 mM GSSG, 1 mM GSH or 40 mM CSH, as further control treatments. Samples were taken from each treatment at the given times for activity assay. Activity is given as [nmol CO_2 fixed/(min · mg of RubisCO)]. Bars represent ± SEM from triplicates.

The fact that all thiols reactivate (to some extent) RubisCO (fig. A4) strongly supports the initial hypothesis that CSSC inactivation results from oxidation of critical cysteines to reversible disulfides (against other alternatives, such as non-covalent CSSC binding to the RubisCO affecting the catalytic site). The partial reactivation provided by GSH (fig. A3) and the partial inactivation produced by GSSG (figs. A1 and A2), cannot be justified by equilibrium constraints in both cases (since the GSH/GSSG redox pair cannot have both too low and too high standard redox potential with regard to the critical cysteines). Moreover, the final effect of GSSG

(and perhaps GSH) on RubisCO activity appears not to be actually restricted but shows a (very slow) progress on a much longer time scale. In addition, because GSSG does not reactivate RubisCO (fig. A5), the hypothesis of protective glutathionylation (that could have been achieved by GSSG through disulfide exchange) is not supported by the data. Therefore, results in figures A1 and A4 suggest that access of GSH and GSSG to the critical Cys was hindered by kinetic barriers, perhaps related to the bulky nature of both of these reagents (GSH and GSSG) compared to those that were effective (CSH, Cys, NAC, EtSH, DTT). Nonetheless, GSH (being half the size of GSSG) may still reduce without hindrance a limited number of relevant Cys, which would be responsible for the slight reactivation achieved by this reagent (fig. A4).

RubisCO modification by GSSG/GSH through intermediary thiols

The fact that RubisCO is neither significantly inactivated by GSSG nor reactivated by GSH *in vitro* appears to rule out a direct control of RubisCO activity by the glutathione pool *in vivo*. Nevertheless, the following experiments were designed to test if the glutathione pool may affect RubisCO activity indirectly by reducing/oxidizing intermediary molecules (such as the small thiol/disulfide compounds that proved effective in figures A1 and A4).

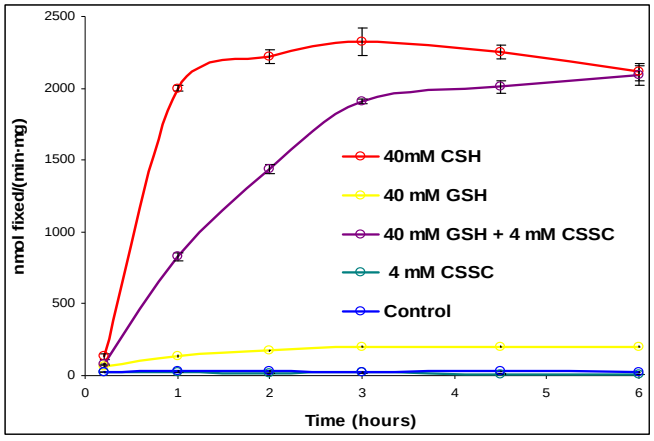


Fig. A6. Time course of reactivation of CSSC-oxidized RubisCO through CSH driven by GSH. RubisCO which had been inactivated with 20 mM CSSC for 2h at 30 ºC under a nitrogen atmosphere, was desalted afterwards to eliminate CSSC. Aliquots of the oxidized RubisCO (0.3 mg/ml) were incubated at 30 ºC under nitrogen atmosphere with 40 mM GSH + 4mM CSSC to attempt reactivation. The time course of activity was compared to that in the absence of redox agents (control). In parallel, other aliquots were incubated with either 40 mM GSH, 4 mM CSSC or 40 mM CSH, as further control treatments. Samples were taken from each treatment at the given times for activity assay. Activity is given as [nmol CO_2 fixed/(min · mg of RubisCO)]. Bars represent ± SEM from triplicates.

Full reactivation of a CSSC-inactivated RubisCO was achieved by GSH in the presence of CSH as an intermediary thiol (introduced in the experiment as a small amount of the disulfide CSSC to discard a possible direct reduction) but not in the absence of it (fig. A6). This result confirms that GSH can drive the reduction of the CSSC-oxidized RubisCO. Therefore, the inability of GSH to reactivate RubisCO directly (without intermediaries) must be of kinetic nature, as postulated above.

RubisCO inactivation by means of GSSG in the presence of a small concentration of thiols has also been attempted. Nevertheless, in this case the addition of thiols did not appreciably accelerate activity loss (fig. A7).

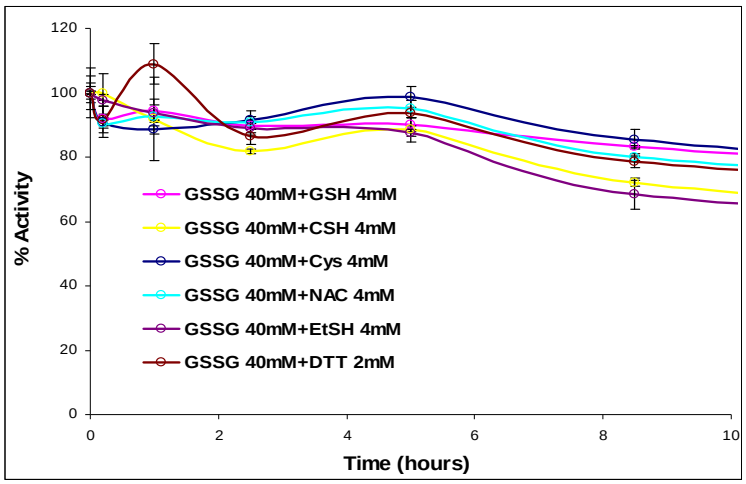


Fig. A7. Time course of RubisCO inactivation by 40 mM GSSG in the presence of different thiols at 4mM concentration. RubisCO (0.2 mg/ml) in activation buffer was incubated with the redox agents at 30 ºC under a nitrogen atmosphere. At given times, samples from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the activity of a non-treated (control) sample incubated in parallel. Bars represent ± SEM.

The fact that GSH was able to reactivate RubisCO rapidly in the presence of CSSC (fig. A6) but GSSG failed to inactivate the enzyme after addition of CSH or other thiols (fig. A7) could be explained considering the intermediary reactions involved:

When GSH (40 mM) is mixed with CSSC (4 mM) the ensuing sequential reactions of disulfide exchange may take place:

CSSC + GSH <======> CSSG + CSH

CSSG + GSH <======> GSSG + CSH

Both reactions produce CSH and, moreover, both reactions are likely to be displaced to the right due to the great excess of GSH (40 mM). Consequently, CSSC is almost fully converted to CSH, which is known to be able to reactivate RubisCO (fig. A4).
On the other hand, when GSSG (40 mM) is mixed with CSH (4 mM) the expected disulfide exchanges are:

GSSG + CSH <=======> GSSC + GSH
GSSC + CSH <=======> CSSC + GSH

Here the first reaction may be displaced to the right due to the excess GSSG (40 mM), but the second is not (because the accumulation of GSSC + CSH is limited to 4 mM at most). Therefore, most of the CSH will probably be converted to GSSC, not to CSSC (which is the form known to inactivate RubisCO, as shown in fig. A1). If the access to the critical cysteines is hindered for GSSC as for GSSG, then the addition of CSH to GSSG would have little effect on RubisCO inactivation as seen in fig.A7.
The above explanation is based on the assumption that the mixed disulfide GSSC cannot inactivate RubisCO. To check this postulate, the kinetics of RubisCO inactivation by CSSC was also tested in the presence of a low amount of GSH or CSH (concentrations at a thiol:disulfide ratio of 1:5).

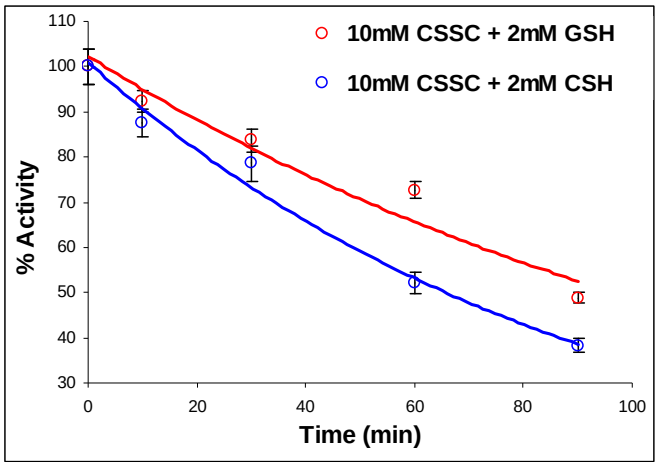
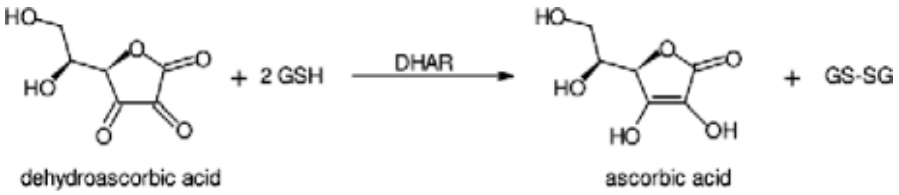


Fig. A8. Time course of RubisCO inactivation by 10 mM CSSC in the presence of 2 mM GSH or CSH. Disulfide/thiol mixtures were allowed to equilibrate for 2 h at 30 ºC in a nitrogen atmosphere before adding to RubisCO. RubisCO (0.25 mg/ml) in activation buffer was incubated with the redox mixtures at 30 ºC under nitrogen atmosphere. At given times, samples from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the initial activity. Bars represent ± SEM from triplicates. An exponential trend line has been fit to the experimental points.

The rationale of this experiment was that the addition of GSH to CSSC would generate a GSSC mixed disulfide, thereby diminishing the total concentration of CSSC. This would slow down RubisCO inactivation if (as postulated) GSSC cannot oxidize the critical cysteines. Fig. A8 shows that the rate of RubisCO inactivation was indeed slower in the presence of GSH when compared to the control in which GSH has been replaced by CSH. This result suggests that the mixed disulfide GSSC has limited access to the critical cysteines that are responsible for the inactivation of RubisCO.

RubisCO modification by GSSG/GSH through DHA/ascorbate

Ascorbate/dehydroascorbate (DHA) is a redox pair which is highly concentrated in the chloroplast stroma (about 10-30 mM) and is naturally connected to the GSH/GSSG pair (Foyer and Noctor, 2011). Electron exchange between these two redox pairs is catalyzed by the GSH-dependent dehydroascorbate reductase (DHAR).



However, this reaction may also take place spontaneously (without enzyme) at a noticeable speed, especially at a basic pH (Jocelyn, 1972) as in the chloroplast stroma. This possibility was tested experimentally (in the reverse direction) by incubating ascorbic acid with GSSG and DTNB (to monitor the production of thiols from disulfides driven by ascorbic acid) in activation buffer (pH 8.2) (fig. A9).
Results demonstrated that the reduction of GSSG by ascorbate takes place in the absence of enzyme at a measurable rate (see legend of fig. A9). Besides, spontaneous cleavage of protein-thiol mixed disulfides by ascorbate has also been reported to occur (Giustarini et al., 2008).
To test if ascorbate could also reactivate RubisCO by reducing the disulfides at critical cysteines generated by a previous treatment with CSSC, we incubated the inactive RubisCO with ascorbate in the presence and absence of GSH (fig. A10).

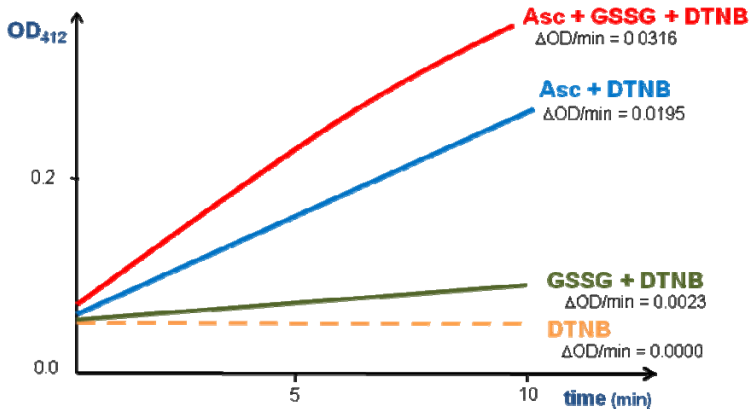


Fig. A9. Time course of the optical density (OD) at 412 nm in 1 ml solutions of 0.4 mM DTNB alone (orange), with 32 mM GSSG (green), with 40 mM ascorbate (blue) or with 32 mM GSSG + 40 mM ascorbate (red) in activation buffer. OD monitoring (against an activation buffer blank) began 0.5 min after mixing the reagents and took place for a further 10 min. Slopes were calculated from the total ΔOD after 5 min. Slopes were almost constant except for some late decay in the Ascorbate + GSSG + DTNB mix. OD increase in the GSSG + DTNB mixture was most likely due to slow disulfide exchange (probably activated by traces of GSH from GSSG impurities). OD increases in the ascorbate + DTNB mix demonstrate that ascorbate can directly reduce the DTNB disulfide. Further increase in the GSSG + DTNB + ascorbate mix indicates reduction of GSSG also by ascorbate. From the net slope increase (net ΔOD/min = 0.0316 – 0.0195 – 0.0023 ≈ 0.01) and knowing that ε_{412} of TNB = $1.36 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ (Russo & Bump, 1988), a concentration increase of ≈ $7 \cdot 10^{-7}$ M/min (corresponding to $7 \cdot 10^{-10}$ mole of GSH/min in 1 ml volume) can be calculated.

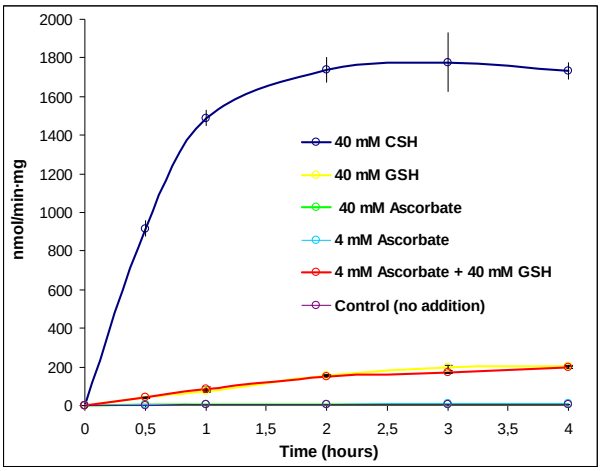
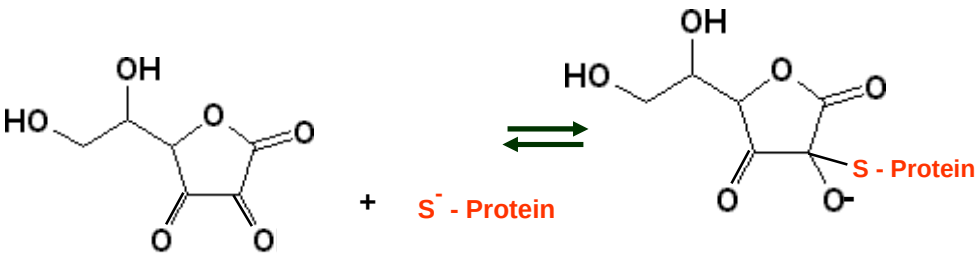


Fig. A10. Time course of the reactivation of CSSC-oxidized RubisCO by different potential reductants. RubisCO which had been inactivated with 20 mM CSSC for 2h at 30 ºC under a nitrogen atmosphere, was desalted afterwards to eliminate CSSC. Inactive RubisCO aliquots (0.3 mg/ml) were treated with either 40 mM CSH, 40 mM GSH, 40 mM ascorbate, 4 mM ascorbate, or 4 mM ascorbate + 40 mM GSH (final concentrations), and kept at 30 ºC under nitrogen atmosphere. A time course of a control recovery (without reductant) is also included. Samples were taken at the given times for activity assay. Activity is given as [nmol CO_2 fixed/(min · mg of RubisCO)]. Bars represent ± SEM from triplicates.

Results indicated that ascorbate was unable to reactivate RubisCO, even at a concentration as high as 40 mM (fig. A10). Thus, in contrast to what happened with small thiols (fig. A6), GSH could not drive RubisCO reactivation using ascorbate as intermediary and, accordingly, the presence of 4 mM ascorbate did not enhance the limited reactivation achieved by 40 mM GSH alone (fig. A10).

The possibility of RubisCO inactivation by DHA, which could be generated through ascorbate oxidation by GSSG, was also tested. DHA seems to be able to oxidize some protein sulfhydryl groups directly. Indeed, studies on the mechanism of the DHAR support a cysteine thiohemiketal intermediate as a result of spontaneous reaction of DHA with a Cys residue of the enzyme (Shimaoka *et al.*, 2003):



DHA thiohemiketal

Thus, DHA could inactivate RubisCO through thiohemiketal derivatives at isolated critical cysteines or even progress to complete the two-electron oxidation (as with the conversion of GSH to GSSG by DHAR) by internal disulfide bonding of vicinal cysteine pairs.

To probe if DHA could act as an intermediary oxidant between GSSG and RubisCO, the enzyme was incubated with 40 mM GSSG in the presence of 4 mM ascorbate. Under these conditions the high concentration of GSSG should increase the DHA/ascorbate ratio significantly. However, after 6 hours, RubisCO inactivation was not promoted by the presence of DHA at any appreciable rate (fig. A11). The slight decrease of activity after 6 hours of exposure to GSSG plus ascorbate took place also with GSSG alone (fig. A11), and it was due to the slow oxidizing effect of GSSG directly on RubisCO (also appreciated in figs. A1, A2 and A7). This indicates that GSSG cannot drive a faster oxidation of the critical cysteines of the enzyme through the intermediation of DHA.

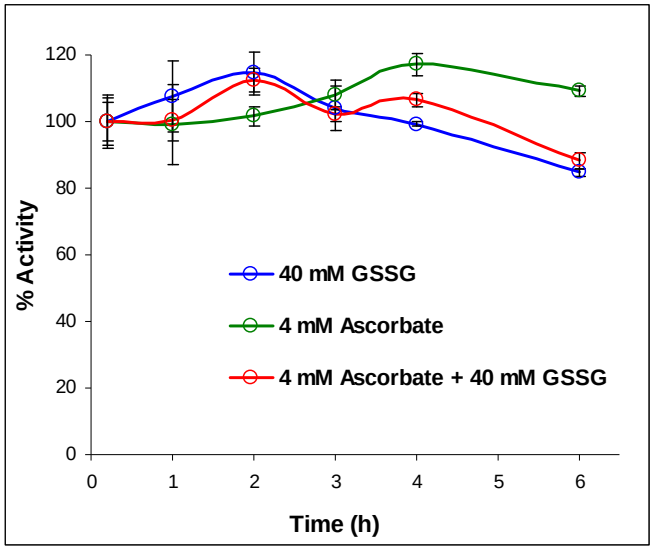


Fig. A11. Time course of RubisCO activity in the presence of 40 mM GSSG, 4 mM Ascorbate, or a mixture of 40 mM GSSG + 4 mM Ascorbate. RubisCO (0.2 mg/ml) in activation buffer was incubated with the redox agents at 30 ºC under nitrogen atmosphere. At given times, samples from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the activity of an untreated sample incubated in parallel. Bars represent ± SEM.

Inactivation of RubisCO by S-nitrosoglutathione (GSNO)

Protein sulfhydryls are also susceptible to combine with nitric oxide (NO) yielding S-nitroso thiol derivatives. Nitric oxide generated in the chloroplast may react first with the abundant GSH pool to give S-nitrosogluthathione (GSNO) which, in its turn, may transfer the NO to a protein thiolate ($P\text{-}S^-$) according to the following exchange reaction:

$$P\text{-}S^- + GSNO \rightarrow P\text{-}SNO + GS^-$$

In order to test if GSNO may act directly as a NO-donor for the S-nitrosylation of RubisCO cysteines, the purified enzyme was incubated with GSNO at different concentrations. Exposure of purified RubisCO to GSNO produced a fast but partial inactivation of the enzyme (fig. A12). Indeed, GSNO produced a faster inactivation of RubisCO than CSSC at equal (5 mM) concentration, but GSNO activity loss was limited to a 30 to 40 % activity loss (fig. A12) whereas CSSC persevered to a slow

but extensive inactivation (figs. A2 and A12 panel A). S-nitrosylation is a reversible modification in most cases (Paige et al., 2008). Accordingly, a previous inactivation of RubisCO by 5 mM GSNO during 29 minutes could be reverted through a further addition of an excess reductant (10 mM DTT or 20 mM GSH) (fig. A12 panel B).

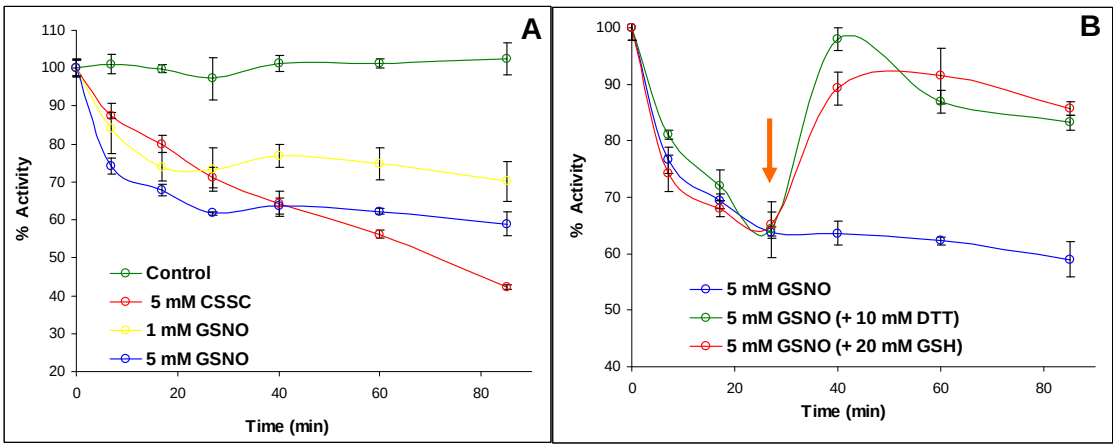


Fig. A12. Time course of RubisCO inactivation by different concentrations of GSNO (A) and reactivation by thiols (B). Time courses corresponding to a Control (no addition) and a 5 mM CSSC treatments are also included in A for comparison. RubisCO (0.2 mg/ml) in activation buffer was incubated with the redox agents at 30 ºC. Reductants (DTT and GSH at 10 and 20 mM final concentration, respectively) were added in B after 29 minutes of inactivation with 5 mM GSNO (orange arrow). At given times, samples from triplicates were taken and assayed for carboxylase activity. Values are expressed as a percentage of the initial activity. Bars represent ± SEM.

DISCUSSION

In vitro inactivation of RubisCO through reagents that modify cysteine thiol groups has been demonstrated for the enzymes of spinach (Sugiyama *et al*. 1968; Schloss *et al*., 1978), *Lemna* (Ferreira and Shaw 1989), orange tree (Peñarrubia and Moreno 1990), *Euglena* (García-Ferris and Moreno, 1993), *Chlamydomonas* (Mehta *et al*., 1992; Moreno and Spreitzer, 1999) and diatoms (Marín-Navarro et al., 2010) suggesting that this is a conserved trait among RubisCOs from photosynthetic eukaryotes. The present work focuses specifically on the inactivating effect of disulfides on RubisCO as has been previously shown using CSSC in several instances (García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999; Marín-Navarro and

Moreno, 2006). In particular, and in view of the reports of a possible RubisCO glutathionylation *in vivo* (Rouhier *et al*., 2005), the aim was to test the possibility of a strict control of RubisCO activity by glutathione, the major disulfide/thiol redox pair in the chloroplast, either by direct action or by indirect means. In previous reports, the inactivating effect of CSSC has been implicitly assumed to proceed through disulfide exchange leading to the oxidation of critical cysteine residues (Moreno *et al.*, 2008). Alternatively, CSSC could bind (non-covalently) to the enzyme at a specific site interfering with the catalytic mechanism. However, the dissimilar chemical structure of the inactivating disulfides (such as CSSC and DTNB) makes this option unlikely. In addition, the pH dependence of the inactivation rate (fig. A3) and the fact that miscellaneous thiols can reverse the inactivation by CSSC (fig.A4), strongly supports the hypothesis that inactivation results indeed from disulfide exchange.

Not all disulfides had the same effect on RubisCO and some of them (GSSG, cystine and oxidized DTT) failed to achieve full activity loss (fig. A1). While in the case of DTT this is likely to result from a strongly displaced equilibrium, disulfide exchange of GSSG with the critical cysteines of RubisCO appears to be hampered by kinetic barriers which slow down the process as to make it physiologically irrelevant. Similarly, some disulfide reducing agents such as GSH, TCEP and, to a lesser extent, NAC had difficulties in recovering the activity of CSSC-treated RubisCO (fig. A2). It is noteworthy (although perhaps coincidental) that reagents that achieve full activity recovery from CSSC-inactivated RubisCO are either positively charged (CSH) or near neutral (EtSH, DTT, Cys), while those that fail to reactivate or lead only to limited reactivation (GSH, NAC, TCEP) carry negative charge at physiological pH. It might be speculated that the net charge of the reducing agent could be crucial for gaining access to the critical cysteines. If true, Cys residues located close to negatively charged groups in the native structure of RubisCO would be good candidates for being critical residues. A survey of the charge environment of Cys residues in the *C. reinhardtii* RubisCO points to Cys 53, 192 and 247 from the large subunit and Cys 41 and 96 fom the small subunit as those surrounded by a higher negative charge density. Alternatively, the inability to inactivate/reactivate RubisCO may have different causes for the different compounds and, in particular, the access of the GSH/GSSG pair to the critical residues may be restricted by steric reasons due to the bulky nature of these reagents (compared, for example, to the CSH/CSSC pair).

The fact that RubisCO is neither inactivated by GSSG (fig. A1) nor significantly reactivated by GSH (fig. A4) at a physiologically significant speed *in vitro* appears to rule out a direct control of RubisCO activity by the glutathione pool. However, small intermediary thiols present in the stroma at a low concentration may serve to reduce back and reactivate the oxidized protein at high GSH/GSSG ratios (as shown in fig. A4). Nevertheless, if the GSH/GSSG ratio drops temporarily to a low value (as a result of prolonged darkness or stress, for example), RubisCO will not be oxidized through the small thiol intermediaries because these will be mostly engaged in mixed disulfides with glutathione and these heterodisulfides (such as GSSC) do not affect RubisCO (fig. A5). This unidirectional effect may help to maintain the RubisCO in the reduced (active) state *in vivo* under a variety of redox environments. However, it might be expected that the small thiol intermediaries will indeed oxidize RubisCO at a low GSH/GSSG ratio if their concentration rise to levels similar to those of glutathione because, under these circumstances, a sufficient amount of homodisulfides (such as CSSC) of these small thiols would be generated at equilibrium. Since the total concentration of non-glutathione thiols plus disulfides have been shown to increase under different stress conditions (reviewed in Moreno *et al.*, 1995), this might be a mechanism for triggering RubisCO catabolism, as has been previously proposed (Moreno *et al.*, 1995).

RubisCO was also partially inactivated by the NO-donor GSNO (fig. A12). Inactivation by S-nitrosylation has been reported already for the *Kalanchoe pinnata* (Abat *et al.*, 2008) and *Brassica juncea* (Abat & Deswal, 2009) RubisCOs. It is noteworthy that both enzymes displayed a higher sensitivity to GSNO than the RubisCO of *C. reinhardtii*, losing about 50 % of their activity around a 0.25 mM concentration of GSNO (to be compared with figure A12 where 5 mM GSNO attains only 40 % inactivation). In case of the *C. reinhardtii* enzyme, it is possible that GSNO does not reach all critical Cys residues or, alternatively, that S-nitrosylation of those Cys does not affect the performance of the catalytic site as much as the disulfide exchange with CSSC. However, because the extent of inactivation produced by GSNO was dependent on its concentration, achieving ~30% of activity loss at 1 mM and ~40% at 5 mM after long-term incubation (fig. A12), it appears that the S-nitrosylation of the critical cysteines might be feasible and actually inactivate the enzyme but the reaction between GSNO and the protein sulfhydryls does not progress further because reaches equilibrium prematurely. Thus, the fact that the inactivation of the *C. reinhardtii* RubisCO needs a higher concentration of GSNO reflects the lesser

tendency of its critical cysteines to exchange NO with GSNO (i.e., NO is more stable combined to GSH than to the critical protein sulfhydryls). It is likely that the stability of the S-nitroso derivatives depends on subtle interactions with the chemical environment surrounding the critical sulfhydryl groups. S-nitrosylation of RubisCO and its subsequent inactivation has been assumed to occur *in vivo* and has been justified as a mean to stop carbon fixation under certain stress conditions (Abat and Deswal, 2009). If this happens to be the case also in *C. reinhardtii*, GSNO does not seem to be an adequate NO-donor for the S-nitrosylation of RubisCO since it would need to accumulate to an unfeasibly high concentration in order to produce a significant inactivation of the enzyme.

The DHA/ascorbate pool, which is the most abundant redox buffer inside the chloroplast, could also potentially control the activity of RubisCO. Indeed, ascorbate has been reported to reduce some small molecular weight disulfides (such as GSSG) and even protein mixed disulfides (Giustarini et al., 2008). In this regard, we have experimentally verified the spontaneous reduction of GSSG by ascorbate (fig. A9). However, our results indicate that ascorbate cannot reactivate the CSSC-oxidized RubisCO (neither directly nor as an intermediary agent for GSH) (fig. A10) while DHA is also unable to drive the oxidative inactivation of the enzyme (fig. A11). The existence of kinetic barriers impeding direct disulfide exchange between critical cysteines of proteins and the main cellular redox pools is not uncommon. Some chloroplastic proteins are known to be regulated by redox through critical cysteines while staying out of equilibrium with the glutathione pool (Clancey & Gilbert, 1987). Indeed, non-equilibrium with the environmental redox ambient appears to be a required feature for a specific redox control mediated by thiol/disulfide exchange (Kemp *et al*.,2008). In that instance, regulation can be kinetically exerted allowing the redox signal to travel only via specific catalyzed pathways (Danon, 2002). In view of the recent reports supporting the mixed disulfide bonding of GSH to RubisCO *in vivo* (Rouhier et al., 2005), the existence of kinetic barriers that prevent spontaneous oxidation of RubisCO by GSSG under most physiological circumstances suggests the possibility of a directed regulation of the enzyme by glutathionylation and deglutathionylation of critical residues catalyzed perhaps by specific glutaredoxins.

CHAPTER B

INDIVIDUAL CONTRIBUTION OF CONSERVED CYSTEINES TO THE REDOX REGULATION OF RUBISCO ACTIVITY IN *CHLAMYDOMONAS REINHARDTII*

INTRODUCTION

The net CO_2-fixing enzyme, ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), from green algae and higher plants is an extraordinarily abundant protein which accumulates in the stroma of chloroplasts at a concentration (about 0.5 mM) which is close to that a pure protein crystal (Pickersgill, 1986). In some green algae (such as *Chlamydomonas reinhardtii*) it may concentrate even further in specialized structures of the chloroplast, called pyrenoids, composed mainly by dense deposits of RubisCO (Borkhsenious *et al*., 1998). The amount of nitrogen invested in the synthesis of this enzyme is substantial, reaching about one fourth of the total content of a typical C_3 higher plant leaf (Evans and Seeman, 1989). While RubisCO accumulates usually in actively growing cells with little turnover, the enzyme is degraded rapidly and selectively in senescent or decaying tissues in a process which is usually understood as a remobilization of nutrients (Ferreira *et al.*, 2000) and which has been shown to play a decisive role in the nutritional economy of different organisms ranging from higher plants (Kang and Titus, 1980; Friedrich and Huffaker, 1980; Mae *et al.*, 1983) to unicellular photosynthetic protists (García-Ferris and Moreno, 1994). Under natural or stress-induced senescence conditions, RubisCO is degraded *in vivo* following different pathways depending on the organism as well as type and intensity of stress (reviewed in Feller *et al*., 2008). However, there is a general consensus in that RubisCO catabolism begins with changes of oxidative nature affecting the structure and activity of RubisCO itself (Ferreira *et al*., 2000; Moreno *et al*., 2008). These changes include cysteine thiol to disulfide oxidation (Garcia-Ferris and Moreno, 1994), carbonylation at certain amino acids (Eckardt and Pell, 1995; Junqua *et al.*, 2000), acidification and multimerization mediated by an enzymatic oxidase system (Ferreira and Davies, 1989; Ferreira and Shaw, 1989), S-nitrosylation (Abat and Deswal, 2009) and non-enzymatic fragmentation caused by reactive oxygen species (Ishida *et al.*, 1998; Nakano *et al.*, 2006). Coincidentally with these oxidative modifications, RubisCO begins to aggregate and to associate with membrane fractions (Ferreira and Shaw, 1989; Mehta *et al*., 1992; García-Ferris and Moreno, 1993; Marín-Navarro and Moreno, 2006) as further steps in the catabolic pathway (Ferreira *et al*., 2000).

RubisCO oxidation by a disulfide such as cystamine (CSSC, 2-mercaptoethylamine disulfide) is a convenient procedure to induce reversible oxidative changes in RubisCO. These changes result in full inactivation of the enzyme and structural shifts that render the enzyme more sensitive to proteolysis upon oxidation (García-Ferris and Moreno, 1993). The progressive transition of the enzyme to the oxidized form may be followed by equilibrating the enzyme with redox buffers made by

mixing CSSC with 2-mercaptoethylamine (CSH) at different ratios (García-Ferris and Moreno, 1993, Moreno and Spreitzer, 1999; Marín-Navarro and Moreno, 2006). When *C.reinhardtii* RubisCO is incubated with CSSC/CSH mixtures at different disulfide/thiol ratios, the enzyme inactivates progressively (as the fraction of the oxidant disulfide increases) until total activity loss (fig. B1).

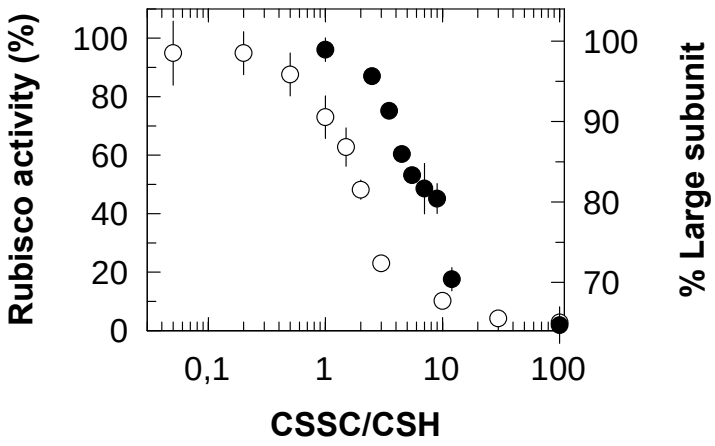


Fig.B1. **Inactivation (○) and proteolytic sensitization (●) of wild type RubisCO incubated in redox buffers at different disulfide/thiol ratios.** Purified RubisCO (ca 0.3 mg/ml) was incubated with mixtures of the disulfide cystamine (CSSC) and the thiol cysteamine (CSH) at different ratios in activation buffer (50 mM Tris HCl, 10 mM Mg Cl_2, 10 mM $NaHCO_3$, pH 8.2) . Constant monomeric concentration (i.e. [CSH] + 2·[CSSC]) was kept constant and equal to 40 mM for all ratios. Incubation took place in a nitrogen chamber at 30 ºC for 2 hours. The residual carboxylase activity, represented as a percentage of that of the fully reduced enzyme, is shown on the left axis. The amount of intact large subunit remaining after 20 minutes of proteolysis with subtilisin (0.5 μg/ml) determined by SDS-PAGE densitometry is indicated on the right axis as a percentage of that obtained with fully reduced RubisCO in the same conditions. Error bars indicate SEM from triplicates. Figure taken from Moreno *et al.* (2008).

As discussed in chapter A, under these mild conditions, inactivation is expected to occur through disulfide exchange with critical cysteines. The CSSC/CSH ratio at which inactivation takes place should reflect the propensity to oxidation (i.e. the redox potential) of these critical residues. Under the experimental conditions described in the legend of figure B1, the transition from active to inactive enzyme takes place around a CSSC/CSH ratio of 1.5. On the other hand, under the same conditions, the conformational change that renders RubisCO sensitive to proteases occurs around a CSSC/CSH ratio of 4 (fig. B1). This fact has been the main support of the hypothesis that changes induced in RubisCO by disulfide exchange are carried out by the oxidation of several (i.e. more than one) cysteine residues displaying different redox potentials (García-Ferris and Moreno, 1993; Moreno *et al*,. 2008). Thus, the cysteines which are crucial for inactivation appear to be more

easily oxidizable than those bringing forth the structural changes that facilitate proteolysis.

In an attempt to identify the critical residues, some of the conserved cysteines of the *C. reinhardtii* RubisCO large subunit have been changed to serines through site-directed mutagenesis and chloroplast transformation. Substitution of Cys 172 produces an enzyme that is somewhat more resistant to proteolysis when oxidized *in vitro*, and shows delayed turnover *in vivo* under stress conditions (Moreno and Spreitzer, 1999). This phenotype was later confirmed when the same residue was replaced by alanine in a cyanobacterial RubisCO (Marcus *et al.*, 2003). In contrast, the substitution of the vicinal Cys 192 in the *C. reinhardtii* enzyme did not change proteolytic susceptibility but shifted oxidative inactivation to a lower CSSC/CSH ratio (fig. B2) (García-Murria, 2006).

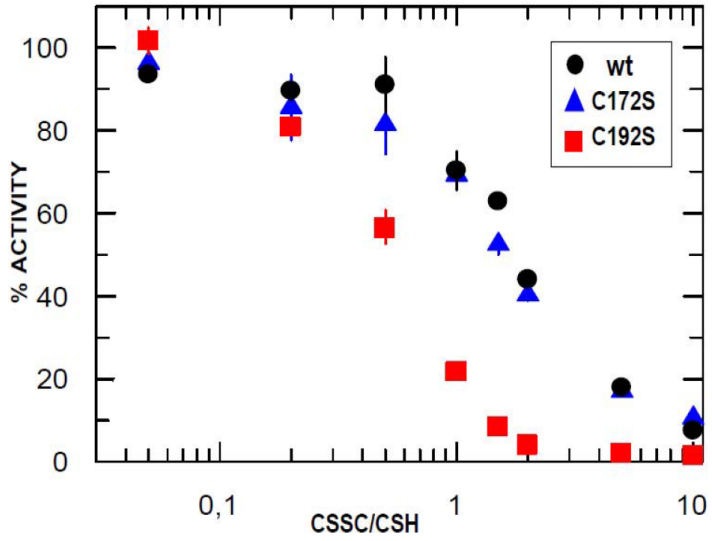


Fig.B2. **Inactivation of C172S, C192S and wild type RubisCO in cystamine/cysteamine redox buffers at different disulfide/thiol ratios.** Purified RubisCO (ca 0.3 mg/ml) was incubated with mixtures of the disulfide cystamine (CSSC) and the thiol cysteamine (CSH) at different ratios in activation buffer (50 mM Tris HCl, 10 mM Mg Cl_2, 10 mM $NaHCO_3$, pH 8.2). Constant monomeric concentration (i.e. [CSH] + 2·[CSSC]) was kept constant and equal to 40 mM for all ratios. Incubation took place in a nitrogen chamber at 30 ºC for 2 hours. The residual carboxylase activity is represented as a percentage of that of the fully reduced enzyme. Error bars indicate SEM from triplicates. Figure taken from García-Murria (2006).

Substitution of the vicinal Cys 449 and 459 resulted in enzymes that retain residual activity at high CSSC/CSH ratios (fig. B3), but experience enhanced aggregation and membrane association under stress (Marín-Navarro and Moreno, 2006).

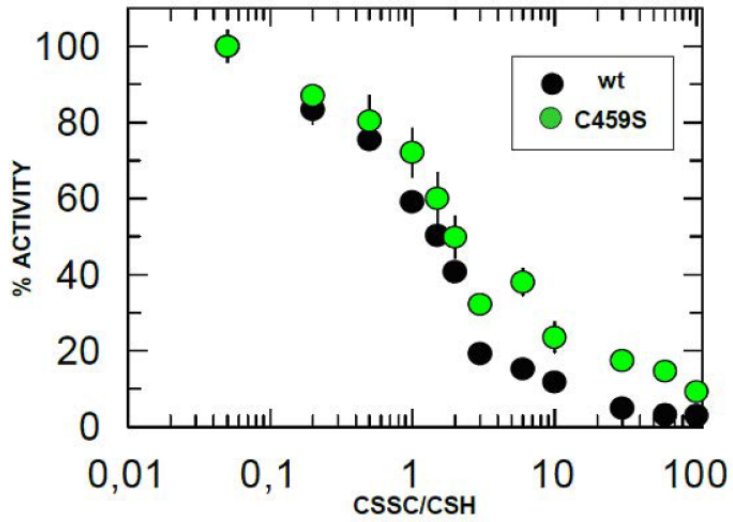


Fig.B3. **Inactivation of C459S and wild type RubisCO in cystamine/cysteamine redox buffers at different disulfide/thiol ratios.** Redox treatment and assay of the purified RubisCO took place as explained in the legend of fig. B2. Figure taken from Marín-Navarro (2004).

In this work, the search for the critical cysteines has been restricted to a set of conserved residues. Moreover, the sequential order in which these residues are oxidized by CSSC has been determined, and the scanning of RubisCO cysteine mutants has been completed by analyzing the site-directed mutants of the relevant cysteines that had not been characterized previously.

MATERIALS and METHODS

***Chemicals*.** References for most chemicals are given in chapter A. Analytical grade guanidinium chloride (GdCl), N-ethylmaleimide (NEM), iodoacetamide (IAM) and 4-vinylpyridine (VP) were obtained from Sigma. Phenylmethylsulfonyl fluoride (PMSF) was purchased from Boehringer. Trypsin and chymotrypsin for protein digestion were from Promega and Roche respectively.

C. reinhardtii strains and culture. The culture medium and maintenance conditions for *Chlamydomonas reinhardtii* have been described already in chapter A. Wild type *C. reinhardtii* strain was 2137 mt + (Spreitzer and Mets, 1981). RubisCO cysteine to serine site-directed mutant strains C459S (Marín-Navarro and Moreno, 2006), C84S, C247S, C284S, C427S, sC41S and sC83S (Marín-Navarro, 2003) have already been characterized.

RubisCO purification and assay. Purification of the *C.reinhardtii* RubisCO was carried out as previously reported in chapter A. Spinach and rice leaves were obtained from local crops, brought to the laboratory under refrigeration, and extracted immediately. Leaves were weighed and minced before being ground with pestle and mortar in 4 ml/(g of fresh weight) of extraction buffer (100 mM Tris-sulfate, 10 mM $MgSO_4$, 20 mM β-mercaptoethanol, pH 8.0). Further purification of RubisCO through ammonium sulphate precipitation, sucrose gradient centrifugation and anion exchange FPLC was carried out as for the *C. reinhardtii* enzyme.
Carboxylase activity assay, using ^{14}C-bicarbonate, in the presence of effectors (CSSC or NEM) has been already described in full detail (chapter A). Exceptionally, the spinach and rice RubiCOs were assayed only for 1 minute to minimize activity decay due to blocking of the catalytic site ("fallover") to which higher plant enzymes are prone when assayed *in vitro*. All inactivation treatments were carried out with the RubisCO dissolved in 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 100 mM Tris-HCl, pH 8.2 (activation buffer).

RubisCO proteolysis. *In vitro* proteolysis of RubisCO with subtilisin has been already described in detail (Marín-Navarro and Moreno, 2003). Briefly, the purified RubisCO (0,3 mg/ml) in activation buffer was incubated with subtilisin (0.5 µg/ml) at 30 ºC in a water bath. Proteolysis was stopped at different times by adding PMSF (at 2 mM final concentration) and chilling on ice. Afterwards, the samples were run

in SDS-PAGE and stained with Coomassie blue as previously reported (Marín-Navarro and Moreno, 2003).

Differential labeling of oxidized and reduced RubisCO cysteines, protein digestion and mass spectrometry analysis. Aliquots (0.8 ml) of purified RubisCO (1.25 mg/ml in activation buffer) were incubated under a nitrogen atmosphere for 2h at 30 ºC with CSSC/CSH mixtures at different ratios but total monomeric concentration (i.e. CSH + 2CSSC) equal to 40 mM in all cases. Afterwards, 0.2 ml of 1M IAM in activation buffer was added to each sample and the mixture was left for 30 minutes a 30 ºC. Then, 0.8 ml were transferred to an Eppendorf vial containing 575 mg of solid guanidinium chloride (GdCl), which was dissolved (final concentration 5.2 M) and the solution was left for a further 30 minutes at 30ºC. After this, 1 ml of the solution was passed through a Sephadex G-25 column (GE Healthcare, NAP 10) equilibrated with 5.2 M GdCl in activation buffer. The protein was recovered in 1.5 ml. Further 30µl of 1M dithiothreitol (DTT) were added to reduce disulfides and the mixture was incubated at 30 ºC for 2 h. Finally, 35 µl of 4-vinylpyridine were mixed in and this was followed by a 30 minute incubation at 20 ºC and storage at 4 ºC overnight.

The derivatized RubisCO was recovered by precipitation in chloroform:methanol (4:1) and redissolved in 25 mM ammonium bicarbonate before being treated either with trypsin (25 µg at 37 ºC for 12h) or chymotrypsin (25 µg at 28 ºC for 2 h). The digestion products were dried in a vacuum centrifuge and resuspended in 0.1% trifluoracetic acid. Peptides were analyzed by liquid chromatography and double mass spectrometry (LC-MS/MS) using an Ultimate nano-LC chromatographic system and a QSTAR XL-QTOF hybrid mass spectrometer. Samples (5 µl) were delivered to the system using a FAMOS autosampler at 30 µl/min and the peptides were trapped onto a PepMap C18 precolumn. Peptides were then eluted onto an analytical PepMap C18 column and separated using a 15-35% acetonitrile gradient at 200 nl/min. The QSTAR XL was operated in the information-dependent acquisition mode in which a 1s TOF MS scan from 400 to 2000 m/z was followed by 3s product ion scans from 60 to 2000 m/z on the two most intense double or triple charged ions. Quantitation of the mass spectrometer detector signals was done using the Progenesis software. Values for the carbamidomethylated peptides were normalized as fractions of the signal obtained for the same peptide in a sample of totally reduced RubisCO. Similarly, pyridylethylated peptides were expressed as fractions of the homologous signals recorded for a fully CSSC-oxidized enzyme.

RESULTS

Inactivation of RubisCO from rice and spinach through disulfide exchange

As shown in figure B1, RubisCO from *C.reinhardtii* becomes inactive when incubated in redox buffers of a CSSC/CSH ratio above 1.5. A similar experiment carried out with the RubisCOs of spinach and rice demonstrated that these enzymes inactivate in the same manner, although the rice RubisCO does so at a slightly lower CSSC/CSH ratio (fig. B4).

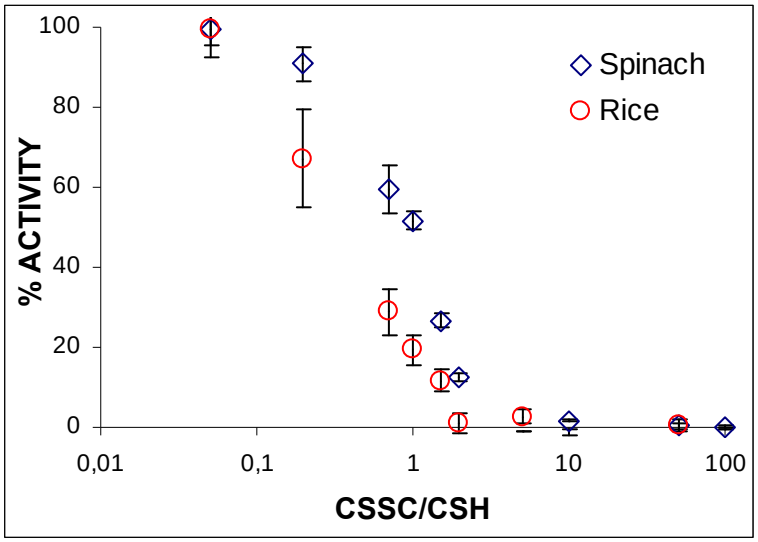


Fig.B4. **Inactivation of spinach and rice RubisCOs in disulfide/thiol redox buffers.** RubisCOs (about 0.2 mg/ml) were incubated with mixtures of the disulfide cystamine (CSSC) and the thiol cysteamine (CSH) at different ratios in activation buffer (50 mM Tris HCl, 10 mM Mg Cl_2, 10 mM $NaHCO_3$, pH 8.2). Constant monomeric concentration (i.e. [CSH] + 2·[CSSC]) was kept constant and equal to 40 mM for all ratios. Incubation took place in a nitrogen chamber at 30 ºC for 2 hours. Afterwards, the carboxylase activity of the RubisCOs was assayed for 1 minute. Activity values are represented as a percentage of that of the RubisCO incubated with 40 mM CSH (CSSC/CSH ratio = 0).

The fact that spinach, rice and *C. reinhardtii* RubisCOs, all of them, experience cysteine-dependent inactivation (through CSSC) suggests that critical cysteines (those that contribute to carboxylase activity loss upon oxidative modification) are conserved residues. According to the sequence of the genes, the RubisCO of *C. reinhardtii* contains 16 cysteines per heterodimer, 12 in the large subunit and 4 in the small subunit. Table BI lists the cysteine residues of both the large and small subunit of the *C. reinhardtii* RubisCO and the corresponding amino acid residues located at the same position in spinach and rice.

Table BI. **Cysteines of the C.reinhardtii RubisCO large (LS) and small (SS) subunits and amino acid residues (one letter code) found at homologous positions in the RubisCOs of spinach and rice.**

Species		*C. reinhardtii*	Spinach	Rice
Cys residue				
LS	53	C	A	A
	84	C	C	C
	172	C	C	C
	192	C	C	C
	247	C	C	C
	256	C (methyl)[a]	F	F
	284	C	C	C
	369	C (methyl)[a]	V	V
	399	C	V	V
	427	C	C	C
	449	C	T	C
	459	C	C	C
SS	41	C	P/C[b]	C
	65	C	(P)[c]	(P)[c]
	83	C	G/C[b]	C
	96	C	V	A

[a] These cysteines are found methylated (thus, redox inactive) in the native *C.reinhardtii* RubisCO

[b] Different residues are found in the products of the various *rbc*S genes.

[c] Cys 65 of the small subunit has no actual homologous residue in rice and spinach because it is included in a sequence stretch which is deleted in higher plants. Proline occupies the numerical position of Cys 65 but in a different conformational context.

Table BI shows that cysteines which are conserved between the RubisCOs of the three species are residues 84, 172, 192, 247, 284, 427 and 459 of the large subunit and 41 and 83 of the small subunit (in the *C. reinhardtii* RubisCO numbering). These residues constitute a highly conserved set of cysteines among eukaryotic green-like RubisCOs (Table BII) with the exception, perhaps, of Cys247 which is only moderately conserved in green algae.

Table BII**. Percentage of species for which a cysteine residue is present at a homologous position for each of the cysteine residues of the *C. reinhardtii* RubisCO**. Sequences (taken from the Gene Data bank) of 495 higher plant and 121 green algae species were tested for the large subunit, and 63 higher plants and 8 green algae for the small subunit. (Number of species in which the particular Cys is present/number total of species in which the sequence at this position is available) is indicated in parentheses. Conserved Cys are indicated in red. Data collected by Dr. Julia Marín-Navarro.

LARGE SUBUNIT		
CYS	HIGHER PLANTS	GREEN ALGAE
53	0% (0/495)	77.5% (93/120)
84	98.8% (489/495)	99.2% (119/120)
172	100% (495/495)	99.2% (119/120)
192	99.4% (492/495)	99.2% (119/120)
247	96.4% (477/495)	53.7% (65/121)
256	3.0% (15/495)	65.3% (79/121)
284	99.4% (492/495)	100% (121/121)
369	0% (151/495)	66.7% (16/24)
399	0% (0/495)	100% (24/24)
427	99.4% (492/495)	100% (24/24)
449	30.5% (151/495)	66.7% (16/24)
459	98.9% (417/422)	100% (14/14)
SMALL SUBUNIT		
CYS	HIGHER PLANTS	GREEN ALGAE
41	100% (63/63)	87.5% (7/8)
65	0% (0/63)	25 (2/8)
83	96.8% (61/63)	100% (8/8)
96	27% (17/63)	87.5% (7/8)

It is likely that the cysteine-dependent redox properties displayed by RubisCO reside in some of the cysteines of this group of conserved residues. Other cysteines are much less conserved. Cys 449 which is present in the enzymes of *C. reinhardtii* and most green algae (but absent in spinach) is potentially relevant to redox regulation because is close to the conserved Cys 459, at a disulfide bonding distance (Taylor *et al*., 2001).

Specific activity of the C. reinhardtii RubisCO cysteine mutants

In order to clarify the individual contribution of the conserved Cys residues to oxidative inactivation, site-directed RubisCO mutants replacing each of the conserved Cys (one at a time) by serine have been constructed (Moreno and Spreitzer, 1999; Marín-Navarro, 2004; Marín-Navarro and Moreno, 2006; García-Murria *et al*., 2008). Because mutants C172S, C192S and C459S have already been characterized, this work focuses on the cysteine mutants at the remaining conserved residues, namely C84S, C247S, C284S and C427S among the large subunit substitutions, and sC41S and sC83S among those from the small subunit.
All these mutants grew autotrophycally (i.e., in minimal medium) at a rate that was similar to the wild type. A survey of the intrinsic specific carboxylase activity of the purified RubisCOs showed that some of the mutant enzymes (in special, C84S, C247S and C427S) had a somewhat lower activity than the wild type (table BIII).

Table BIII. **Intrinsic specific carboxylase activity of the mutant RubisCOs with single cysteine replacement**. Activity values are given ± SEM from five determinations.

Mutant	**Activity [µmol CO_2 fixed((min·mg)]**
C84S	1.10 ± 0.04
C247S	1.41 ± 0.16
C284S	1.93 ± 0.08
C427S	1.25 ± 0.28
sC41S	1.75 ± 0.11
sC83S	1.63 ± 0.12
Wild type	1.86 ± 0.06

The specific activity of C84S was particularly low (60 % of the wild type) and this mutant had also a reduced content of RubisCO per cell (one third of the wild type). Further information was sought by analyzing the properties of the purified mutant RubisCOs *in vitro*. Each mutant was assayed for thermal stability, activity remaining after equilibration with redox buffers at different CSSC/CSH ratios, and kinetics of inactivation by several thiol-directed reagents. In the following sections, the main results showing differences between mutant and wild type enzymes are presented.

Thermostability and structure changes of the cysteine mutant RubisCOs

To check possible structural roles of the conserved cysteines, the thermal stability of all mutants RubisCOs was tested by measuring the carboxylase activity of samples that had been previously incubated at different temperatures for 30 minutes. Noticeable changes of stability were detected in the C84S, C284S and C427S mutants. C284S and C427S denatured around 54 ºC, some 2 ºC below the wild type (fig. B5). The highest thermal sensitivity was displayed by the C84S RubisCO, which experienced thermal denaturation around 51 ºC, about 5 ºC lower than the wild type (fig. B5).

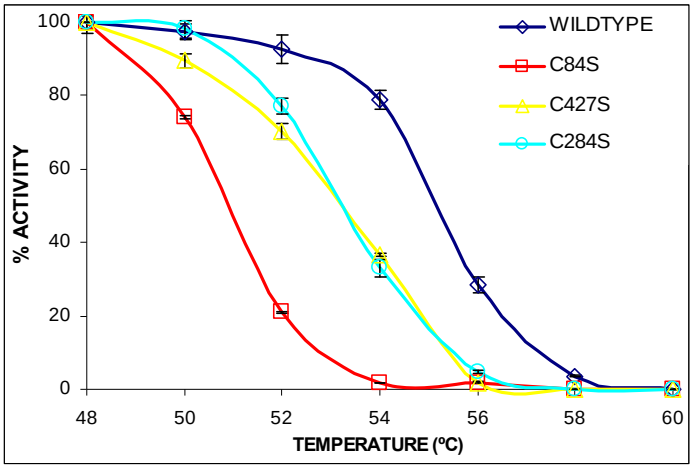


Fig.B5. **Thermal inactivation of the wild type and the C84S, C284S and C427S mutant RubisCOs.** Purified wild type and mutant RubisCO samples (ca 0.2 mg/ml in activation buffer) were incubated for 30 minutes at the given temperatures, Afterwards, they were chilled on ice for 5 min, transferred to a water bath at 30 ºC and, after a further 10 min, assayed for carboxylase activity at 30 ºC. Activity values are given as a percentage of that of the sample incubated at 48 ºC. Error bars indicate ± SEM from triplicates.

Furthermore, the C84S mutation caused a remarkable conformational distortion which could be detected by a change in the fragmentation pattern resulting from the treatment of the native holoenzyme with subtilisin (fig. B6).

The C84S mutant was, in general, more intensely degraded that the wild type enzyme treated in parallel and displayed a new and characteristic proteolytic band of about 47 kDa (fig. B6). All these features of the C84S mutant suggest a significant alteration of the three-dimensional structure which is likely to be the cause of its low specific carboxylase activity (table BIII).

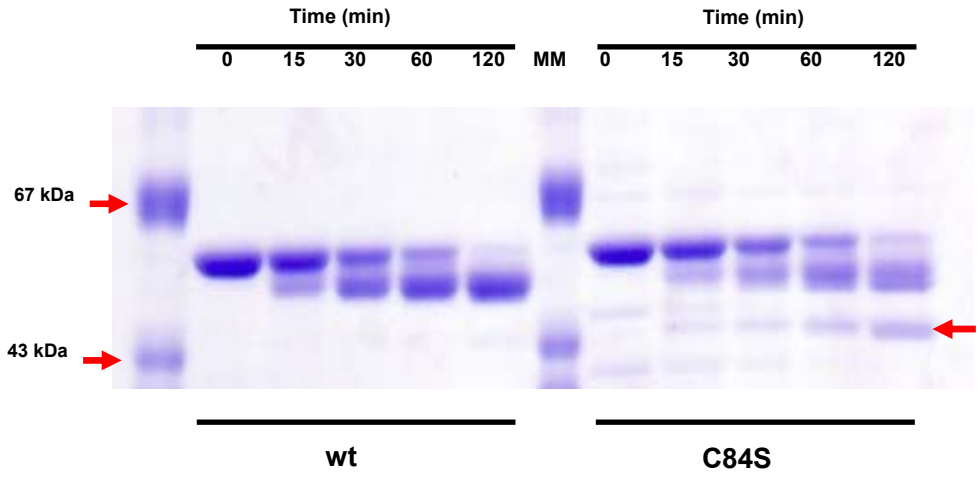


Fig.B6. **SDS-PAGE electrophoregrams showing the fragmentation of the large subunit (ca 55 kDa) of the wild type (wt) and C84S mutant RubisCOs after incubation of the purified native holoenzyme with subtilisin for the indicated times**. Mass of molecular markers (MM) is indicated on the left. The C84S mutation promotes a distinctive 47 kDa fragmentation band (indicated by the red arrow on the right).

Inactivation of the mutant RubisCOs by cysteine-directed reagents

In order to check the redox properties of the purified mutant RubisCOs *in vitro*, each mutant was assayed for activity remaining after equilibration with redox buffers at different CSSC/CSH ratios. Besides, the kinetics of RubisCO inactivation by CSSC and by the thiol-directed reagent N-ethyl maleimide (NEM) was also determined. Significant differences between the wild type enzyme and the mutants will be presented in this section. Full details for all mutants are given in the Supplementary Information section at the end of the chapter.

All of the studied mutants were fully inactivated by CSSC. Scanning the progressive inactivation with CSSC/CSH redox buffers for differences between wild type and mutants revealed only a slight shift to a lower CSSC/CSH ratio in the case of the C427S mutant, which showed a transition midpoint around a CSSC/CSH ratio of 1 (fig. B7).

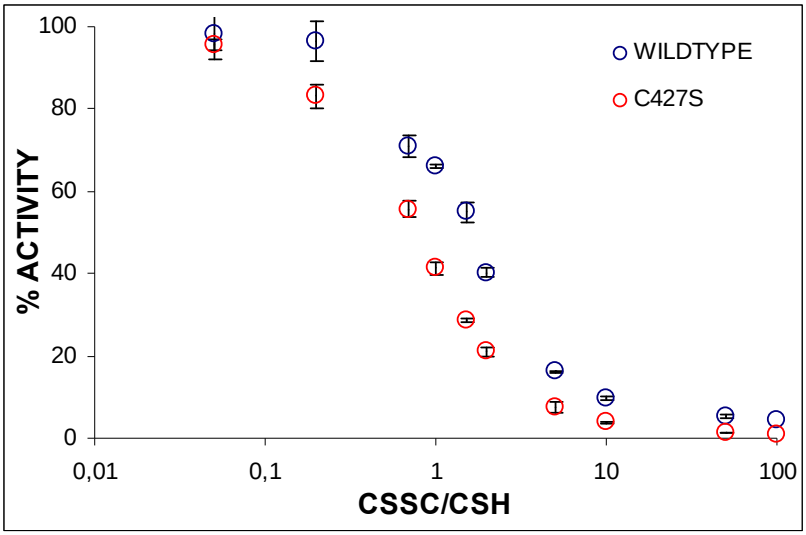


Fig.B7. **Inactivation of C427S and wild type RubisCO in cystamine/cysteamine redox buffers at different disulfide/thiol ratios.** Purified RubisCO (ca 0.3 mg/ml) was incubated with mixtures of the disulfide cystamine (CSSC) and the thiol cysteamine (CSH) at different ratios in activation buffer (50 mM Tris HCl, 10 mM Mg Cl_2, 10 mM $NaHCO_3$, pH 8.2). Constant monomeric concentration (i.e. [CSH] + 2·[CSSC]) was kept constant and equal to 40 mM for all ratios. Incubation took place in a nitrogen chamber at 30 ºC for 2 hours. The residual carboxylase activity is represented as a percentage of that of the fully reduced enzyme. Error bars indicate ± SEM from triplicates.

The kinetics of inactivation by CSSC was also somewhat faster for the C427S mutant than for the wild type (fig. B8, panel A).

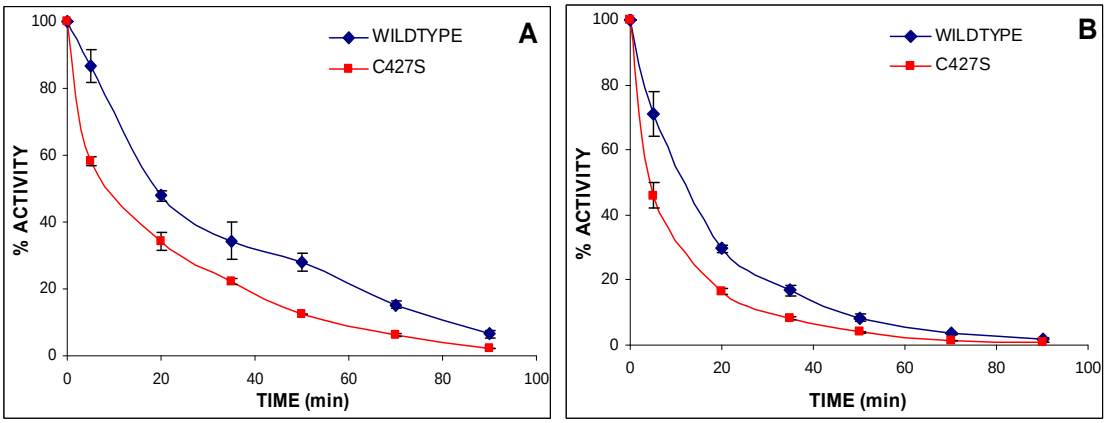


Fig.B8. **Time course of the carboxylase activity decay for the C427S and wild type RubisCOs incubated with 20 mM CSSC (A) or 0.1 mM NEM (B).** Activity is expressed as a percentage of the initial value. Error bars indicate ± SEM from triplicates.

Similarly, inhibition by NEM was faster in the C427S mutant when compared to the wild type enzyme (fig. B8, panel B). These results suggest an increased accessibility to the critical residues in the mutant enzyme. Remarkably, no single cysteine substitution suppressed the sensitivity of RubisCO to any of these reagents, reaching always full inactivation in all mutants.

Sequential oxidation of RubisCO cysteines followed by mass spectrometry

Inactivation and proteolytic sensitization of RubisCO is known to take place by the progressive oxidation of cysteines with different redox potential (García-Ferris and Moreno, 1993; Moreno *et al*., 2008) (fig. B1). To attempt the identification of the cysteines involved in those changes, a chemical labelling protocol has been followed to react free sulfhydryl cysteines with iodoacetamide (IAM) and disulfide cysteines (after reduction) with vinylpyridine (VP). This protocol has been applied to purified RubisCO samples that had been exposed to five different CSSC/CSH ratios (0.01, 1.5, 2.8, 4, and 80). The enzyme was simultaneously denatured and treated with IAM to carbamidomethylate the cysteines remaining as free sulfhydryls after the redox treatment. Then, the potential disulfides were reduced with dithiotreitol and the newly reduced cysteines were pyridylethylated with VP. Afterwards, the modified RubisCO was subjected to proteolytic fragmentation (either by an extensive treatment with trypsin or a time-limited partial proteolysis with chymotrypsin) and the resulting peptides were separated by liquid chromatography and analyzed by mass spectrometry. Mass determination of identified peptides that contained one cysteine indicated the type of derivatization (carbamidomethylation or pyridylethylation) suffered by that residue. Full details of the identified peptides are given as Supplementary Information at the end of the chapter.

Among the group of conserved cysteine residues, no pyridylethylated peptides were found for cysteines 284 from the large subunit and 83 from the small subunit implying, perhaps, that these residues are internal and not engaged in redox exchanges (therefore, being always carbamidomethylated at the first step of denaturation and treatment with IAM). On the other hand, no carbamidomethylated peptide for Cys 247 from the large subunit was detected either, suggesting that this residue is almost always found as a disulfide. Indeed, disulfide bonding between Cys247 residues from adjacent large subunits has been reported to take place spontaneously during purification of RubisCO (Ranty *et al*., 1991). All other conserved cysteines were found in both modifications revealing a certain degree of redox modulation.

Representing the fraction of oxidized residue against the oxidation state (corresponding to the different CSSC/CSH ratios tested) showed the progression of the modification for each of the residues (fig. B9).

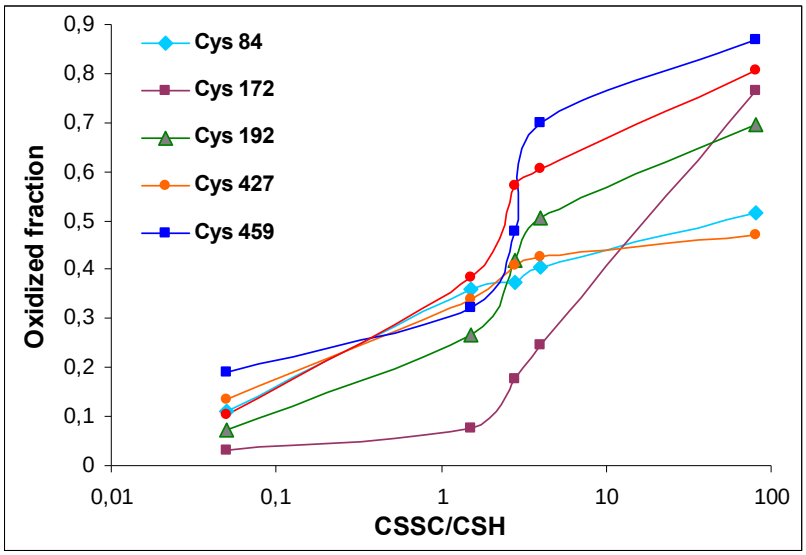


Fig.B9. **Fraction of RubisCO cysteine residues that became oxidized after equilibration with different CSSC/CSH ratios at a fixed 40 mM total monomeric (CSH + 2 CSSC) concentration of redox agents.** The fraction of oxidized residues was calculated from the signals of carbamidomethylated and pyridylethylated peptides containing the corresponding cysteine as described in Matherias and Methods

Most of the residues experienced a clear transition from reduced to oxidized at a CSSC/CSH ratio near 1.5. Only Cys172 displayed a distinctly delayed transition. Cys 84 and 427 progressed only to partial oxidation (about half of the residues) even at the highest CSSC/CSH ratios (fig. B9). These cysteines showed, however, a definite stepwise increase of oxidation around the same CSSC/CSH ratio of 1.5 as most other residues. A plausible explanation for this result would be that the oxidation of certain cysteines taking place around that particular CSSC/CSH ratio promotes conformational changes that hinder further oxidation of Cys 84 and 427 in the same or a neighboring subunit of the holoenzyme. Other cysteines (41 from the small subunit and 172, 192 and 459 from the large) experienced extensive oxidation and, among these, an ordered sequence of oxidation can be tentatively read from figure B9. The sequence runs sCys 41 - Cys 459 - Cys 192 - Cys 172 from most to least easily oxidizable.

It is noteworthy that Cysteines 172 and 192, integrating one of the vicinal pair of residues, showed a dissimilar oxidation course. Cys 172 became abruptly oxidized at high CSSC/CSH ratios (above 4) while Cys 192 displayed a more progressive oxidation with a lower transition midpoint (fig. B10).

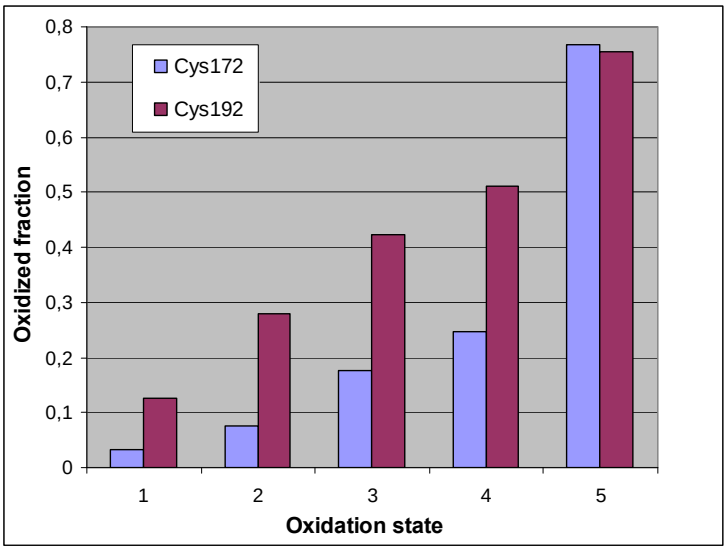


Fig.B10. **Fraction of oxidized Cys172 and Cys192 residues after equilibration with different CSSC/CSH ratios.** Oxidation states marked 1 to 5 correspond to CSSC/CSH ratios of 0.01, 1.5, 2.8, 4 and 80, correlatively. Data are singled out from fig. B9.

Some conclusions can be drawn from this result. First, these vicinal cysteines do not appear to engage in an internal disulfide bond when oxidized (otherwise, the oxidation of the two residues would have run parallel). This was already predicted from the unfavourable orientation of the thiol groups in the structural model obtained by X-ray crystallography (Taylor *et al*., 2001; García-Murria *et al*., 2008). Second, the late oxidation of Cys172 is coincident with the conformational changes that trigger the proteolytic susceptibility of the enzyme (fig. B1). Thus, the oxidation of Cys172 could contribute to the induction and/or stabilization of these changes, as might also be expected from the proteolysis-resistant phenotype displayed by the RubisCO mutants in which this specific residue has been substituted (Moreno and Spreitzer, 1999: Marcus *et al*., 2003).

DISCUSSION

While the redox state of RubisCO cysteines is known to regulate the activity and conformation of the enzyme (Moreno *et al*., 2008), the precise identity of the residues that promote the functional changes has remained elusive hitherto. The fact that redox regulation is present in several species (fig.B4) suggests that the relevant changes are carried out by the oxidation of conserved residues. To investigate the role of the individual residues, a set of *C. reinhardtii* RubisCO mutants has been constructed in which conserved cysteines have been replaced, one at a time, by serine. This is a very conservative substitution that abolishes

redox activity by exchanging a single atom (sulfur for oxygen). Although the mutant RubisCOs display some differences in specific carboxylase activity *in vitro* (table BIII), all single cysteine mutants grow autotrophically without noticeable difficulties at a rate similar to the wild type, suggesting that these cysteines do not play a prominent role in catalysis and, therefore, may be highly conserved because of additional advantages.

A possible role for these residues could be to establish interactions that contribute to the structural stability of the holoenzyme. This might be the case of Cys247 linking the two large subunits that integrate the catalytic homodimer through a disulfide bond. This residue is deeply buried in the holoenzyme structure and does not apparently participate in redox exchange with the surrounding aqueous environment while the enzyme remains in the native folding (Ranty *et al*. 1991). In addition, results presented here indicate a remarkable structural role for Cys 84 from the large subunit, whose replacement by serine produced thermosensitivity (fig.B5) and structural shifts which were detected by a change of the subtilisin fragmentation pattern (fig.B6). Cys 284 and 427 appear to play also a modest role in structural stability, their replacement being only noticeable as a decrease of a few degrees in the critical denaturation temperature (fig.B5).

The other obvious role for the cysteines is to participate in the redox modulation of RubisCO activity and conformation related to the catabolism of the enzyme. The main result of this work in this respect is that none of the single cysteine replacements eliminated the sensitivity of RubisCO activity to disulfide exchange with CSSC. Similarly, inactivation through sulfhydryl-directed modification with NEM could also not be abolished by single substitutions. This strongly suggests that oxidative inactivation is the result of a cooperative effort of a set of cysteine residues with highly redundant roles.

It might be noted that the simultaneous oxidation of the different cysteines may create interferences between them. The close transitions of cysteines s41, 84, 192, 427 and 459 around a CSSC/CSH ratio of 1.5 (fig.B9) can lead to structural changes which affect each other accessibility to disulfide exchange. This may explain why cysteines 84 and 427 do not progress to the full oxidation of all residues even at high CSSC/CSH ratios (fig.B9). Moreover, the substitution of two of the conserved residues, Cys 192 (García-Murria, 2006) and Cys 427 (described in this work), results in an increased sensitivity of the enzyme to oxidation, shifting the inactivation curves to lower CSSC/CSH ratios (figs. B2 and B7). Thus, Cys 192 and Cys 427 appear to protect the enzyme against oxidation. In order to explain this result one has to assume that these "protective" residues oxidize without inhibiting the carboxylase activity, but their oxidation impedes a further disulfide

exchange by other critical residues, thereby postponing inactivation. Cys 172, being the last to oxidize in the wild type enzyme and at a distinctly high CSSC/CSH ratio (fig. B9), is the most likely residue to experience a delayed transition. The interfering action could be exerted through long range conformational effects but also through direct obstruction of the access to a neighbor residue. This might be the case with Cys192 which is vicinal to Cys 172. When Cys 192 is present, its disulfide exchange with CSSC may easily restrict a further mixed disulfide with Cys 172 because of steric or charge (since cysteamine is positively charged) constraints. Therefore, Cys172 could only oxidize after substantial conformational changes at a high CSSC/CSH ratio (fig.B9). In the absence of Cys192 (i.e. in the C192S mutant), the open access to Cys 172 could lead to a premature inactivation of RubisCO (fig. B2) if the disulfide exchange of Cys 172 with CSSC inhibits the enzyme, as has been shown to occur when this particular residue is specifically modified by an affinity label (Schloss *et al.*, 1978). In the case of Cys 427, the protective effect is more difficult to explain since this residue does not appear to complete its oxidation (fig.B9), even at high CSSC/CSH ratios. Nevertheless, it is possible that a partial oxidation of Cys 427 (i.e oxidation of this residue only at some of the large subunits integrating RubisCO) may block the access to other critical cysteines in the whole holoenzyme because of the cooperative interactions between subunits.

Summarizing published and present observations, a tentative role may be assigned to every conserved cysteine regarding the redox properties of RubisCO (table BIV).

Table BIV. **Presumed roles for the conserved cysteine residues of RubisCO in structural support and redox regulation by disulfide exchange**.

Residue	Role
Cys 84	Weighty structural. Absence produces gross conformational distortions
Cys172	Critical for inducing proteolytic susceptibility and probably for activity
Cys192	Protective and structural
Cys247	Secluded by spontaneous oxidation without affecting activity
Cys284	Not accessible. Does not exchange disulfides
Cys427	Protective and structural
Cys459	Critical for redox regulation of activity. Needed for full inactivation
sCys41	Probably critical for redox regulation of activity
sCys83	Not accessible. Does not exchange disulfides

Three residues appear not to be involved redox regulation: Cys 84 has a definite structural function (figs. B5 and B6) and does not reach full oxidation (fig.B9),

while Cys284 and sCys83 do not engage in disulfide exchange with CSSC remaining always reduced. Cys 247 oxidizes spontaneously without activity loss and thereafter becomes isolated from the redox environment (Ranty *et al*., 1991). Therefore, this one must also be discarded as critical. Two residues, Cys 192 and Cys 247, appear to protect the enzyme from premature inactivation (fig. B2 and B7) as discussed above. The three remaining cysteines, sCys 41, Cys 172 and Cys 459 are the most likely to be involved in redox regulation of RubisCO. Cys 172 is clearly related to conformational changes leading to enhanced proteolytic susceptibility (Moreno and Spreitzer, 1999). While the oxidation of this residue might perhaps cause RubisCO inactivation when the protective Cys192 is absent (fig.B2), its oxidation (about at CSSC/CSH ratio of 4) occurs much too late to contribute to the inactivation transition seen in the wild type (about a CSSC/CSH ratio of 1.5) (fig.B9). In contrast, Cys 459 is definitely involved in RubisCO inactivation since its absence prevents full activity loss (fig.B3) (Marín-Navarro and Moreno, 2006). Accordingly, Cys 459 oxidizes around a CSSC/CSH ratio of 1.5 (fig.B9), which is coincident with wild type RubisCO inactivation (fig.B1). However, because there is still a very substantial activity loss when Cys 459 is absent (fig.B3), another Cys residue oxidizing at a similar CSSC/CSH ratio must be responsible for the transition to the inactive state of the C459S mutant. This is likely to be sCys41. Therefore, the simplest hypothesis that fits the currently accumulated experimental facts is that inactivation of the wild type RubisCO by CSSC results from the oxidation of sCys 41 and Cys459 by disulfide exchange. Oxidation of any of these residues produces extensive inactivation (indeed full inactivation for Cys 459 and almost full for sCys41), thereby playing redundant roles. Further oxidation of Cys 172 at higher CSSC/CSH ratios fulfils conformational changes that render the enzyme sensitive to proteases. Only when one of the protective residues, Cys 192 or Cys 427, is absent, inactivation may result from the premature oxidation of other residues (such as Cys 172) which become unprotected. In this manner, the interacting network of critical and protective cysteines ensure a robust and fine-tuned switching of RubisCO activity in response to a changing redox environment.

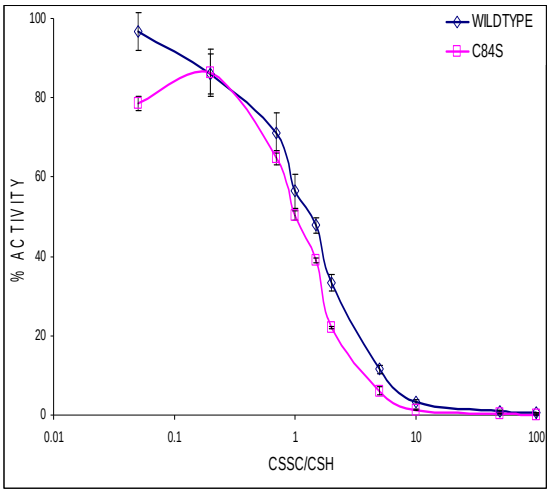
Acknowledgements

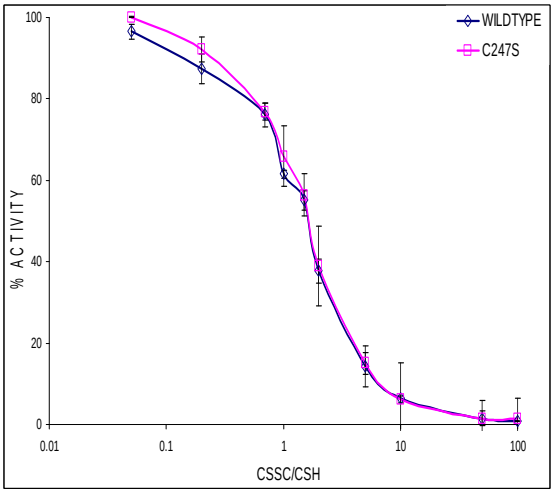
Dr. Julia Marín-Navarro (Dept. Biotechnology, IATA, CSIC) is thankfully acknowledged for gently sharing the cysteine RubisCO mutants and the data of table BII. Drs. María Jesús García-Murria, Luz Valero and Manuel Sánchez del Pino, from the Proteomics Service at Instituto Príncipe Felipe (Valencia), are also thanked for carrying out the mass spectrometry analysis of the derivatized RubisCO samples.

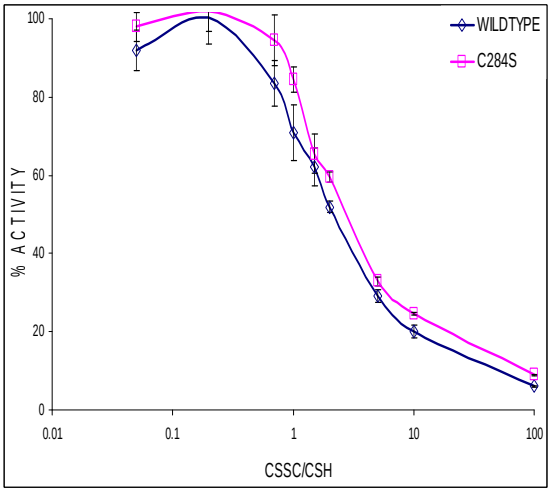
SUPPLEMENTARY INFORMATION

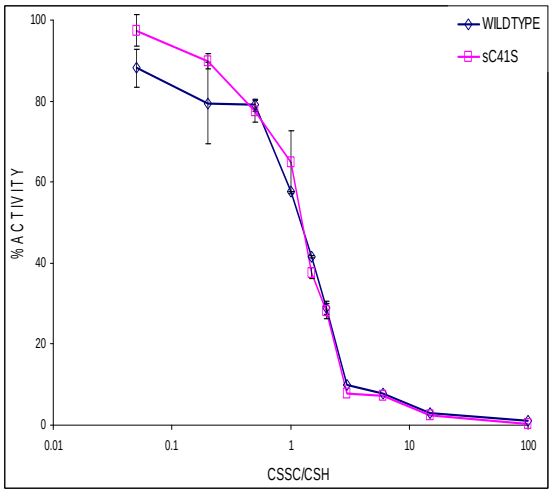
A. Redox Inactivation of the cysteine mutants of RubisCO

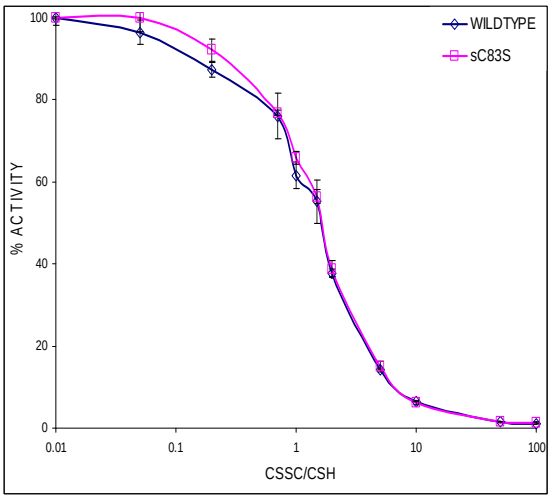
INACTIVATION IN CSSC/CSH REDOX BUFFERS



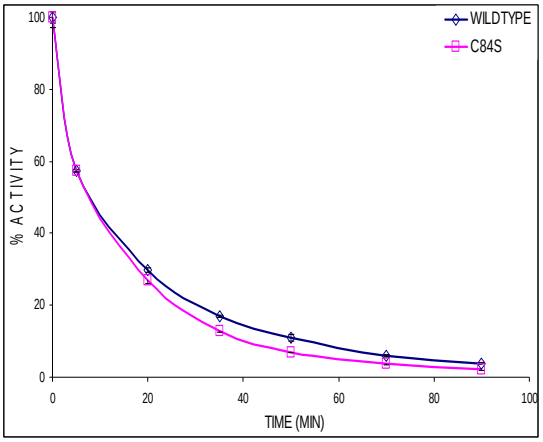


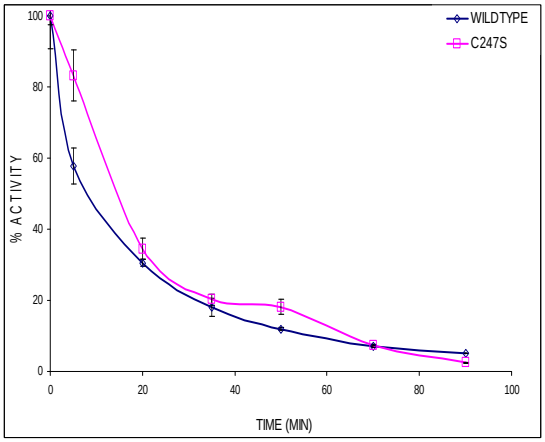


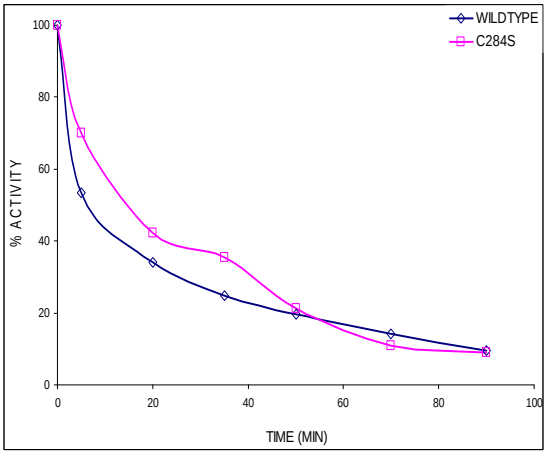


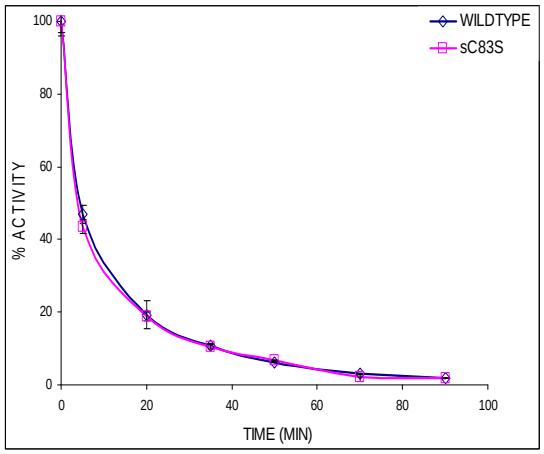


INACTIVATION WITH 20mM CSSC

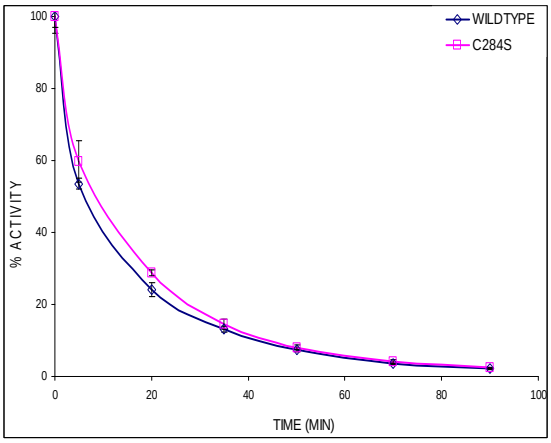


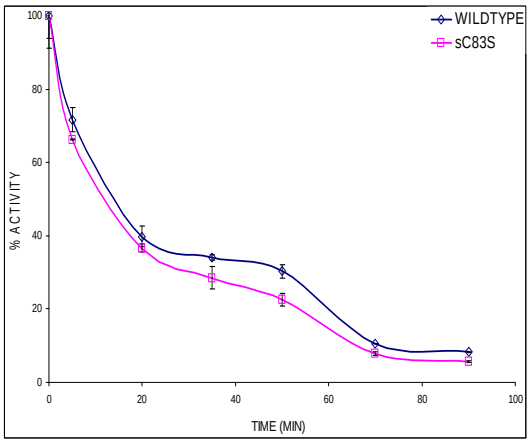


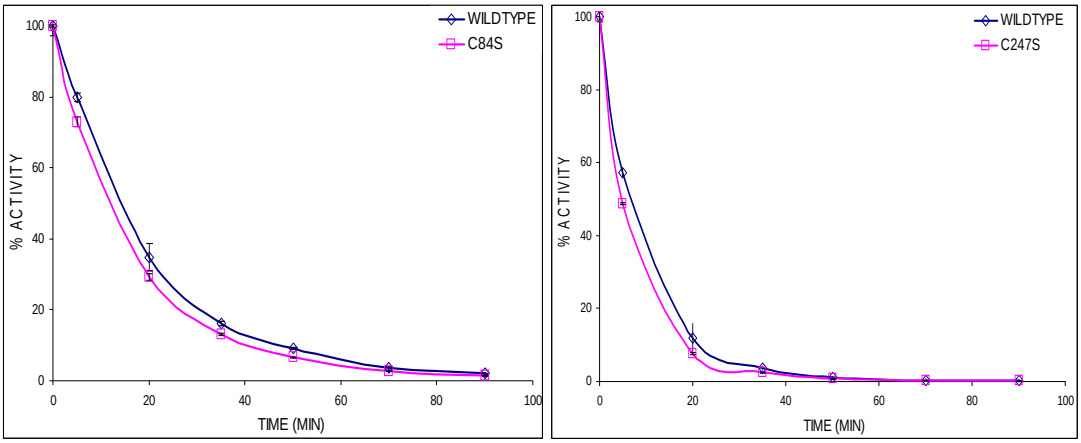




INACTIVATION WITH 0.1mM NEM







B. RubisCO peptides identified by mass spectrometry for each of the conserved cysteine residues

Subunit	Cysteine	Digestion[1]	Derivatization[2]	Identified peptide
Large	Cys 84	Chym	CAM	LS 81-100
		Chym	PE	LS 81-100
	Cys 172	Tryp	CAM	LS 168-177
		Tryp	PE	LS 168-177
	Cys 192	Tryp	CAM	LS 188-194
		Tryp	PE	LS 188-194
	Cys 247		CAM	Not found
		Tryp	PE	LS 237-252
	Cys 284	Chym	CAM	LS 281-290
			PE	Not found
	Cys 427	Tryp	CAM	LS 422-431
		Tryp	PE	LS 422-431
		Chym	CAM	LS 425-437
		Chym	PE	LS 425-437
	Cys 459	Tryp	CAM	LS 451-463
		Tryp	PE	LS 451-463
Small	sCys41	Chym	CAM	SS 39-60
		Chym	PE	SS 39-60
	sCys83	Tryp	CAM	SS 78-84
		Chym	CAM	SS 81-90
			PE	Not found

[1] Chym = chymotrypsin; Tryp = trypsin
[2] CAM = carbamidomethylated; PE = pyridylethylated

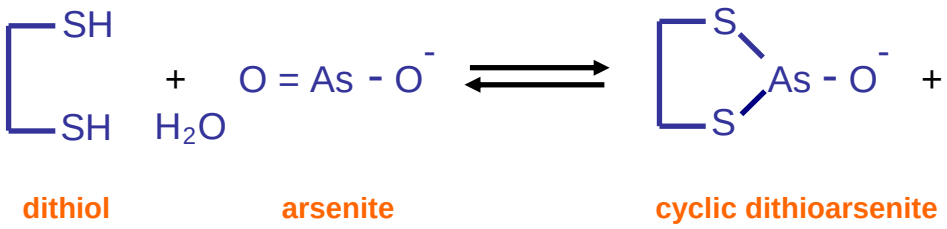
CHAPTER C

REVERSIBLE INHIBITION OF CO_2 FIXATION BY RUBISCO THROUGH THE SYNERGIC EFFECT OF ARSENITE AND A MONOTHIOL

INTRODUCTION

The net fixation of CO_2 is the crucial event recycling carbon into the biosphere. In higher plants, algae, cyanobacteria and most chemiosynthetic bacteria, carbon assimilation proceeds through a unique metabolic pathway known as the Calvin cycle. The actual fixation of CO_2 is carried out by the catalytic activity of the ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO, EC 4.1.1.39). It has been estimated that the worldwide CO_2 fixation by RubisCO introduces 10^{11} tons of carbon per year into the biosphere (Field et al., 1998). The activity of RubisCO is known to be regulated *in vivo* by different mechanism, including reactivation of the catalytic site (which requires a subservient enzyme, the RubisCO activase) after spontaneous deactivation and blocking by specific inhibitors (reviewed by Parry *et al.*, 2008). Moreover, under conditions that promote oxidation of sulfhydryl groups the activity of RubisCO is also lost because of oxidation of cysteine residues to disulfides. This modification leads to conformational changes that promote enzyme inactivation but also facilitate holoenzyme disassembly and proteolysis (Marín-Navarro and Moreno, 2003). Therefore, cysteine-dependent oxidative modification of RubisCO has been related to the catabolism of the enzyme during natural or stress-induced senescence (Ferreira *et al.*, 2000; Moreno *et al.*, 2008).

Arsenite salts (such as sodium arsenite, AsO_2Na) and other trivalent arsenic compounds react specifically with vicinal dithiols (i.e. two sulfhydryl groups in close proximity) to form cyclic dithioarsenites (Scheme 1):



Scheme 1. **Reaction of arsenite with vicinal dithiols**.

Arsenite may also react with one or two monothiol molecules to form (non-cyclic) mono- or dithioarsenites, but these are usually unstable and easily hydrolyzable (Torchinsky, 1981). Therefore, arsenite is considered a specific reagent for dithiols. The stability of the cyclic dithioarsenites depends on its geometry. When the two sulfhydryl groups are on adjacent C atoms (such as in 2,3-dimercaptopropanol) the resulting five-membered ring is specially stable. 7-membered rings are also stable, more than 6-membered (Jocelyn, 1972; Torchinsky, 1981). In the case of

conformationally vicinal thiols (as in sulfhydryls from Cys residues that are closely positioned within a protein structure), the stability of the cyclic dithioarsenite may vary widely depending on subtle geometric details and it is difficult to predict. Arsenicals (including arsenite) are well known poisons (Jomova *et al*., 2011). Inactivation of different key enzymes upon reaction of protein sulfhydryls with arsenite have been advanced as a possible justification of the toxic effects of this compound. In plants, arsenite is known to inhibit carbon fixation (Anderson and Avron, 1976; Marques and Anderson, 1986), decrease growth (Shri *et al.*, 2009), and cause oxidative stress which induces the the upregulation of antioxidants (Shri *et al.*, 2009), phytochelatins (Mishra *et al.*, 2011) and components of the ubiquitin/proteasome pathway (Santos *et al*., 2006).

Incubation of purified RubisCO with arsenite *in vitro* has been shown to produce a partially inactive (retaining about 60 to 70 % activity) form of the enzyme when tested at millimolar concentration (García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999). RubisCO from *C. reinhardtii* is known to contain 3 pairs of Cys residues which are structurally vicinal (Taylor *et al.*, 2001). These are the Cys172-Cys192, the Cys449-Cys459 and the Cys247-Cys247 (from adjacent subunits) pairs, all of them located in the large subunit (numbering of the *C.reinhardtii* enzyme). Because a RubisCO C172S site-directed mutant (in which the Cys172 residue was replaced by serine) proved insensitive to arsenite, it was suggested that the partial inactivation of the wild type enzyme caused by arsenite was exclusively due to reaction with the Cys172-Cys192 pair (Moreno and Spreitzer, 1999). Furthermore, it was subsequently found that the inactivating effect of arsenite could be increased in the presence of thiols (García-Murria, 2006). This effect was tentatively explained assuming that the thiol would reduce a pre-existing disulfide (presumably between Cys172 and Cys192), releasing a dithiol that would be subsequently targeted by arsenite (García-Murria, 2006). In this work, the inhibition of RubisCO by arsenite has been reconsidered. The results advanced here demonstrate that, while arsenite alone produces a partial inactivation of RubisCO at a relatively high concentration, it can also eliminate the carboxylase activity completely at a much lower concentration when combined with monothiols (which do not affect RubisCO activity by themselves) and RubisCO in a ternary complex. Moreover, this arsenite-monothiol synergism represents a new mode of enzyme inhibition which targets protein vicinal dithiols and allows full (but reversible) suppression of CO_2 fixation both *in vitro* and *in vivo*, under very mild conditions.

MATERIALS and METHODS

Chemicals

Analytical grade sodium arsenite (AsO_2Na), cysteamine (2-mercaptoethylamine) (CSH), reduced glutathione (GSH), N-acetylcysteine (NAC), and tris (2-carboxyethyl) phosphine (TCEP) were purchased from Sigma. Cysteine and dithiothreitol (DTT) were obtained from Merck. $NaH^{14}CO_3$ (51.7Ci/ mol) were from Perkin-Elmer. All other common chemicals were also analytical grade. Solutions used in oxidative inactivation experiments were made with highly purified water (Milli Q from Millipore).

C. reinhardtii strains and growth conditions

The culture medium and growth and maintenance conditions for *Chlamydomonas reinhardtii* have already been described in chapter A. Wild type *C. reinhardtii* strain was 2137 mt + (Spreitzer and Mets, 1981). RubisCO cysteine to serine site-directed mutant strains C172S (Moreno and Spreitzer, 1999), C192S, C172S/C192S (García-Murria *et al*. 2008), C449S, C459S (Marín-Navarro and Moreno, 2006), C84S, C247S, C284S, C427S, sC41S, sC83S (Marín-Navarro, 2003) as well as a wild type revertant of the mutant strain used for hosting the mutations (García-Murria, 2006) have already been described.

Purification and assay of RubisCO.

Purification of the *C.reinhardtii*, spinach and rice RubisCOs was carried out as previously reported in chapters A and B. Carboxylase activity assay, using ^{14}C-bicarbonate, in the presence of effectors (thiols and/or arsenite, in this case) has been already described in full detail (chapter A). All inactivation/reactivation treatments were carried out with the RubisCO dissolved in 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 100 mM Tris-HCl, pH 8.2 (activation buffer). RubisCO reactivation after arsenite inhibition was performed by passing the inactive enzyme through a Sephadex G-25 (GE Healthcare, NAP-5) desalting column equilibrated with the adequate activation buffer solution to eliminate either arsenite, either the potentiating monothiol, or both. Afterwards, the carboxylase activity was periodically monitored to follow the time course of enzyme recovery.

In vivo CO_2 fixation assay

Cell cultures of *C. reinhardtii* used for *in vivo* fixation experiments were grown in minimal medium (i.e modified TAP medium as described in chapter A, without acetate) for several days under constant light at 28-30 ºC. Cultures were kept at

the logarithmic growth phase by periodic dilution and, in particular, they were diluted two hours before the assay to about $2 \cdot 10^6$ cells/ml. 0.5 ml of minimal medium containing the inhibiting chemicals (3x concentrated) were delivered to glass tubes in a water bath at 30 ºC under constant light (100 W white light bulb placed at 20 cm from the tubes). Each treatment was assayed in three independent tubes. Then, 0,9 ml of a growing culture of cells (about $1.8 \cdot 10^6$ cells) were added to each tube. After exactly 10 min of pre-incubation, the fixation assay was started by adding 100 µl of 40 mM ^{14}C-bicarbonate (specific radioactivity ca 650 dpm/nmol) dissolved in water. The tubes were sampled periodically by transferring 200 µl aliquots of the fixing culture to a vial with 50 µl of 2% SDS in 2 M HCl. Vials were then evaporated twice before counting. Tubes containing the fixing cultures were periodically stirred and permuted as to being placed equal times at equivalent positions (with regard to the light source) in between two consecutive samplings.

RESULTS

Inactivation of RubisCO by arsenite and enhancement through monothiols

Arsenite at 4 mM concentration induced partial (about 30-35%) activity loss of the purified *C.reinhardtii* RubisCO, as has been already reported in other instances (García-Ferris and Moreno, 1993; Moreno and Spreitzer, 1999). However, at the same concentration, arsenite achieved full inactivation of the enzyme in the presence of all tested monothiols [cysteamine (CSH), cysteine (Cys), N-acetylcysteine (NAC), 2-mercaptoethanol (EtSH)] except for reduced glutathione (GSH), which did not potentiate the inactivating effect of arsenite alone (fig.C1). Activity loss took place rapidly (within 20 minutes) with all other monothiols except for NAC, which produced full inactivation only after 2 hours of treatment (not shown). In contrast to most monothiols, dithiotreitol (DTT; a dithiol with vicinal sulfhydryl groups) did not enhance activity loss but, on the contrary, appeared to protect RubisCO from arsenite inactivation (fig.C1).

Because thiols do not inactivate RubisCO by themselves but rather reactivate the oxidized enzyme (see Chapter A), arsenite and monothiols appear to inhibit the carboxylase in a synergic manner.

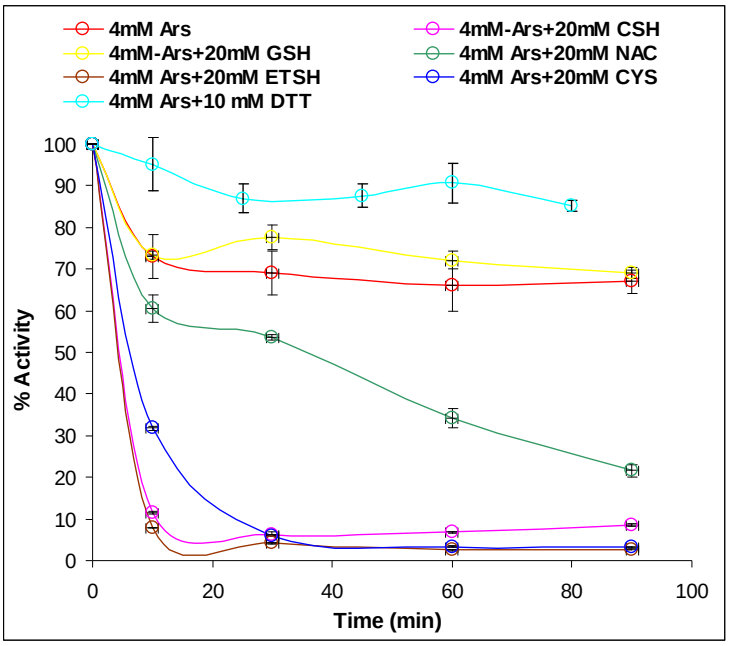


Fig.C1 Time course of RubisCO inactivation by 4 mM sodium arsenite alone and in the presence of several thiols at 20 mM concentration of sulfhydryl groups. RubisCO (0.3 mg/ml) was incubated with the indicated reagents at 30 ºC in a nitrogen atmosphere (inside a N_2 purged oven) and assayed at indicated times. Activity is represented as percentage of the initial value [2.0 µmol CO_2 fixed/(min·mg RubisCO)]. Error bars indicate ± SEM from triplicates.

The synergic effect of arsenite and certain monothiols on RubisCO was not a particular feature of the *C. reinhardtii* enzyme, as witnessed by the inhibition of RubisCOs from other species, such as rice and spinach, under the same conditions (fig. C2). The sequences of the rice, spinach and *C. reinhardtii* enzymes share only a core of 10 Cys residues which are conserved in all form I green-like RubisCOs (see Chapter B). These are Cys 84, 172, 192, 247, 284, 427, 449 and 459 from the large subunit and Cys 41 and 83 from the small subunit (*C. reinhardtii* numbering).

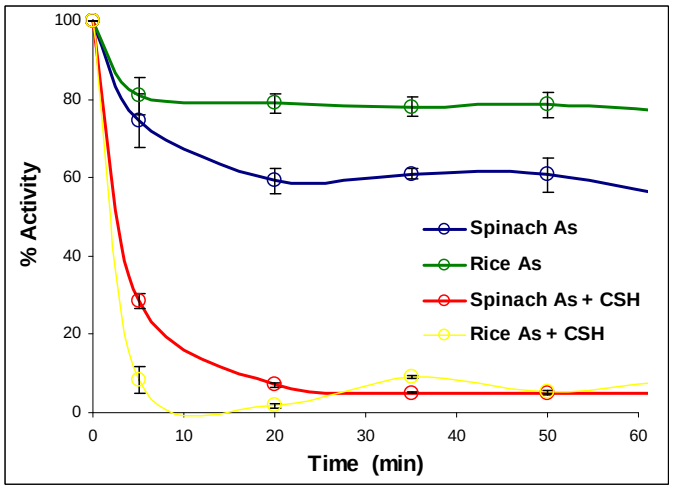


Fig.C2.Time course of inactivation of spinach and rice RubisCO by arsenite. RubisCO (0.2 mg/ml in activation buffer) was incubated either with 2 mM sodium arsenite or 2 mM sodium arsenite + 40 mM CSH at 30 ºC. At given times, samples (0.02 ml) from triplicates were taken and assayed for carboxylase activity during 1 minute. Values are expressed as a percentage of the initial activity. Error bars indicate ± SEM.

Inactivation appears to result from a specific interaction with the Cys172-Cys192 pair of vicinal residues because mutant RubisCOs with any (or both) of these Cys replaced by Ser resisted the combined effect of arsenite and a monothiol (fig. C3).

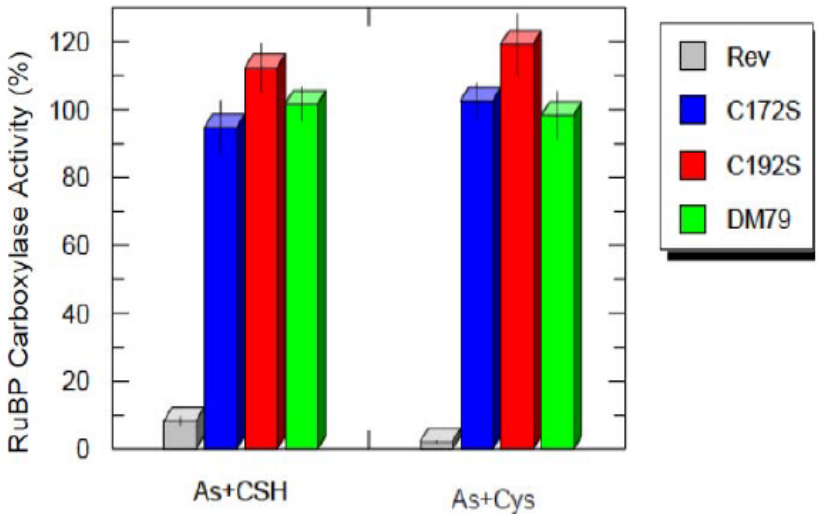


Fig.C3. Effect of arsenite on wild type and cysteine-mutant RubisCOs. Purified RubisCOs from a wild type revertant strain (Rev) and from mutants C172S, C192S and doble mutant C172S/C192S (DM79), were incubated with 2 mM sodium arsenite with either 40 mM CSH (As + CSH) or 2 mM Cys (As + Cys) in activation buffer at 30 ºC under a nitrogen atmosphere. The activity remaining after 2h is represented as a percentage of the activity of an untreated sample. Error bars represent ± SD from triplicates. [Figure taken from García-Murria (2006)].

Moreover, Cys172 and Cys192 were the only residues (among cysteines that are conserved in eukaryotic green-like RubisCOs) that prevented arsenite inactivation upon replacement by serine (fig. C4).

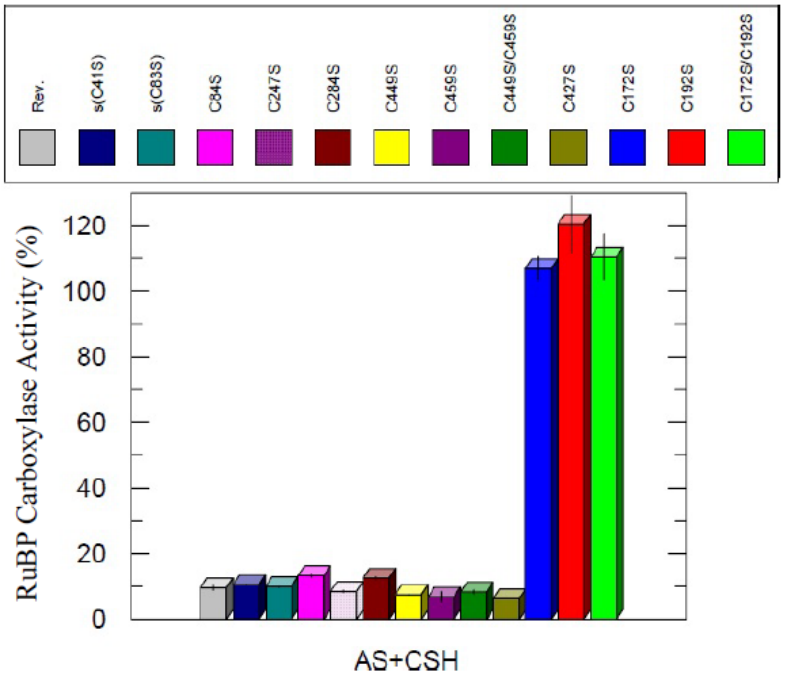


Fig.C4. Effect of arsenite and CSH on different cysteine-mutant RubisCOs. Total cell extracts from *C. reinhardtii* mutants [in which one (or two) of the conserved RubisCO cysteines have been replaced by serine through site-directed mutagenesis] were assayed for RuBP carboxylase activity after 1 hour of incubation with 2 mM sodium arsenite and 40 mM CSH in activation buffer at 30 ºC. The remaining activity is represented as a percentage of that of an untreated sample incubated in parallel. A wild type revertant (Rev) of the mutant strain hosting the mutations was used as a control. Error bars represent ± SD from 6 replicas. [Figure taken from García-Murria (2006)]

Because arsenite is usually considered a reagent against vicinal dithiols, these result strongly support the notion that RubisCO inactivation by the combined effect of arsenite and a monothiol is exclusively due to reaction with the highly conserved Cys172-Cys192 pair, thereby discarding other residues which are known to be conformationally vicinal such as Cys247-Cys247 (from adjacent large subunits) and Cys449-Cys459.

The mechanism of the monothiol enhancement of RubisCO inhibition by arsenite

Does the monothiol reduce an internal disulfide bond of RubisCO?

The potentiation of the inactivation by arsenite through the addition of a free thiol has been observed in a number of different enzymes. In most instances this has been justified by the need of reducing an internal disulfide of the enzyme to generate a vicinal dithiol group that would afterwards react with arsenite. However, in other cases the explanation is not so straightforward and some previous complex between arsenite and the free thiol has been postulated to overcome an undefined kinetic barrier (Fluharty and Sanadi, 1961; Bagui *et al*., 1996). In order to distinguish between these two alternatives (in the first CSH would act independently of arsenite and before it, while in the second both reagents would act simultaneously) RubisCO was treated with arsenite before and after being reduced by incubating it with 20 mM CSH for 2 hours and removing the excess thiol (through a desalting column). Results in figure C5 show that the previous reduction of the RubisCO had no effect on arsenite inactivation once the CSH had been removed. This suggests that CSH (or another monothiol) must be present together with arsenite to potentiate its inhibitory effect, discarding the possibility that the thiols might solely reduce an internal disulfide bond of RubisCO thereby releasing vicinal cysteine dithiols that could be subsequently targeted by arsenite alone.

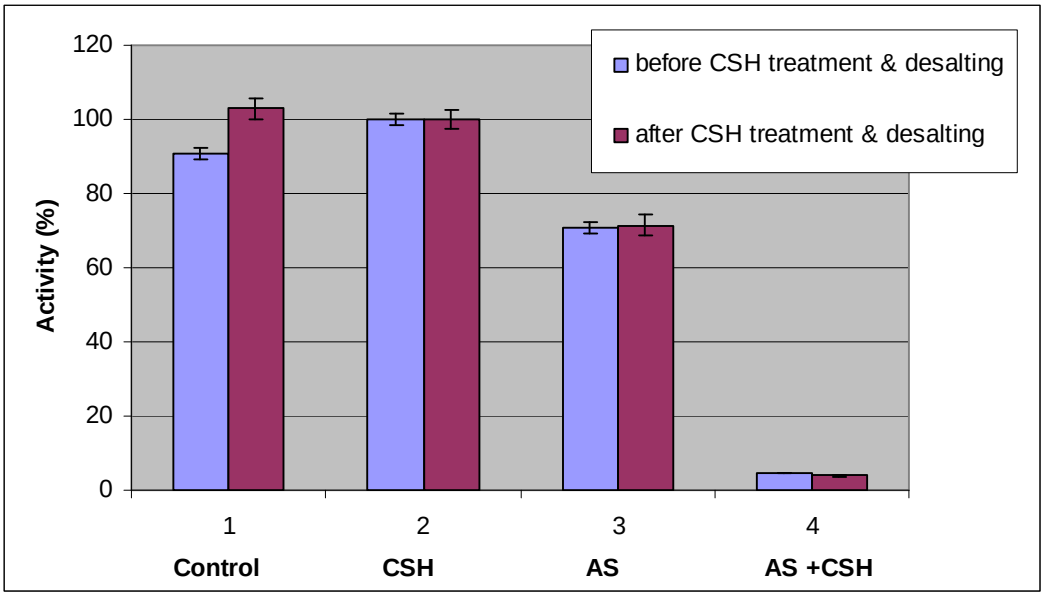


Fig.C5. Carboxylase activity of arsenite and/or CSH-treated RubisCO before (blue) or after (magenta) reduction of the enzyme with 20 mM CSH for 1 hour and desalting in a NAP-10 column. Purified RubisCO was incubated with activation buffer (Control) 20 mM CSH (CSH), 20 mM sodium arsenite (AS), or 20 mM sodium arsenite + 20 mM CSH (AS + CSH) in activation buffer for 1 h at 30 ºC under nitrogen. Afterwards, an aliquot (20 µl) of each mixture was assayed for activity. Next, the sample treated with CSH was passed through NAP-10 equilibrated with activation buffer. Then, the eluted RubisCO (desalted from CSH) was again incubated with activation buffer (Control), 20 mM CSH (CSH), 20 mM arsenite (AS) or 20 mM arsenite + 20 mM CSH (AS + CSH) in activation buffer at 30 ºC. After 10 minutes, each mixture was assayed again for carboxylase activity. Activity is given as a percentage of that of the CSH-treated sample. Error bars indicate ± SEM from triplicates.

Dependence of the degree of inhibition on arsenite and thiol concentration

Inactivation by arsenite alone took place over wide range of concentrations (between 0.6 and 600 mM) following a biphasic transition until total activity loss (fig.C6). Inhibition at concentrations below 100 mM were probably caused by specific binding of arsenite to RubisCO, apparently leading to a partially inactive enzyme. On the other hand, arsenite produced further inactivation above 100 mM, as experienced also with other similar salts (such as nitrite and acetate) at the same concentration (fig.C6). This second step appears to result from a nonspecific inhibitory effect of high ionic concentration on RubisCO as has been already reported to occur with the enzyme of a higher plant species (Peñarrubia and Moreno, 1988).

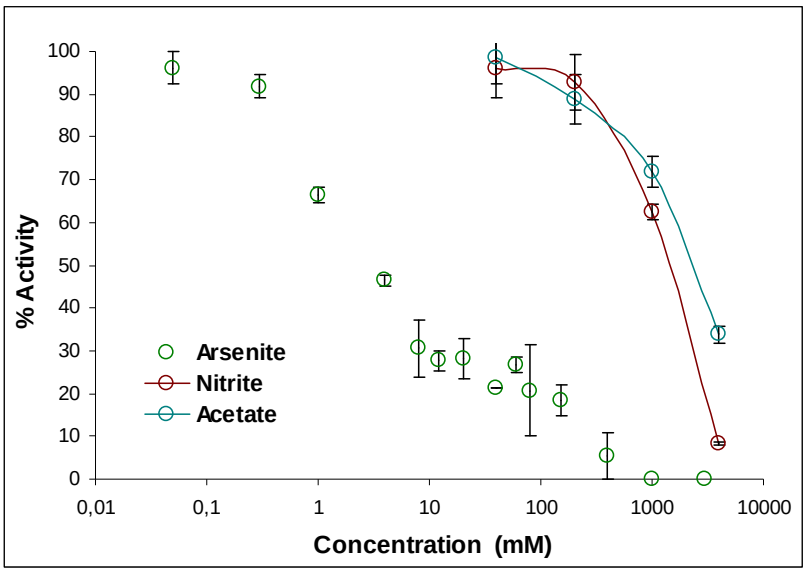


Fig.C6. **Dependence of RubisCO inactivation on the concentration of sodium arsenite and other sodium salts (nitrite and acetate)**: Purified RubisCO (0.16 mg/ml) was incubated with the salts for 2h at 30 ºC under a nitrogen atmosphere. Immediately afterwards triplicate 20 µl aliquots vere assayed for RuBP carboxylase activity. Activity is represented as a percentage of that of a non-treated sample. Error bars represent ± SEM.

RubisCO inactivation by arsenite at concentrations below 100 mM may occur as a result of a single equilibrium step of arsenite reaction with the Cys172-Cys192 dithiol leading to a partially inactive RubisCO-Arsenite complex:

$$\text{RubisCO} + \text{Arsenite} \underset{}{\overset{K}{<=====>}} \text{RubisCO-Arsenite complex}$$

If so, the following relation might be expected between the fraction of active enzyme (**f**) and the arsenite concentration (see Appendix):

$$f = (1 + a \cdot K \cdot [\text{Arsenite}]) / (1 + K \cdot [\text{Arsenite}]) \qquad \text{Eq.1}$$

where **a** is the fraction of carboxylase activity retained by the RubisCO when complexed with arsenite, and **K** is the association equilibrium constant. Eq. A1 can be written also as:

$$(1-f) / [\text{Arsenite}] = K \cdot f - a \cdot K \qquad \text{Eq.2}$$

Therefore, a plot of **(1-f)/[Arsenite]** against **f** should yield a straight line. Indeed, experimental points below 100 mM from the inactivation curve by arsenite in fig.C6 fit eq. 2 reasonably well (fig. C7).

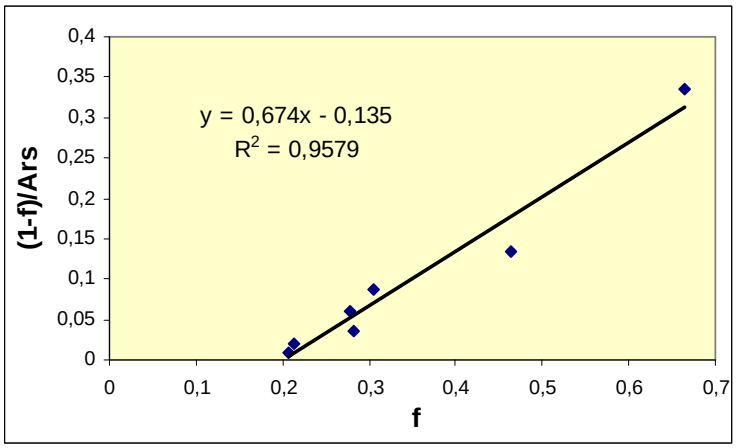


Fig.C7. Plot of (1- f)/[Arsenite] against f for points below 100 mM arsenite: **f** is the fraction of RubisCO activity remaining at each arsenite concentration. Data were taken from the inactivation curve by arsenite alone (fig. C6). The experimental points were adjusted to a straight line with values of 0.674 and -0.135 for slope and intercept, respectively. Correlation coefficient (R^2) is also given.

The slope of the straight line (fig.C7) gives an estimate of the equilibrium constant (**K** ≈ 0.67 mM^{-1}), while the fraction of RubisCO activity remaining when bound to arsenite can be determined from the ratio between ordinate intercept and slope to be **a** ≈ 0.20. Therefore, it might be concluded that the specific binding of arsenite to RubisCO that takes place at concentrations below 100 mM eliminates 80% of the enzymatic activity.

Inactivation of RubisCO by arsenite in the presence of 40 mM CSH occurs at a range of arsenite concentration (around 0.06 mM) which is about three order of magnitude lower than in the absence of the thiol (fig. C8). Moreover, in the presence of CSH, RubisCO appears to be completely inactivated by arsenite above a 10 mM concentration (fig. C8).

The effect of CSH concentration on RubisCO inactivation by 40 mM arsenite has also been studied (fig. C8). The transition from active to inactive RubisCO takes place around a CSH concentration near 0.6 mM, which is one order of magnitude higher than the midpoint arsenite concentration for the symmetric titration (i.e. varying arsenite at constant 40 mM CSH concentration) (fig. C8).

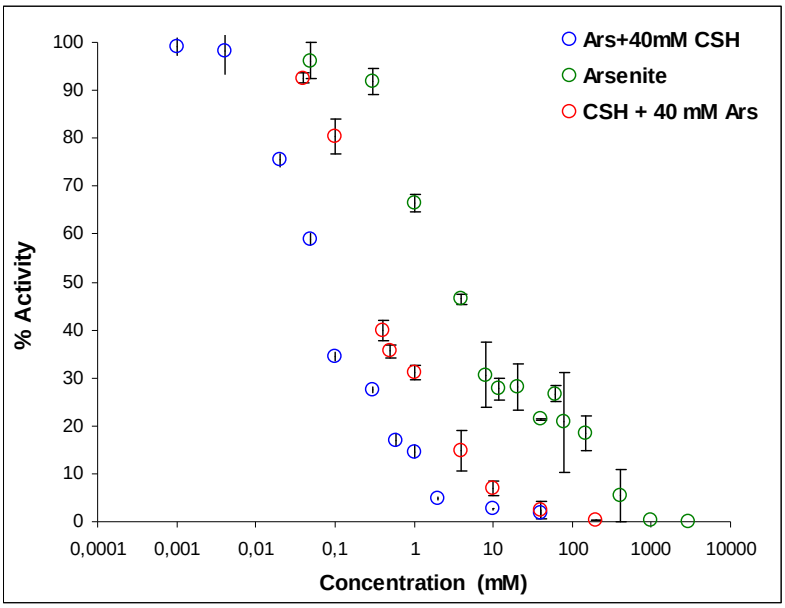


Fig.C8. Dependence of RubisCO inactivation on the concentration of arsenite (at a constant 40 mM concentration of CSH) or of CSH (at a constant 40 mM concentration of arsenite). For comparison, inactivation of RubisCO at different concentrations of arsenite alone (green points taken from fig.C6) is also represented. Purified RubisCO (0.16 mg/ml) was incubated with the reagents at 30 ºC for 2h under a nitrogen atmosphere. Afterwards triplicate 20 µl aliquots were assayed for RuBP carboxylase activity Activity is represented as a percentage of that of a sample incubated with 40 mM arsenite (red), or 40 mM CSH (blue), or without treatment (green). Error bars represent ± SEM.

A model for RubisCO-arsenite-monothiol interaction

Because CSH must be integrated in the final complex and cannot simply act as a catalyst facilitating the arsenite-RubisCO adduct (otherwise changes in CSH concentration would not displace equilibrium as seen in fig. C8), all the above results suggest that arsenite, CSH and the RubisCO Cys172-Cys192 dithiol assemble in a stable ternary complex. The presence of CSH could shift the equilibrium by transforming the RubisCO-arsenite complex in a more stable RubisCO-arsenite-CSH ternary adduct, thereby allowing arsenite to be associated to RubisCO at much lower concentrations.

A plausible model scheme would be the following:

$$\text{RubisCO} + \text{Arsenite} \xleftrightarrow{K_1} \text{partially active RubisCO-Arsenite complex}$$

$$\text{RubisCO-Arsenite} + \text{CSH} \xleftrightarrow{K_2} \text{inactive RubisCO-Arsenite-CSH complex}$$

On the other hand, it is known that arsenite and thiols can loosely combine as dithioarsenites (Torchinsky, 1981):

$$2\ \text{CSH} + \text{Arsenite} \quad \overset{K_0}{<=====>} \quad (CS)_2\text{-dithioarsenite} + H_2O$$

A model that takes into account the above three equilibria (see Appendix) predicts changes in RubisCO activity (when scanning CSH or arsenite concentrations) which fit the experimental points (from fig. C8) in an apparently unbiased manner (fig. C9) and with a global determination coefficient of 0.985 (i.e. the model explains 98.5% of the observed variability). Moreover, adjusting the model to the experimental points delivers values for the relevant constants (a = 0.20, K_1 = 0.63 mM^{-1}, K_2 = 3.45 mM^{-1}, K_0 = 0.0035 mM^{-1}). Values for a and K_1 are close to those independently determined from the inactivation curve for arsenite alone (fig. C7). Comparison of the values obtained for K_1 (= 0.63 mM^{-1}) and K_2 (= 3.45 mM^{-1}) supports the view that the binding of CSH to the RubisCO-arsenite complex results in a remarkable stabilization of the interaction.

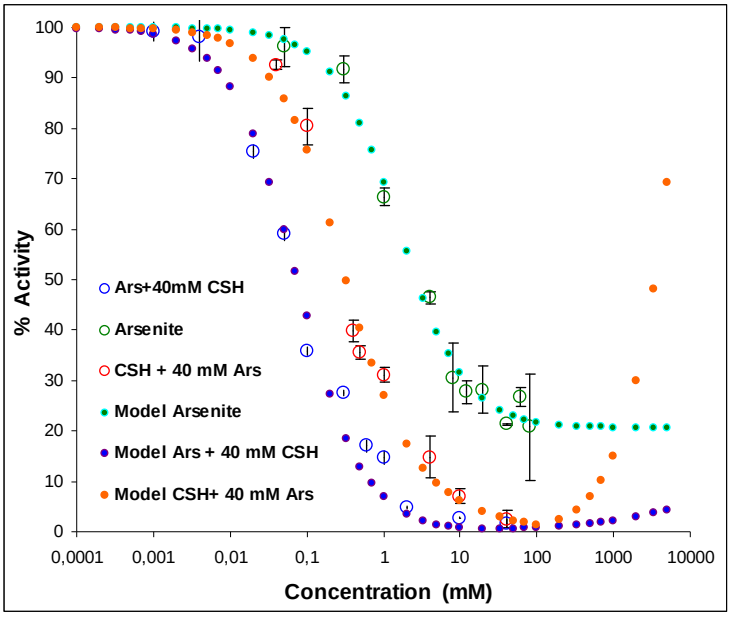


Fig.C9. Fitting of the model of RubisCO-arsenite-CSH interaction to the experimental data. The model assumes a stable ternary complex in competition with CSH-dithioarsenite (see a detailed description in the Appendix). Model predictions (closed circles) were adjusted to experimental data (open circles) taken from figure C8 after eliminating points above 100 mM arsenite or CSH concentration to avoid unspecific inhibitory effects of ionic strength not considered in the model. Error bars on experimental points represent ± SEM

In order to assess the general validity of the model, the inhibition of RubisCO by arsenite combined with another thiol was also addressed. When EtSH was used as the potentiating thiol instead of CSH, synergic inhibition took place at even lower

concentrations of arsenite and thiol. Representative activity titration curves were obtained scanning arsenite concentrations at 5 mM EtSH or EtSH concentrations at 1 mM arsenite (fig. C10). A preliminary fit showed that experimental points did not unbiasedly follow the model proposed above for CSH. Rather it was necessary to assume a trivalent arsenic complex with EtSH:

$$3\ \text{EtSH} + \text{Arsenite} \quad \overset{K_0'}{<=====>} \quad (\text{EtS})_3\text{-arsenic} + H_2O + OH^-$$

in order to fit the data to the model (see Appendix) (fig. C10). This suggests that EtSH can combine with arsenite with a 3:1 stoichiometry, as has been also shown to happen with other monothiols such as glutathione (Han *et al.*, 2007). Otherwise, the model postulated again binary RubisCO-arsenite and ternary RubisCO-arsenite-EtSH complexes with equilibrium constants K_1 and K_2', as in the case of CSH (see Appendix). Fitting of the data to the final model attained a global determination coefficient of 0.98 and produced estimates for the relevant equilibrium constants (K_1 = 0.68 mM^{-1} ; K_2' = 176 mM^{-1}; K_0' = 0.15 mM^{-1}). The elevated value of K_2', compared to the K_2 (3.45 mM^{-1}) obtained with CSH, denotes that the stability of the RubisCO-arsenite-thiol complex is much higher with EtSH than with CSH.

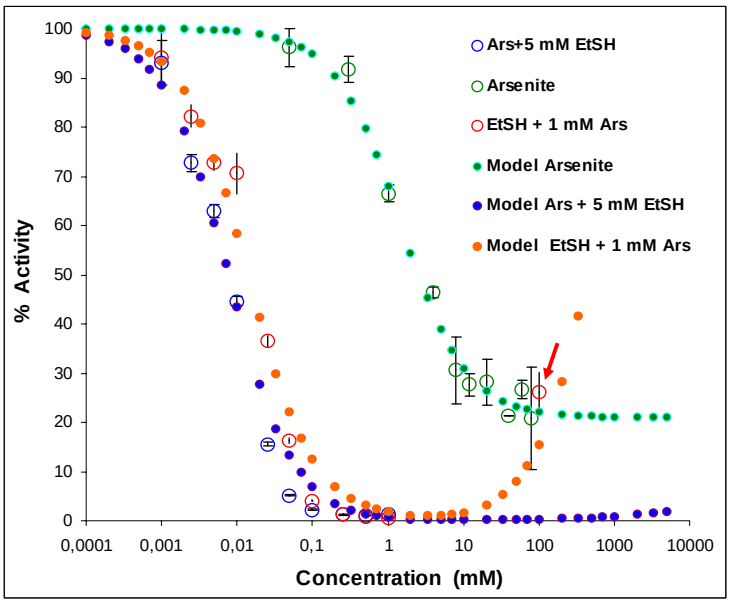


Fig.C10. Fitting of the model of RubisCO-arsenite-EtSH interaction to the experimental data. The model assumes a stable ternary complex in competition with EtSH-trithioarsenic compound (see a detailed description in the Appendix). Model predictions (closed circles) were adjusted to experimental data (open circles) of arsenite inhibition (green points taken from figure C8, but eliminating those above 100 mM arsenite concentration to avoid unspecific inhibitory effects of ionic strength not considered in the model) or obtained by determining RubisCO activity in the presence of 5 mM EtSH and given concentrations of arsenite (blue points) or, conversely, in the presence of 1 mM EtSH and indicated concentrations of EtSH (red points). An experimental point showing activity recovery at high EtSH concentration is indicated by a red arrow. Error bars on experimental points represent ± SEM.

It is noteworthy that the models predict a recovery of RubisCO activity at high concentrations of thiol when the arsenite concentration is kept fixed (figs. C9 and C10). This is because, under these conditions, almost all arsenite is sequestered as a di- or tri-thioarsenite complex with the excess thiol, leaving only a reduced amount of arsenite to combine with RubisCO. This prediction could be tested in the case of EtSH (since the concentrations were unfeasible high for CSH) and the result (100 mM point in fig. C10, maked by an arrow) fully confirmed the hypothesis. This fact supports the assumption of the model that RubisCO and the free monothiol compete for arsenite binding.

Reversibility of the arsenite inhibition of RubisCO

Arsenite inactivation of RubisCO proved fully reversible. Indeed, the activity of RubisCO was spontaneously recovered upon elimination of the inactivating reagents by desalting in a Sephadex G-25 column (fig. C11).

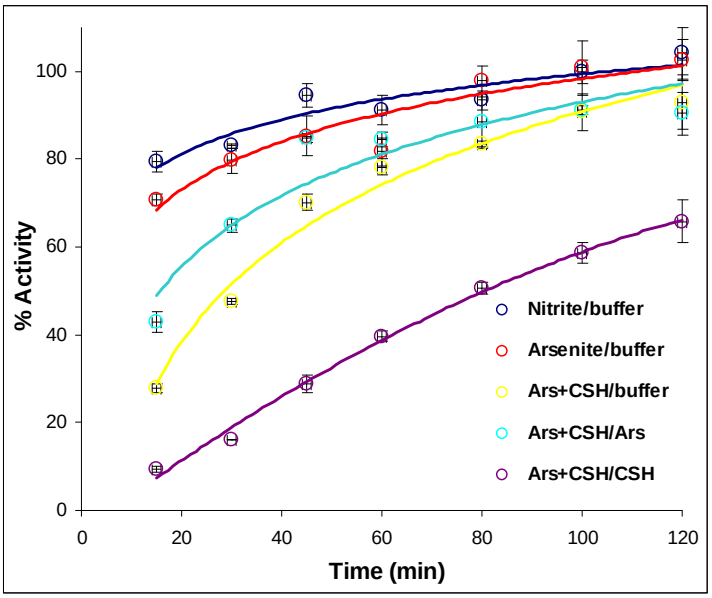


Fig.C11. **RubisCO activity recovery after inhibition with 4 M sodium nitrite (dark blue), 4M sodium arsenite (red) or 40 mM sodium arsenite + 40 mM CSH (all other samples) and desalting in a Sephadex G-25 column.** Purified RubisCO (0.3 mg/ml) was incubated with the inactivating reagents at 30 ºC for 2h under a nitrogen atmosphere. Afterwards, it was passed through a desalting column, equilibrated with activation buffer (dark blue, red and yellow points), 40 mM arsenite in activation buffer (light blue points) or 40 mM CSH in activation buffer (magenta ponts). At given times (counting from the moment that the sample finished to enter the desalting column) triplicate 20 µl aliquots were assayed for RuBP carboxylase activity Activity is represented as a percentage of the maximum value obtained after total recovery. Trend lines were drawn by fitting the points to logarithmic functions. Error bars represent ± SEM.

Reactivation after treatment with sodium arsenite alone was relatively fast (average time of 10 minutes), and proceeded at a similar rate than the recovery from salt inhibition with sodium nitrite (fig. C11). Recovery after inhibition with arsenite plus

CSH was slower. Eliminating both arsenite and CSH, or only CSH, had almost the same effect on the kinetics of RubisCO reactivation. In these cases, the enzyme regained activity in an average time of about 35 minutes (fig. C11). This may represent the mean time interval needed for CSH release from the complex. In contrast, if arsenite was withdrawn but CSH remained in the medium, reactivation was much slower (average time of 105 minutes) (fig. C11). Thus, the presence of CSH substantially delayed arsenite exchange from the complex with RubisCO increasing its average residence time from 10 to 105 minutes. These results support the view that CSH binding stabilizes the RubisCO-arsenite complex both thermodynamically and kinetically.

In the case of EtSH (fig. C12), reactivation was much slower (average recovery time of about 3 hours) than with CSH. Thus, here again, a higher thermodynamic stability (higher K_2') was accompanied by a slower kinetic exchange. Besides, as in the case of CSH, the recovery of activity was faster when the thiol was present than when it was absent from the medium (fig. C12).

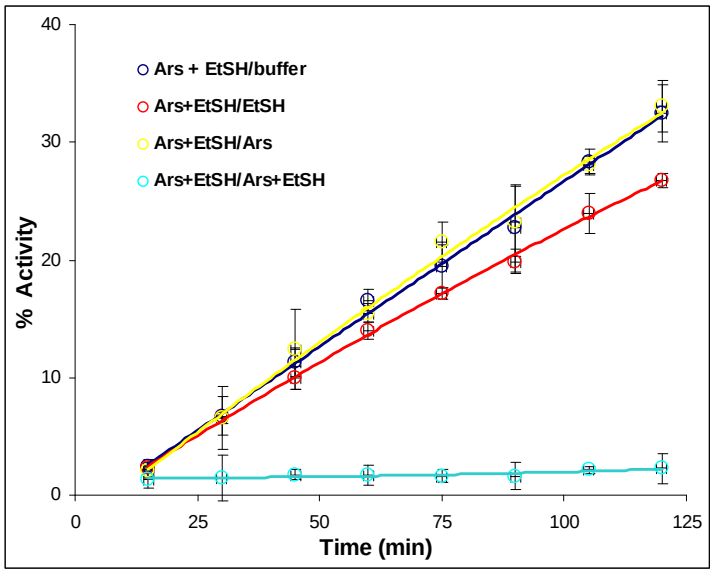


Fig.C12. RubisCO activity recovery after inhibition with 1 mM sodium arsenite + 5 mM EtSH and desalting in a Sephadex G-25 column. Purified RubisCO (0.3 mg/ml) was incubated with the inactivating reagents at 30 ºC for 2h under a nitrogen atmosphere. Afterwards, it was passed through a desalting column, equilibrated with activation buffer (dark blue points), 1 mM arsenite (yellow points), 5 mM EtSH (red points) or 1 mM arsenite + 5 mM EtSH (light blue points) in activation buffer. At given times (counting from the moment that the sample finished to enter the desalting column) triplicate 20 µl aliquots were assayed for RuBP carboxylase activity Activity is represented as a percentage of the maximum value obtained after total recovery. Trend lines were drawn by fitting the points to logarithmic functions. Error bars represent ± SEM.

Arsenite inhibition of CO_2 fixation in vivo

Fixation of CO_2 by illuminated cell cultures of wild type *C. reinhardtii* in minimal medium was totally inhibited by arsenite at a relatively high (40 mM) concentration

(fig. C13). However, at 1 mM concentration, arsenite inhibition was not longer noticeable (fig. C13a). On the other hand, 5 mM EtSH produced only a very slight decrease of the fixation rate of the cells (fig. C13a). However, when both 1 mM arsenite and 5 mM EtSH were present in the medium, CO_2 fixation by the cells was strongly inhibited (fig. C13).

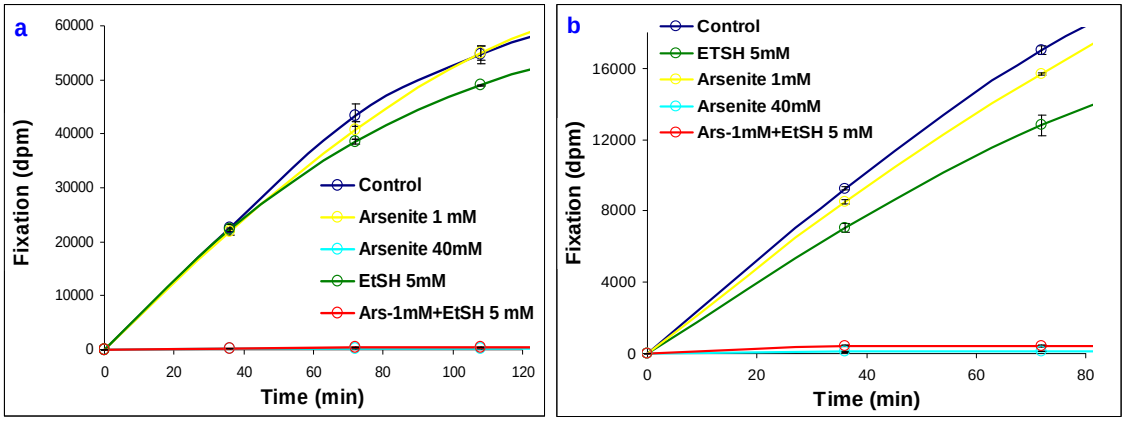


Fig. C13. Effect of arsenite and EtSH on the $^{14}CO_2$ fixation rate by an illuminated culture of the wild type (a) or a C172S/C192S RubisCO double mutant strain (b) of *C.reinhardtii* growing in minimal medium. Cell cultures in logarithmic growth phase were diluted with minimal medium containing EtSH, arsenite or both (at the indicated final concentrations) and 2 mM ^{14}C-bicarbonate (about 650 dpm/nmol) to a cell density of about $1.5 \cdot 10^6$ cells/ml. Cultures were placed at 30 ºC under constant light and aliquots of it were harvested at the given times to determine the carbon fixation (represented as acid-stable radioactivity in dpm). A control treatment (without arsenite or EtSH) was run in parallel. The initial slope is representative of the fixation rate of the cells. The progressive decline of the slope with time results from specific radioactivity loss due to unavoidable exchange of CO_2 with the atmosphere. Error bars represent ± SEM from triplicate independent cultures.

On the other hand, when the experiment was repeated with C172S/C192S mutant cells, containing RubisCO which is not inactivated by arsenite-thiol mixtures *in vitro* (see fig. C3), the *in vivo* CO_2 fixation was nevertheless blocked by the arsenite-EtSH combination as in the case of the wild type cells (fig. C13b). This suggests that *in vivo* CO_2 fixation is controlled by another factor which can sense the synergic effect of arsenite and EtSH as well as RubisCO.

A scanning of the *in vivo* effect of different concentrations of arsenite at a constant (5 mM) concentration of EtSH revealed that all tested strains (the wild type and the RubisCO mutants at the Cys 172 and/or Cys 192 residues) showed a similar sensitivity, displaying half-inhibition of CO_2 fixation around 0.06 mM of arsenite (fig. C14). This was somewhat lower than the concentration of arsenite needed for half-inactivation of RubisCO *in vitro* under the same conditions (i.e. in the presence of 5 mM EtSH) (reproduced in the same figure C14 for comparison). Therefore, the apparent binding affinity of arsenite-EtSH for the putative sensor that arrests carbon fixation *in vivo* seems to be slightly higher than the one for RubisCO.

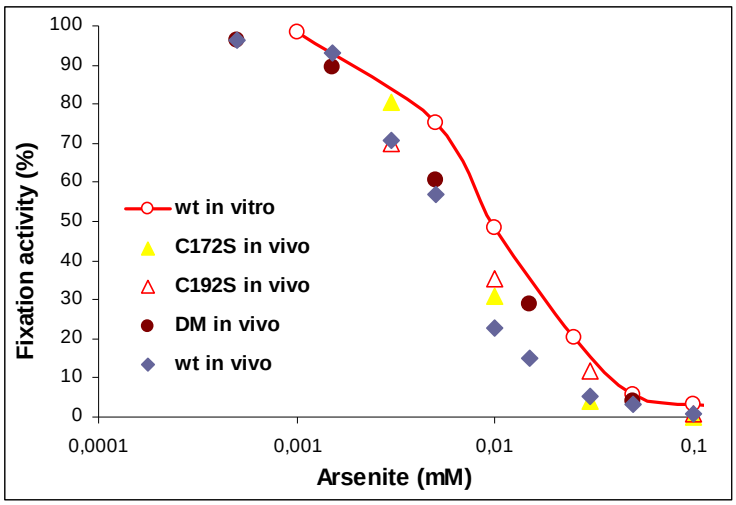


Fig.C14. Effect of arsenite concentration on CO_2-fixation activity of various *C. reinhardtii* strains in the presence of 5 mM EtSH in the medium. *In vivo* carbon fixation activity was determined for illuminated cultures of the wild type, as well as of the C172S, C192S and C172S/C192S (DM) RubisCO mutant strains, grown at 30 ºC in minimal medium by labeling with ^{14}C-bicarbonate (about 650 dpm/nmol) and determining the initial slope of acid-stable radioactivity accumulation. Data are represented as a percentage of the initial slope displayed by an untreated (control) culture run in parallel. *In vitro* fractional activity points (obtained with purified wild type RubisCO) are taken from fig. C10 and are plotted here (joined by a red line) for comparison.

Besides, after removing arsenite and EtSH (through centrifugation of the cells and replacement of the supernatant by fresh medium) the cells recovered full CO_2 fixation activity (Fig. C15) demonstrating that the inhibition was reversible *in vivo*, as has been shown to be *in vitro* (Fig. C12).

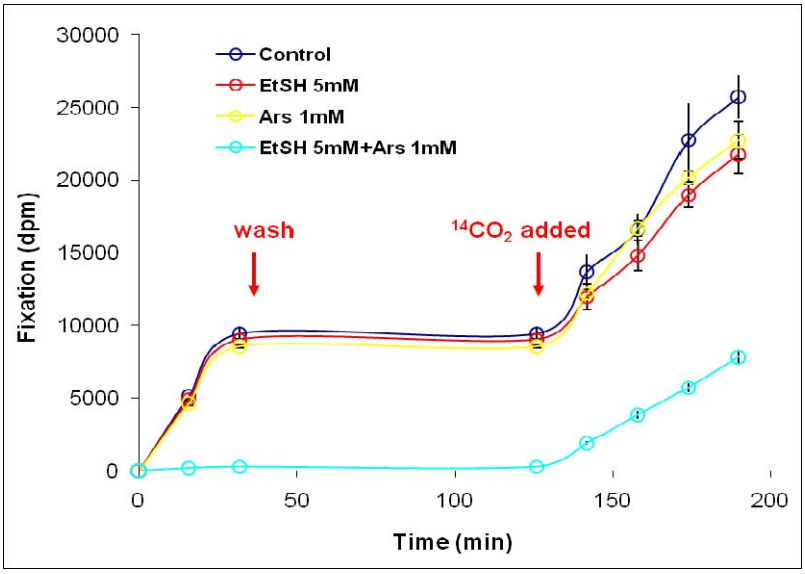
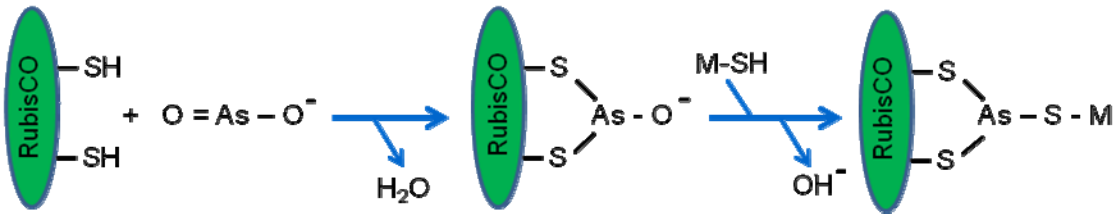


Fig.C15. Time course of radioactivity fixed by wild type *C. reinhardtii* cells exposed to 5 mM EtSH, 1 mM arsenite or both, before and after washing the cells and resuming growth in regular minimal medium. An untreated (control) culture was run in parallel. All cultures were kept illuminated at 30 ºC in minimal medium. At time 0 (start of the treatments),^{14}C-bicarbonate was given to the cells and the fixed radioactivity was monitored up to 40 min. Immediately afterwards, the cells were washed by centrifuging the cultures and resuspending the cells twice in fresh minimal medium (without EtSH, arsenite or radioactivity). At 125 minutes, ^{14}C-bicarbonate was given again to the cells and fixation was monitored for a further 65 minutes. Error bars represent ± SEM from triplicate independent cultures.

DISCUSSION

The potentiation of arsenite toxicity by dithiols is well documented (Jan *et al.*, 2006) although not always well understood. For example, the uncoupling effect of arsenite on oxidative phosphorylation in rat liver mitochondria respiring citrate or malate has long been know to be potentiated by dithiols, while monothiols are ineffective (Fluharty and Sanadi, 1961). In that case, it was assumed that the final product was a cyclic dithioarsenite formed with two vicinal cysteines at a functional site of the target protein, but a previous complex with the potentiating dithiol was needed in order to have arsenite transported across an undefined barrier (Fluharty and Sanady, 1961). Adenosine kinase has also been reported to be inhibited by arsenite and 2,3-dimercaptopropanol acting together, but not separately, on a pair of conformationally vicinal cysteines (Bagui *et al.*, 1996). Here again it was suggested that a previous arsenite-dithiol complex could neutralize the charge of arsenite allowing it to penetrate a hydrophobic barrier. Potentiation of arsenite by monothiols is much less frequent and should lead to a different molecular organization of the protein-arsenite complex. In the case of RubisCO, it might be envisaged that the monothiol could reduce a hypothetical internal disulfide bond between a pair of vicinal cysteines that would subsequently react with arsenite to produce an inactive enzyme. However, the evidence presented here (fig. C5) does not support this assumption. Moreover, because the thiol concentration displaces the equilibrium of the inactive RubisCO (fig. C8), it appears that the potentiating monothiol does not play merely a catalytic role but rather it is incorporated in the final inhibitory complex. Results are consistent with a model in which monothiols combine to a previous arsenite-RubisCO adduct (most likely a cyclic dithioarsenite with the 172 and 192 cysteine residues of the large subunit) thereby stabilizing the arsenite-RubisCO interaction. However, our equilibrium model cannot resolve binding priority. Therefore, it cannot distinguish between a sequential binding of arsenite and CSH to RubisCO, and a previous arsenite-CSH complex binding the enzyme, both possibilities being plausible. The final ternary complex is suggested to be a trithioarsenic adduct in which the trivalent arsenic atom is bonded to the sulfur atoms from the three thiolates (the two RubisCO cysteines and the potentiating monothiol) (scheme 2).



Scheme 2. **Proposed sequence of reactions involved in RubisCO inactivation by arsenite and a monothiol**. In the first step, the sulfhydryl groups from the vicinal Cys172 and Cys192 of RubisCO large subunit react with arsenite to form a cyclic dithioarsente producing a partially inactive enzyme. In a second step, a monothiol (M-SH) combines with the dithioarsenite as a trithioarsenic adduct that renders RubisCO fully inactive. A previous combination of arsenite with the monothiol before reacting with RubisCO is also plausible.

These triothioarsenic compounds are known to be spontaneously formed by arsenite with GSH (Han et al., 2007). For some monothiols (or combinations of them), these complexes might be thermodynamically favoured over the labile mono or dithioarsenite alternatives. In contrast, vicinal dithiols (such as in DTT) usually bind to arsenite with high affinity as stable dithioarsenites (Jocelyn, 1972; Torchinsky, 1981) and, therefore, would not be able to engage in a ternary complex with RubisCO and arsenite. Indeed, in the presence of competing DTT at a relatively high concentration, the amount of free arsenite available to react with RubisCO would be severely diminished and this could be the reason why DTT protects RubisCO from arsenite inactivation (fig. C1).

While the binary arsenite–RubisCO adduct is already partially inactive, the incorporation of the potentiating monothiol to the ternary complex may further distort the enzyme structure near the catalytic site resulting in total inactivation of the enzyme. Cys 172 resides in the β1 strand of the α/β barrel that scaffolds the active site of RubisCO. Subtle mutations of this residue are known to affect the kinetic parameters of the enzyme remarkably (García-Murria *et al*. 2008), while substitutions in the neighboring 171 and 173 positions render a fully inactive enzyme (Spreitzer, 1993). Thus, it appears that arsenite binds at a site which is critical for maintaining the functional structure of the catalytic center of RubisCO.

The stability of arsenite binding to dithiols is known to be strongly dependent on the geometry and relative orientation of the two sulfhydryls (Jocelyn, 1972; Torchinsky, 1981). It is likely that the same happens with the trivalent thio-arsenic adducts. Thus, the sensitive binding of arsenite to RubisCO may be a highly specific feature. Moreover, the nature of the potentiating monothiol is also remarkably relevant. In this study the cases of CSH and EtSH have been compared showing that EtSH binds to arsenite-RubisCO with a much (two orders of magnitude) higher affinity than CSH. Nevertheless, other proteins which are relevant for

photosynthetic carbon assimilation may also have appropriate sites for binding arsenite and a monothiol. In fact, it should be so because *in vivo* inhibition of CO_2 fixation appears to be driven by an undetermined sensor which is sensitive to the synergic effect of arsenite and a monothiol with an even higher affinity than RubisCO for these agents (fig. C14). It might be assumed that this sensor possesses also vicinal dithiols, similar to the Cys172-Cys192 pair of RubisCO. Candidates include regulatory thioredoxins as well as thioredoxin-regulated Calvin cycle enzymes. In fact, the analysis of the levels of chloroplastic metabolites suggest that these enzymes are the targets of the inhibition of carbon fixation when pea chloroplasts are treated with 0.5 mM arsenite (Marques and Anderson, 1986). At any rate, the identification of this sensor would shed light on the potential targets for the toxicity of arsenite in photosynthetic organisms and the mechanisms that regulate CO_2 fixation through redox stress perception.

In summary, we have uncovered a chemical mechanism for inhibition of CO_2 fixation of a new type, involving a ternary binding between arsenite, a free monothiol and a vicinal dithiol from a target protein. This can be used as a tool for specific RubisCO inhibition *in vitro* or photosynthetic studies *in vivo*, whenever CO_2 fixation should be reversibly inhibited under relatively mild chemical conditions (i.e. with no general effects on proteins or not triggering extended chemical stress responses). Moreover, it has been recently proposed that a periodic and reversible inhibition of CO_2 fixation by RubisCO could be an efficient strategy to boost hydrogen production by *C. reinhardtii* under illuminated anaerobic conditions (Marín-Navarro *et al.*, 2010). The sensitivity and specificity of the arsenite-thiol synergism, as well as its reversibility, offer an ideal choice to attempt that goal.

Finally, after the early report of the sensitivity of the mitochondrial activity to the combination of arsenite with dithiols (Fluharty and Sanadi, 1961) and the present communication, it might be noted that two of the most fundamental biochemical processes sustaining life on Earth (namely, oxidative phosphorylation and photosynthetic carbon fixation) are now known to be targets of the toxic effects of arsenite-thiol synergisms.

Acknowledgements

Dr. María Jesús García Murria, who discovered the synergic effect of arsenite and thiols on RubisCO, is gratefully acknowledged for advice and sharing the C192S and C172S/C192S mutants.

APPENDIX

Reaction of RubisCO with Arsenite

Assuming a 1:1 stoichiometry in the bonding of arsenite to RubisCO (to form a cyclic dithioarsenite with residues Cys172 and Cys 192), the reaction reads:

$$\text{RubisCO } (\mathbf{R}) + \text{Arsenite } (\mathbf{A}) \xleftrightarrow{\mathbf{K}} \text{RubisCO-Arsenite complex } (\mathbf{C})$$

where **K** is the equilibrium constant.
If **C** is partially active as catalyst (by a factor **a** of the activity of the untreated enzyme), the fractional activity (**f**) of RubisCO at equilibrium will be:

$$\mathbf{f} = ([\mathbf{R}] + \mathbf{a}\cdot[\mathbf{C}])/([\mathbf{R}] + [\mathbf{C}]) \qquad \text{Eq. A1}$$

and since

$$[\mathbf{C}] = \mathbf{K}\cdot[\mathbf{R}]\cdot[\mathbf{A}] \qquad \text{Eq. A2}$$

substituting Eq. A2 in Eq. A1 yields

$$\mathbf{f} = (1 + \mathbf{a}\cdot K\cdot[\mathbf{A}])/(1 + K\cdot[\mathbf{A}]) \qquad \text{Eq. A3}$$

which is Eq.1 in the main body of the chapter. Here **A** is the free-arsenite concentration but, in practice, this equals the total initial concentration of arsenite because the total RubisCO concentration is much smaller (in the μmolar range) and we are assuming that only one arsenite molecule will react per RubisCO heterodimer, thereby inactivating one catalytic site

Reaction of RubisCO with arsenite and CSH

In this case, the presumed reaction scheme would be:

$$\text{RubisCO } (\mathbf{R}) + \text{Arsenite } (\mathbf{A}) \xleftrightarrow{\mathbf{K_1}} \text{partially active RubisCO-Arsenite complex } (\mathbf{C})$$

(activity factor = **a**)

$$\text{RubisCO-Arsenite } (\mathbf{C}) + \text{CSH } (\mathbf{S}) \xleftrightarrow{\mathbf{K_2}} \text{inactive RubisCO-Arsenite-CSH complex } (\mathbf{I})$$

where K_1 and K_2 are the equilibrium constants of the two consecutive steps.

Besides, CSH may compete with RubisCO for arsenite combining as a dithioarsenite:

$$2\ \text{CSH}\ (\mathbf{S}) + \text{Arsenite}\ (\mathbf{A}) \xleftrightarrow{K_0} (CS)_2\text{-dithioarsenite}\ (\mathbf{D}) + H_2O$$

While this global reaction could also be split in two sequential steps (i.e. the reaction of the first CSH molecule to form a monothioarsenite, followed by the reaction of the second), there was no substantial increase of the model fitness by considering this decomposition. Therefore, the two reactions were lumped into one, neglecting the amount of intermediary monothioarsenite and reducing the number of adjustable parameters of the model by introducing a single global equilibrium constant K_0.

Under these conditions the fractional activity (**f**) is expected to be:

$$\mathbf{f} = ([\mathbf{R}] + \mathbf{a}\cdot[\mathbf{C}])/([\mathbf{R}] + [\mathbf{C}] + [\mathbf{I}]) \qquad \text{Eq. A4}$$

and since, at equilibrium,

$$[\mathbf{C}] = \mathbf{K_1}\cdot[\mathbf{R}]\cdot[\mathbf{A}] \qquad \text{Eq. A5}$$

$$[\mathbf{I}] = \mathbf{K_2}\cdot[\mathbf{C}]\cdot[\mathbf{S}] = \mathbf{K_2}\cdot\mathbf{K_1}\cdot[\mathbf{R}]\cdot[\mathbf{A}]\cdot[\mathbf{S}] \qquad \text{Eq. A6}$$

the substitution of Eqs. A5 and A6 into Eq. A4 leads to

$$\mathbf{f} = (1 + \mathbf{a}\cdot\mathbf{K_1}\cdot[\mathbf{A}])/(1 + \mathbf{K_1}\cdot[\mathbf{A}] + \mathbf{K_2}\cdot\mathbf{K_1}\cdot[\mathbf{A}]\cdot[\mathbf{S}]) \qquad \text{Eq. A7}$$

On the other hand,

$$[\mathbf{S}] = \mathbf{S_0} - 2\cdot[\mathbf{D}] - [\mathbf{I}] \approx \mathbf{S_0} - 2\cdot[\mathbf{D}] \qquad \text{Eq. A8}$$

$$[\mathbf{A}] = \mathbf{A_0} - [\mathbf{D}] - [\mathbf{C}] - [\mathbf{I}] \approx \mathbf{A_0} - [\mathbf{D}] \qquad \text{Eq. A9}$$

because the total concentration of RubisCO is low (in the micromolar range) and, therefore, [**C**] and [**I**] are negligible compared to the initial (i.e total) concentrations of CSH ($\mathbf{S_0}$) and arsenite ($\mathbf{A_0}$) (both in the millimolar range).
Besides, at equilibrium,

$$[D] = K_0 \cdot [A] \cdot [S]^2 \qquad \text{Eq. A10}$$

Eliminating [D] and [A] from Eqs. A8 to A10, yields

$$[S]^3 + (2 \cdot A_0 - S_0) \cdot [S]^2 + (1/K_0) \cdot [S] = S_0/K_0 \qquad \text{Eq. A11}$$

This cubic equation can be solved (analytically or numerically) for [S]. Once [S] is known, [A] can also be obtained by solving from Eqs. A9 and A10:

$$[A] = A_0 / (1 + K_0 \cdot [S]^2) \qquad \text{Eq. A12}$$

After determining the values of [S] and [A], **f** can be calculated from Eq. A7.
The adjustable constants of the model (K_0, K_1, K_2 and **a**) were determined by fitting the theoretical f to the experimental values (at known A_0 and S_0) minimizing square deviations.

Reaction of RubisCO with arsenite and EtSH

As in the former case with CSH, the RubisCO-Arsenite-EtSH complex is presumed to result from two sequential reactions:

$$\text{RubisCO (R) + Arsenite (A)} \xleftrightarrow{K_1} \text{partially active RubisCO-Arsenite complex (C)}$$

(activity factor = **a**)

$$\text{RubisCO-Arsenite (C) + EtSH (E)} \xleftrightarrow{K_2'} \text{inactive RubisCO-Arsenite-EtSH complex (I)}$$

where K_1 and K_2' are the equilibrium constants of the two consecutive steps.

However, in contrast with the case of CSH, EtSH is assumed to react with arsenite to form a trivalent thioarsenic compound:

$$3\ \text{EtSH (E) + Arsenite (A)} \xleftrightarrow{K_0'} (EtS)_3\text{-arsenic (T)} + H_2O + OH^-$$

Here again the intermediaries are neglected by considering a single equilibrium with a global constant K_0' (including, in this case, not only the water but also the OH^- concentration, which should be nearly constant in a pH-buffered medium).

Under these conditions, Eq. A4 still holds for the fractional activity (**f**) and, considering the equilibrium mass law relations as above, it can be rewritten as:

$$f = (1 + a \cdot K_1 \cdot [A])/(1 + K_1 \cdot [A] + K_2' \cdot K_1 \cdot [A] \cdot [E]) \qquad \text{Eq. A13}$$

Here

$$[E] = E_0 - 3 \cdot [T] - [I] \approx E_0 - 3 \cdot [T] \qquad \text{Eq. A14}$$

$$[A] = A_0 - [T] - [C] - [I] \approx A_0 - [T] \qquad \text{Eq. A15}$$

and

$$[T] = K_0' \cdot [A] \cdot [E]^3 \qquad \text{Eq. A16}$$

Eliminating [**T**] and [**A**] from Eqs. A14 to A16, gives

$$[E]^4 + (3 \cdot A_0 - E_0) \cdot [E]^3 + (1/K_0') \cdot [E] = E_0/K_0' \qquad \text{Eq. A17}$$

This quartic equation can be solved for [**E**]. Thereafter, [**A**] may be obtained from Eqs. A15 and A16:

$$[A] = A_0 /(1 + K_0' \cdot [E]^3) \qquad \text{Eq. A18}$$

After determining the values of [**E**] and [**A**], **f** can be calculated from Eq. A13. Fitting f to the experimental values (at given A_0 and E_0) yields the unknown constants of the model (K_0', K_1, K_2' and **a**).

CONCLUSIONS

CONCLUSIONS

The main conclusions of the present work are:

A. Regarding the role of glutathione in the redox regulation of RubisCO activity.

1. RubisCO can be inactivated by disulfide exchange with several disulfides, and subsequently reactivated by several thiols. However, neither reduced nor oxidized glutathione are able to modify RubisCO activity through direct disulfide exchange because of the existence of kinetic barriers.

2. Ascorbate cannot mediate the redox modulation of RubisCO activity by glutathione *in vitro*. Nevertheless, reduced glutathione can drive the reactivation of oxidized RubisCO by means of a limited amount of small intermediary thiols (such as cysteamine or cysteine). The reverse reaction (i.e., the inactivation of RubisCO by oxidized glutathione using small disulfides as mediators) does not progress if the intermediaries are kept at a lower concentration than glutathione.

3. S-nitrosoglutathione can act as a nitric oxide donor to inactivate RubisCO by transnitrosation of cysteine residues. However, in the case of the enzyme from *Chlamydomonas reinhardti*, S-nitrosoglutathione is not an efficient donor for this reaction, needing an exceedingly high concentration (not plausible *in vivo*) to achieve extensive inactivation of RubisCO.

B. Regarding the identity of the critical cysteine residues of RubisCO.

4. The sensitivity to cystamine displayed by RubisCOs obtained from *C. reinhardtii*, rice and spinach suggests that enzyme inactivation results from disulfide exchange with cysteines which are among the common conserved residues. These are cysteines 84, 172, 192, 247, 284, 427 and 459 from the large subunit, and cysteines 41 and 83 from the small subunit.

5. None of the single cysteine site-directed substitutions of the conserved residues abolishes RubisCO inactivation by disulfide exchange with cystamine. This result suggests that inactivation results from the oxidation of several cysteines with redundant contributions to Rubisco inhibition.

6. The single replacement of cysteine 427 (or of cysteine 192, which has been already described) renders a mutant RubisCO which is more sensitive to oxidative inactivation. This suggests that these two residues play a protective role interfering with the modification of critical residues, or with the structural changes induced by them, thereby delaying inhibition.

7. A review of all available data indicates that cysteines 172 and 459 from the large subunit and 41 from the small subunit are the most likely candidates to drive RubisCO inactivation and proteolytic sensitization upon oxidation of these residues to mixed disulfides.

C. Regarding the mechanism by which thiols increase the inhibitory effect of arsenite on RubisCO activity.

8. Arsenite alone inhibits up to 80% of Rubisco activity at concentrations above the millimolar range. However, when combined with certain monothiols (such as cysteamine, cysteine, 2-mercaptoethanol, N-acetylcysteine, but not reduced glutathione) arsenite achieves full inactivation of Rubisco at submillimolar concentration. In contrast, dithiols such as dithiothreitol do not enhance arsenite inhibition.

9. The monothiol does not reduce a previous disulfide or play a catalytic role but rather integrates a ternary complex with arsenite and RubisCO which inhibits the enzyme. In the final complex the arsenic atom is suggested to bind three sulfur atoms belonging to the monothiol and the vicinal cysteines 172 and 192 from the large subunit of RubisCO.

10. The stability of the ternary complex is highly dependent on the particular monothiol involved, the association equilibrium constant with 2-mercaptoethanol being two orders of magnitude higher than with cysteamine. In any case, the association is fully reversible and the complex can be easily dismantled by desalting to eliminate the free forms of arsenite, monothiol or both.

11. The combination of arsenite and a monothiol can also suppress carbon fixation by photosynthesizing cultures of *C. reinhardtii* in a fully reversible manner. However, the *in vivo* inhibition does not occur through RubisCO inactivation but affects an undetermined sensor that is even more sensitive than RubisCO to the arsenite-thiol synergism.

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RESUMEN de la MEMORIA de TESIS DOCTORAL titulada:

CONTROL OF THE ACTIVITY OF RUBISCO FROM CHLAMYDOMONAS REINHARDTII THROUGH THE REDOX STATE OF ITS CYSTEINE RESIDUES.

presentada por HEMANTH PHANI KUMAR SUDHANI para optar al título de DOCTOR dentro del Programa Oficial de Posgrado en BIOTECNOLOGÍA (D030-01) de la Universitat de València.

Tema: La actividad del enzima fijador de carbono en eucariotas fotosintéticos, la ribulosa 1,5-bisfosfato carboxilas/oxigenasa (Rubisco), puede regularse in vitro por efectores redox. Esta capacidad existe en los enzimas de todas las especies eucarióticas ensayadas y se supone que reside en un número relativamente reducido de residuos de cisteína conservados, cuyo estado redox influye en la conformación del sitio catalítico a través efectos estructurales a larga distancia. Este mecanismo de regulación parece hayarse implicado en el control del catabolismo de la Rubisco durante procesos de senescencia natural e inducida por situaciones ambientales adversas que generan condiciones oxidantes. Sin embargo, se desconocen detalles acerca de los posibles efectores redox que pudieran modular la actividad del enzima in vivo, así como de los residuos de cisteína implicados. Para clarificar el papel que juega cada uno de los residuos conservados de cisteína en este mecanismo de regulación se ha iniciado la obtención y el estudio de mutantes del alga unicelular *Chlamydomonas reinhardtii* en los que se ha sustuido algún residuo conservado de cisteína de la Rubisco por serina. Previamente se han estudiado ya los mutantes en los residuos 172, 192, 449 y 459, habiéndose encontrado que las mutaciones producen alteraciones fenotípicas como el retraso en la degradación del enzima (caso del mutante Cys 172) o su agregación en condiciones oxidativas (caso de los mutantes en las Cys 449 y 459). Estos resultados abren la posibilidad de una posible manipulación biotecnológica de la degradación de la Rubisco durante la senescencia, un proceso de enorme importancia nutricional para las plantas superiores ya que el catabolismo de esta abundante proteína proporciona hasta un 50 % del nitrógeno y azufre movilizado desde las hojas a los nuevos tallos, flores y frutos en fases de crecimiento.

Objetivos: El objetivo de la tesis es continuar la elucidación del mecanismo redox que controla la actividad y estabilidad de la Rubisco, identificando y caracterizando el papel de los diferentes residuos críticos de cisteína implicados en la regulación,

determinando la naturaleza de los efectores redox que pueden controlar la actividad del enzima (revisando especialmente el papel del glutatión, que es el tampón redox más importante del cloroplasto) e investigando el mecanismo de inhibición del enzima pot arsenito.

Metodología: En los experimentos se ha utilizado la Rubisco del alga unicelular *Chlamydomonas reinhardtii*, un organismo modelo para estudios del cloroplasto, y que, en la actualidad, es uno de los pocos que admite la transformación del genoma cloroplástico.

El estudio se ha llevado a cabo mediante varios abordajes distintos:

1) Análisis de mutantes con sustitución de cisteínas de Rubisco en *C. reinhardtii*.

Se ha analizado el fenotipo de mutantes ya obtenidos (con sustituciones simples de las cisteínas 84, 247, 284 y 427 de la subunidad grande y 41 y 83 de la subunidad pequeña) y las propiedades del enzima purificado de ellos con objeto de caracterizar la contribución de cada residuo al mecanismo de regulación. Para ello se han purificado las Rubiscos de cada uno de los mutantes y se han sometido a incubación con tampones redox y tratamientos con agentes modificadores de cisteína, para detectar diferencias en los efectos de estos agentes sobre la actividad en comparación con el enzima silvestre.

2) Estudio de la secuencia temporal de oxidación de cisteínas

Se ha realizado un marcaje químico diferencial de las cisteínas reducidas y oxidadas de la Rubisco a lo largo de un barrido de condiciones redox (logrado mediante equilibrado del enzima con un mezcla de un oxidante y un reductor en distintas proporciones) y se ha analizado el progreso de la oxidación de cada uno de los residuos mediante fragmentación tríptica y análisis de los fragmentos por espectrometría de masas. El objeto de este experimento es establecer una secuencia temporal de oxidación de cisteínas, tal como puede ocurrir in vivo cuando, en condiciones de senescencia, el entorno del enzima se vuelve progresivamente más oxidante.

3) Estudio de la inactivación de la Rubisco por distintos efectores redox.

Se ha probado la inactivación y reactivación del enzima por diferentes disulfuros y tioles y otros reactivos específicos de cisternas. Específicamente se ha estudiado la posibilidad de que el glutatión pueda controlar la actividad de la Rubisco directamente por intercambio de disulfuros o de forma indirecta, a través de efectores redox mediadores. Se ha investigado también el mecanismo de inhibición

de la Rubisco por arsenito y el efecto potenciador que producen los tioles en este proceso.

Conclusiones:

A. Respecto al papel del glutatión en la regulación redox de la actividad de la Rubisco:

1. La Rubisco puede inactivarse por intercambio de disulfuros mediante diversos compuestos disulfuros y, posteriormente, puede ser reactivada por diversos tioles. Sin embargo, ni el glutatión oxidado ni el reducido puede afectar a la actividad de la Rubisco por intercambio directo de disulfuros debido a la existencia de barreras cinéticas.

2. El ascorbato no puede mediar la modulación redox de la Rubisco por el glutatión *in vitro*. Sin embargo, el glutatión reducido puede impulsar la reactivación de la Rubisco oxidada mediante una pequeña cantidad de pequeños tioles intermediarios (como la cysteamina o la cisteína). El proceso contrario (la inactivación de la Rubisco por el glutatión oxidado utilizando pequeños disulfuros como mediadores) no progresa si los intermediarios se mantienen a una concentración inferior a la del glutatión.

3. El S-nitrosoglutatión puede actuar como donante de óxido nítrico inactivando a la Rubisco por transnitrosación de sus residuos de cisteína. Sin embargo, en el caso del enzima de *Chlamydomonas reinhardtii*, el S-nitrosoglutatión no es un donador eficiente para esta reacción ya que se necesita una concentración demasiado alta (no esperable *in vivo*) para producir una inactivación mayoritaria de la Rubisco.

B. Respecto a la identidad de las cisteínas críticas de la Rubisco:

4. La sensibilidad a cistamina que muestran las Rubiscos de *C. reinhardtii*, espinaca y arroz sugiere que la inactivación ocurre por intercambio de disulfuros con cisteínas que se encuentran entre los residuos comunes conservados. Estas son las cisteínas 84, 172, 192, 247, 284, 427 y 459 de la subunidad grande, y las cisteínas 41 y 83 de la subunidad pequeña.

5. Ninguna de las sustituciones simples de las cisteínas conservadas pormutagénesis elimina la inactivación de la Rubisco por intercambio de disulfuros con cistamina. Esto sugiere que la inactivación se produce por oxidación de varias cisteínas que contribuyen de forma redundante a la inhibición de la Rubisco.

6. La sustitución de la cisteína 427 (o de la cisteína 192 que ya se ha descrito anteriormente) produce una Rubisco mutante que es más sensible a la inactivación oxidativa. Esto sugiere que estos dos residuos juegan un papel protector, interfiriendo con la modificación de las cisteínas críticas (o con las cambios conformacionales a los que dan lugar) y, de esta manera, retrasando la inhibición.

7. Una revisión de todos los datos actualmente recogidos indica que las cisteínas 172 y 459 de la subunidad grande y 41 de la subunidad pequeña son los candidatos más probables a impulsar la inactivación y susceptibilización proteolítica de la Rubisco cuando se oxidan por intercambio de disulfuros.

C. Respecto al mecanismo por el que los tioles incrementan el efecto inhibitorio del arsenito sobre la Rubisco:

8. El arsenito por sí solo inhibe el 80% de la actividad de la Rubisco a concentraciones que se hallan por encima del rango milimolar. Sin embargo, cuando se combina con ciertos monotioles (como cisteamina, cisteína, N-acetilcisteína y 2-mercaptoetanol, pero no glutatión reducido) alcanza una inhibición completa a concentraciones en el rango submilimolar. En cambio, ditioles como el ditiotreitol no potencian la inhibición del arsenito.

9. El monotiol no actúa reduciendo un disulfuro previo ni juega un papel catalítico sino que se integra con el arsenito y la Rubisco en un complejo ternario que inactiva al enzima. Se sugiere que, en el complejo final, el átomo de arsénico está enlazado a tres átomos de azufre correspondientes al monotiol y a las cisteínas vecinas 172 y 192 de la subunidad grande de la Rubisco.

10. La estabilidad del complejo ternario es muy dependiente del monotiol particular que lo integre, siendo la constante de asociación dos órdenes de magnitud mayor cuando el tiol es mercaptoetanol que cuando es cisteamina. En cualquier caso, la

asociación es totalmente reversible desmantelándose el complejo al desalar para eliminar las formas libres de arsenito, monotiol o ambos.

11. La combinación de arsenito y un monotiol puede también suprimir la fijación de carbono por parte de un cultivo fotosintetizante de *C. reinhardtii* de forma totalmente reversible. Sin embargo, la inhibición *in vivo* no es causada por la inactivación de la Rubisco sino que afecta a un sensor indeterminado que es todavía más sensible que la Rubisco al sinergismo entre arsenito y monotiol.