

SPECIAL ISSUE REVIEW PAPER

# Catalysis and regulation in Rubisco

Inger Andersson\*

Department of Molecular Biology, Swedish University of Agricultural Sciences, BMC Box 590, S-751 24 Uppsala, Sweden

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## Abstract

**Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the incorporation of inorganic CO<sub>2</sub> into the organic molecules of life. Rubisco is extremely inefficient as a catalyst and its carboxylase activity is compromised by numerous side-reactions including oxygenation of its sugar phosphate substrate by atmospheric O<sub>2</sub>. The reduction in the catalytic efficiency as a result of these processes has implications for crop yield, nitrogen and water usage, and for the global carbon cycle. Several aspects of Rubisco including its complex biosynthesis and multi-step catalytic reaction are subject to tight control involving light, cellular metabolites, and molecular chaperones. Numerous high-resolution crystal structures of different forms of Rubisco are now available, including structures of mutant enzymes. These provide a molecular framework for the understanding of these processes at the molecular level.**

Key words: Carbon fixation, CO<sub>2</sub>/O<sub>2</sub> specificity, light-regulation, Rubisco, structure–function studies.

## Introduction

The importance of the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39, Rubisco) would be difficult to exaggerate. Rubisco catalyses the primary photosynthetic CO<sub>2</sub> reduction reaction, the fixation of atmospheric CO<sub>2</sub> to ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate (3PGA), which is subsequently used to build the organic molecules of life. Due to its key role in photosynthetic carbon fixation, Rubisco is found in most autotrophic organisms

including photosynthetic bacteria, cyanobacteria, algae, and plants. It has been estimated that Rubisco constitutes up to half of the soluble protein in the plant leaf (Ellis, 1979). Of equal importance for the global carbon cycle is its presence in oceanic phytoplankton, which are estimated to provide more than 45% of global net primary production annually (Field *et al.*, 1998).

Carbon fixation resulting from Rubisco's activity amounts to more than 10<sup>11</sup> tons of atmospheric CO<sub>2</sub> annually (Field *et al.*, 1998). This is an impressive feat, however, the characteristics of Rubisco as a catalyst are less so: Rubisco is extremely inefficient and its carboxylation activity is compromised by numerous side-reactions, the most notable with another atmospheric gas, O<sub>2</sub>. The oxygenation of RuBP produces 2-phosphoglycolate, a molecule of limited use to most organisms. Although some of the carbon of 2-phosphoglycolate is rescued by photorespiration, an energy-requiring salvage pathway, the result of the oxygenase reaction is a constant drain on the pool of RuBP and a decrease of the efficiency of carbon fixation by up to 50%. This has implications for crop yield, plant nitrogen and water usage, and for the global carbon cycle. From another perspective, it has been argued that such energy consumption may be beneficial in some circumstances. For instance, it may function as a sink, dissipating excess photosynthetic reductant under photoinhibitory conditions when CO<sub>2</sub> availability is limited (Osmond, 1981).

As befits an enzyme of this dignity and abundance, Rubisco depends on effector molecules to modulate its activity and on ancillary proteins to control its activation state (Rubisco activase) and to mediate its correct folding and assembly in the cell (chaperones). The exact details of these processes vary in enzymes from different origins. High-resolution three-dimensional structures of Rubisco

\* E-mail: inger@xray.bmc.uu.se

Abbreviations: Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose-1,5-bisphosphate; 3PGA, 3-phospho-D-glycerate; RLP, Rubisco-like protein; L, large subunit; S, small subunit; *rbcL*, Rubisco large subunit gene; *rbcS*, Rubisco small subunit gene; 2CABP, 2'-carboxyarabinitol-1,5-bisphosphate; CA1P, 2'-carboxyarabinitol-1-phosphate; XuBP, D-xylulose-1,5-bisphosphate.

from a number of organisms are now available (reviewed in Andersson and Taylor, 2003; Andersson and Backlund, 2008). While the structural information has been necessary to understand important aspects of Rubisco's biochemistry, catalysis, and evolution, it is not yet sufficient to understand, for example, its regulation at the molecular level (including the interaction with Rubisco activase) or the molecular basis for the partitioning between its carboxylation and oxygenation reactions. In-depth discussions of several aspects of Rubisco research are available (Spreitzer, 1993, 1999, 2003; Hartman and Harpel, 1994; Cleland *et al.*, 1998; Spreitzer and Salvucci, 2002; Andersson and Taylor, 2003; Andrews and Whitney, 2003; Houtz and Portis, 2003; Parry *et al.*, 2003, 2008; Portis and Parry, 2007; Moreno *et al.*, 2008). The present article highlights recent advances in the knowledge of the molecular basis for catalysis and regulation of Rubisco.

## Structure overview

### Molecular forms of Rubisco

All Rubisco enzymes are multimeric. Two different types of subunits occur: catalytic large (L, 50–55 kDa), and small (S, 12–18 kDa) subunits. Different molecular forms of Rubisco are distinguished by the presence or absence of the small subunit. The most common form (form I) of Rubisco is composed of large and small subunits in a hexadecameric structure, L<sub>8</sub>S<sub>8</sub> (Fig. 1). This form is present in most chemoautotrophic bacteria, cyanobacteria, red and brown algae, and in all higher plants. Form I Rubisco consists of a core of four L<sub>2</sub> dimers arranged around a 4-fold axis, capped at each end by four small subunits (Knight *et al.*, 1990). The small subunit is not essential for catalysis, because the large subunit octamer still retains some carboxylase activity (Andrews, 1988; Gutteridge, 1991; Lee *et al.*, 1991; Morell *et al.*, 1997). A further distinction of the form I enzymes can be made (Delwiche and Palmer, 1996; Tabita, 1999) between green-type enzymes (forms IA and B from cyanobacteria, eukaryotic algae, and higher plants) and red-type enzymes (forms IC and D from non-green algae and phototrophic bacteria). Crystal structures of the form IA/B hexadecameric enzyme have been determined from spinach, tobacco, rice, *Synechococcus* PCC6301, and *Chlamydomonas reinhardtii* (Knight *et al.*, 1990; Curmi *et al.*, 1992; Newman and Gutteridge, 1993; Taylor *et al.*, 2001; Mizohata *et al.*, 2002). Crystal structures of form IC/D Rubisco have been determined from the enzymes from *Ralstonia eutropha* and *Galdieria partita* (Hansen *et al.*, 1999; Sugawara *et al.*, 1999).

The form II enzyme is a dimer of large subunits (L<sub>2</sub>)<sub>n</sub> and lacks small subunits. The form II enzyme was initially discovered in purple non-sulphur bacteria, but has also been found in several chemoautotrophic bacteria (reviewed

in Shively *et al.*, 1998) and in eukaryotic dinoflagellates (Morse *et al.*, 1995; Whitney *et al.*, 1995; Rowan *et al.*, 1996). Several non-sulphur phototrophic bacteria, i.e. *Rhodobacter sphaeroides*, *R. capsulatus*, *Thiobacillus* sp., and *Hydrogenovibrio marinus* contain both form I and form II enzymes (Gibson and Tabita, 1977; Hayashi *et al.*, 1998). The first crystal structure of Rubisco was from the dimeric form II enzyme from *Rhodospirillum rubrum* (Schneider *et al.*, 1986, 1990a).

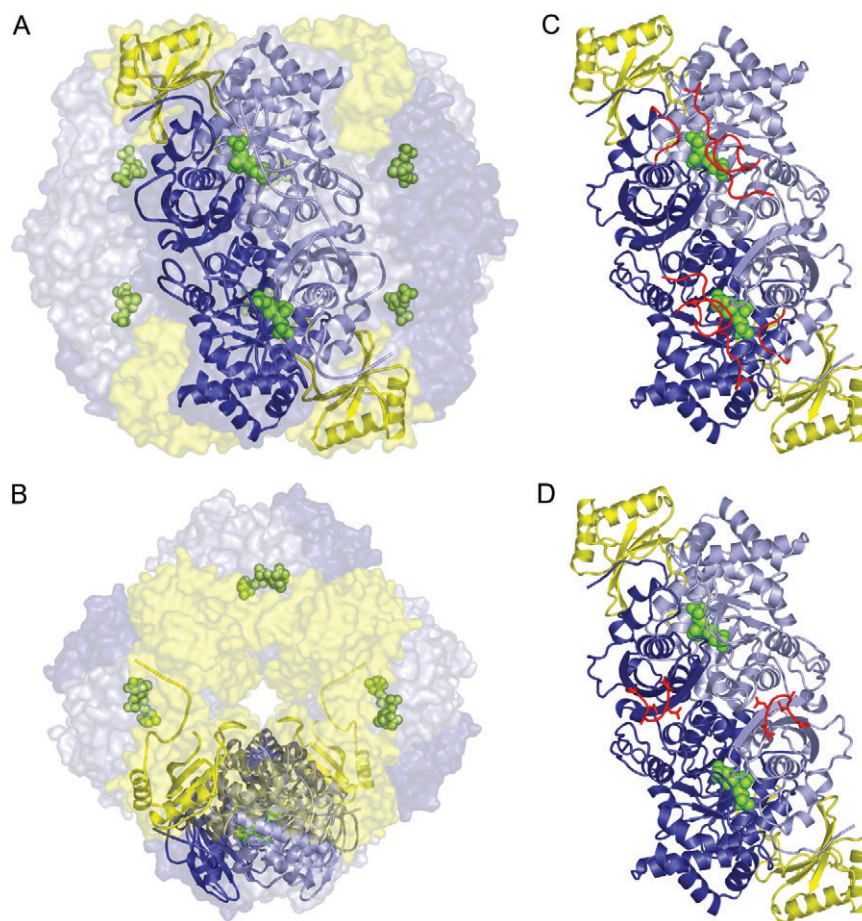
*RbcL* sequences have also been identified in archaea and assigned to a separate group, form III. With respect to quaternary structure, the archaea are diverse and comprise L<sub>2</sub>, L<sub>8</sub>, and L<sub>10</sub> enzymes. Rubisco from *Thermococcus kodakaraensis* (Kitano *et al.*, 2001) features a pentameric structure composed of five L<sub>2</sub>-units, L<sub>10</sub>. The crystal structure of Rubisco from *Pyrococcus horikoshii* consists of an octamer of large subunits, L<sub>8</sub> (PDB codes 2cxe, 2cwx, 2d69).

Putative Rubisco sequences differing from form I and II sequences have been identified in organisms that do not use CO<sub>2</sub> as the major source for carbon (Tabita, 1999). The homologues were termed Rubisco-like proteins (RLPs), form IV (reviewed in Tabita *et al.*, 2007) and are involved in sulphur metabolism (Hanson and Tabita, 2001; Murphy *et al.*, 2002). Crystal structures have been reported for the RLPs from *Geobacillus kaustophilus* (Imker *et al.*, 2007), *Rhodopseudomonas palustris* (Tabita *et al.*, 2007), and the green sulphur bacterium *Chlorobium tepidum* (Li *et al.*, 2005).

### A fold for all Rubisco enzymes

Despite apparent differences in amino acid sequence and function (in the case of the RLPs), the secondary structure of the large (catalytic) subunit is extremely well conserved throughout different forms of Rubisco (reviewed in Andersson and Taylor, 2003; Andersson and Backlund, 2008). The overall fold features a smaller amino-terminal domain consisting of a four-to-five-stranded mixed  $\beta$  sheet with helices on one side of the sheet and a larger carboxy-terminal domain featuring an eight-stranded  $\alpha/\beta$  barrel structure. Overall, large subunits of forms I–IV display 25–30% sequence identity. Despite this relatively large divergence on the level of sequence, differences are localized to a few loops (Andersson and Backlund, 2008).

The active site is located at the intra-dimer interface between the carboxy-terminal domain of one large subunit and the amino-terminal domain of the second large subunit in the L<sub>2</sub> dimer. In the hexadecameric molecule, the dimers are arranged such that the eight active sites face the outside solvent (Fig. 1) (Andersson *et al.*, 1989; Knight *et al.*, 1990). Loops 1, 2, and 5–8 (connecting  $\beta$ -strand 1 with  $\alpha$ -helix 1, etc) at the carboxy-terminal end of the  $\beta$ -strands contribute residues involved in catalysis and substrate binding. Two loop regions in the amino-terminal domain of the second large subunit in the dimer



**Fig. 1.** The most common form of Rubisco from plants, cyanobacteria, and green algae is a hexadecamer of eight large and eight small subunits. (A) The L8S8 hexadecamer from spinach viewed along the 2-fold symmetry axis. Large subunits are blue, small subunits are yellow, and the substrate mimic (2CABP) is displayed as green spheres. The fold of one L2S2 unit is highlighted. (B) View of the hexadecamer along the 4-fold axis. This view is obtained from (A) by a 90° rotation around the *x*-axis. (C) The L2S2 unit in (A) with mobile elements (amino-terminal loop, loop 6, carboxy-terminus) highlighted in red. (D) The L2S2 unit with the  $\beta$ C– $\beta$ D loop highlighted in red. Residues in the  $\beta$ C– $\beta$ D loop are implicated to interact with Rubisco activase.

contribute additional residues to the active site. Thus, the functional unit of Rubisco is an L2 dimer of large subunits containing two active sites. The substrate binds in an extended conformation across the opening of the  $\alpha/\beta$ -barrel and is anchored at two distinct phosphate-binding sites at opposite sides of the  $\alpha/\beta$ -barrel and in the middle at the magnesium-binding site.

The small subunit is more diverse. The common core structure consists of a four-stranded anti-parallel  $\beta$ -sheet with two helices on one side (Knight *et al.*, 1989, 1990). The most significant variations occur in two distinct regions, the loop between  $\beta$  strands A and B, the so-called  $\beta$ A– $\beta$ B-loop, and the carboxy-terminus (reviewed in Spreitzer, 2003). The  $\beta$ A– $\beta$ B loops of four small subunits line the openings of the solvent channel that traverses the holoenzyme and are wedged between two different large subunits making numerous interactions both to large and small subunits. The length of the loop is variable, the shortest occurs in Rubisco from prokaryotes and non-green algae, and the longest in green algal

Rubisco (Karkehabadi *et al.*, 2005). In non-green algae and some prokaryotes the short loops are accompanied by carboxy-terminal extensions that form  $\beta$  hairpin structures ( $\beta$ E– $\beta$ F loop) in the spaces that are normally occupied by the longer  $\beta$ A– $\beta$ B loops (Hansen *et al.*, 1999; Sugawara *et al.*, 1999). The function of the small subunit is enigmatic. Its structural arrangement, covering a substantial area at two opposite ends of the L-subunit octamer makes it reasonable to assume a structural (chaperone-like) function of the small subunit. In this capacity it influences both holoenzyme stability and catalytic performance (Spreitzer *et al.*, 2001; reviewed in Spreitzer, 2003).

### Synthesis and assembly

The Rubisco large subunit is encoded by a single gene in the chloroplast genome and is synthesized by the plastid ribosome. In plants, the small subunit is coded by a family of closely related nuclear genes and synthesized in the cytosol (reviewed in Spreitzer, 2003). The synthesis and



assembly of the Rubisco holoenzyme, involving the coordinated control of chloroplastic and cytosolic processes, have been shown to require the assistance of ancillary proteins termed molecular chaperones (Barracough and Ellis, 1980; reviewed in Gatenby and Ellis, 1990). The plastid chaperones belong to a large family named chaperonins (Hemmingsen *et al.*, 1988). Two major types are encountered, the chaperonin 60 (cpn60) and chaperonin 10 (cpn10). The best-studied chaperonins are encoded by the *groEL/ES* genes of *Escherichia coli* (Goloubinoff *et al.*, 1989). Despite the close homology between the bacterial and plastid chaperonins (Hemmingsen *et al.*, 1988), all attempts to assemble catalytically active Rubisco enzymes from crop plants in *E. coli* have failed, suggesting that the plant system is highly specific and/or that additional factors are required in the process (Andrews and Whitney, 2003).

Studies on the structure and function of a cyanobacterial protein, RbcX, point to a role for RbcX as an assembly chaperone (Saschenbrecker *et al.*, 2007; Tanaka *et al.*, 2007). RbcX has been shown to promote the synthesis and assembly of active recombinant cyanobacterial holoenzyme in *E. coli* (Li and Tabita, 1997; Onizuka *et al.*, 2004; Emlyn-Jones *et al.*, 2006; Saschenbrecker *et al.*, 2007). In several species of  $\beta$ -cyanobacteria, the *rbcX* gene encoding RbcX is located in the *rbcLXS* operon between the *rbcL* and *rbcS* genes encoding Rubisco large and small subunits (Larimer and Soper, 1993). This is the case for instance in *Anabaena* sp. strain CA (Li and Tabita, 1997), *Synechococcus* PCC7002 (Onizuka *et al.*, 2004; Saschenbrecker *et al.*, 2007), and *Synechocystis* sp. PCC6803 (Tanaka *et al.*, 2007). In contrast, in *Synechococcus* PCC7942 *rbcX* is found >10 kb away from the *rbcLS* genes and here the absolute requirement for RbcX for assembly seems to be relaxed and may not be necessary for *in vivo* assembly (Emlyn-Jones *et al.*, 2006).

Saschenbrecker *et al.* (2007) were able to show that RbcX from *Synechococcus* sp. PCC7002 functions by binding and stabilizing Rubisco large subunits (RbcL) following their interaction with the GroEL/ES chaperonin. The crystal structure of the RbcX monomer is a four-helix bundle. Formation of a homodimer of the 15 kDa protein creates a central peptide-binding groove that recognizes a conserved and partially unfolded carboxy-terminal peptide of the large subunit. Based on their studies, Saschenbrecker *et al.* (2007) propose a model where RbcX assists in the initial formation of the *rbcL8* core complex by binding the carboxy-terminal peptide. Displacement of RbcX in the RbcX-RbcL complex by Rubisco small subunits (RbcS) produces the active holoenzyme RbcL8S8. This process, in turn, is facilitated by the dynamic assembly between RbcX and the core complex RbcL8. The degree of sequence conservation in cyanobacterial RbcX homologues suggest they have similar architecture and this was confirmed by crystal structures of

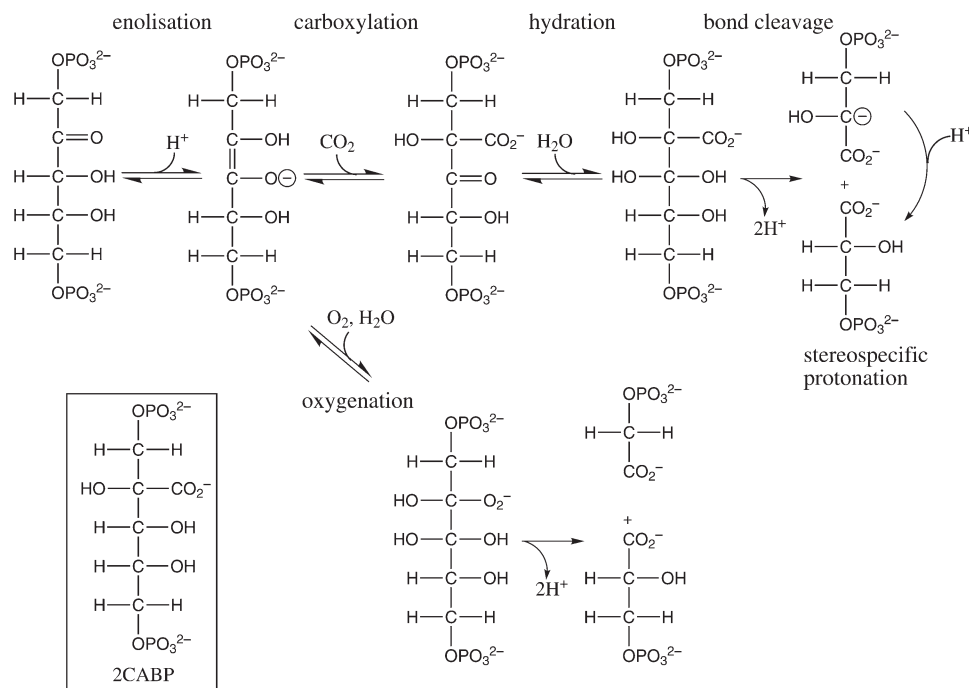
RbcX from *Anabaena* sp. strain CA (Saschenbrecker *et al.*, 2007) and *Synechocystis* sp. PCC6803 (Tanaka *et al.*, 2007) revealing nearly identical structures for the homologues. RbcX homologues identified in plants are more distant, but share the register and structural residues.

Sequence comparison showed that the peptide recognized by RbcX contains a highly conserved carboxy-terminal motif, EIKFEFD in form I Rubisco large subunits, including those of higher plants. Form II and archaeal Rubisco lack this motif as well as the gene encoding RbcX. Three residues downstream from the EIKFEFD motif is the conserved residue Asp473 (spinach RbcL numbering; Schneider *et al.*, 1990b), which is intimately involved in the formation of the closed state required for catalysis to occur (Schreuder *et al.*, 1993; Taylor and Andersson, 1996; Duff *et al.*, 2000, see also below). In the crystal structure of the D473E mutant of Rubisco from *Chlamydomonas reinhardtii* (Satagopan and Spreitzer, 2004) the carboxy-terminus is disordered in seven of the eight large subunits (Karkehabadi *et al.*, 2007). However, in one large subunit, it is stabilized by a crystal contact in a way that may mimic the interaction with a chaperone. Thus the RbcL carboxy-terminus appears to have multiple functions; it participates in conformational transitions during catalysis and also in forming and stabilizing the RbcL8 core complex during assembly. The role of RbcX appears to be to protect this sequence from incorrect interactions during these processes.

## Catalysis

### Reaction pathway

The main reaction catalysed by Rubisco (the addition of CO<sub>2</sub> and H<sub>2</sub>O to RuBP to yield two molecules of 3PGA) involves multiple discrete steps and associated intermediates of variable stability (Fig. 2). To be functional, Rubisco requires prior activation by carbamylation of the  $\epsilon$ -amino group of active-site Lys201 (Lorimer and Miziorko, 1980) by a CO<sub>2</sub> molecule, which is distinct from the substrate-CO<sub>2</sub>. The carbamylated Lys201 is stabilized by the binding of magnesium ion to the carbamate. The carboxylation involves at least four, perhaps five discrete steps and at least three transition states; enolization of RuBP, carboxylation of the 2,3-enediolate, and hydration of the resulting ketone, carbon-carbon scission, and stereospecific protonation of the resulting carboxylate of one of the product 3PGA. Several, if not all, of these steps involve acid-base chemistry. Considerable time and effort has been spent to identify enzyme residues that participate in catalysis. High-resolution crystal structures have provided steric constraints, while chemical modification, site-directed mutagenesis, molecular dynamics calculations, and quantum chemical analyses have added mechanistic and energetic constraints (reviewed in Andrews and Lorimer, 1987; Hartman and Harpel, 1994; Cleland *et al.*, 1998; Andersson



**Fig. 2.** The main reactions catalysed by Rubisco, carboxylation and oxygenation of RuBP.

and Taylor, 2003). Here the main points are recapitulated as far as they are known.

#### *In search of the essential base*

In order to direct the attack of  $\text{CO}_2$  to the C2 carbon atom of RuBP, the ketone form of RuBP needs to be converted to an enediol. This requires removal of the proton at C3 and protonation of the carbonyl group at C2. Deprotonation by an enzyme base is a typical feature of enzymes that catalyse enolization reactions (Knowles, 1991) and, in the case of Rubisco, the nature of the base has been intensely debated (Hartman and Harpel, 1994; Cleland *et al.*, 1998). The steric constraints imposed by the crystal structures singled out the non-Mg-co-ordinated carbamate oxygen on K201 as the most likely candidate for the base (Andersson *et al.*, 1991; Newman and Gutteridge, 1993; Andersson, 1996; Taylor and Andersson, 1997a). This conclusion assumes a *cis* conformation of the O2 and O3 oxygen atoms of RuBP. This may now seem trivial, but required X-ray data to high resolution that could only be obtained with the introduction of larger and better detectors (Andersson *et al.*, 1991). The involvement of K201 would be difficult to prove or disprove by mutagenesis, because mutation of K201 would render the enzyme inactive.

Computational methods have been used to probe the energetics of the reaction either using minimal 3-carbon or 5-carbon transition structures *in vacuo* (Tapia *et al.*, 2000) or abbreviated systems including groups on the protein (King *et al.*, 1998; Mauser *et al.*, 2001; Tapia *et al.*, 2002). These calculations stress the importance carbamylated

Lys201 in the enolization. Calculations by King *et al.* (1998) also suggest how the seemingly disparate results on the involvement of Lys175 in the enolization reaction obtained from chemical and mutagenesis studies and X-ray crystallography (reviewed in Hartman and Harpel, 1994) may be reconciled by assigning a different role to Lys175 as an acid for the protonation of O2 of the enediolate (King *et al.*, 1998). Lys175 may thus correspond to the essential acid implicated in measurements of the pH profile in the deuterium isotope effect (Van Dyk and Schloss, 1986). Deprotonation of C3 by Lys201 and protonation of O2 by Lys175 is in accordance with the crystal structures that place Lys175 on the opposite side of the transition state analogue with respect to the carbamylated Lys201 (Knight *et al.*, 1990). Presumably, these steps help to direct the gaseous substrate to the C2 atom, otherwise C3 would be as likely to react. The moulding of the C2 and C3 centres in a *cis* out-of-plane conformation around the C2–C3 bond in the transition structure provides the necessary activation for the reaction to proceed (Andres *et al.*, 1993; Tapia *et al.*, 2002). In the next step,  $\text{CO}_2$  or  $\text{O}_2$  compete for the enediolate.

#### *Variation in catalytic efficiency of Rubisco enzymes from diverse origins*

The competing reaction of  $\text{O}_2$  with RuBP appears to be an inevitable consequence of the mechanism of carboxylation (Andrews and Lorimer, 1987; Cleland *et al.*, 1998) and results in the synthesis of one molecule each of 3PGA and 2-phosphoglycolate. The oxygenation reaction is the first

step in photorespiration, a process that salvages some of the carbon of 2-phosphoglycolate at the cost of energy and evolved CO<sub>2</sub> (Andrews and Lorimer, 1987). The oxygenase reaction is an intrinsic characteristic of Rubisco, the extent of which depends on the properties of the particular enzyme studied. 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 to improve crop yields (reviewed in Spreitzer and Salvucci, 2002; Parry *et al.*, 2003).

The efficiency with which CO<sub>2</sub> is able to compete with O<sub>2</sub> is quantified by the CO<sub>2</sub>/O<sub>2</sub> specificity factor (often referred to as  $\tau$ , or  $\Omega$ ) and is defined as  $V_c K_o / V_o K_c$ , where  $V_c$  and  $V_o$  are the maximal velocities of carboxylation and oxygenation, respectively, and  $K_c$  and  $K_o$  are the Michaelis constants for CO<sub>2</sub> and O<sub>2</sub>, respectively (Laing *et al.*, 1974). Thus the relative rates for carboxylation and oxygenation are defined by the product of the specificity factor and the ratio of CO<sub>2</sub> to O<sub>2</sub> concentrations at the active site. The specificity values of Rubisco enzymes from different origins differ substantially (Jordan and Ogren, 1981). Some photosynthesizing bacteria have the lowest specificity values (5–40), red algae have the highest (180–240), whereas higher plants and green algae have intermediate specificity values in the range of 60–100. However, it appears that positive selection towards higher specificity has occurred at the cost of overall carboxylation rate, because an inverse correlation between specificity and turnover rate ( $V_c$  or  $k_{cat}$  for carboxylation) has been observed (Jordan and Ogren, 1981; Bainbridge *et al.*, 1995) with, for example, bacteria displaying low specificity values and high turnover rates whereas higher plants have high specificity values coupled to low turnover rates. Some organisms have evolved mechanisms (carboxysomes, pyrenoids, C<sub>4</sub>- and CAM metabolisms) that concentrate CO<sub>2</sub> at the carboxylation site (Dodd *et al.*, 2002; von Caemmerer and Furbank, 2003; Giordano *et al.*, 2005; Price *et al.*, 2008) in order to increase carbon fixation efficiency. Thus the specificity factor is but one parameter that determines the net efficiency of Rubisco enzymes, but it can serve as an important first diagnostic parameter to indicate changes in efficiency of engineered Rubisco enzymes. McNevin *et al.* (2007) have shown how differences in catalytic mechanisms can be examined through the utilization of carbon isotope discrimination. In their study, significant deviations from the mean  $\delta^{13}\text{C}$  value measured in C<sub>3</sub> plants (form I enzyme) were observed in a tobacco mutant enzyme and in a form II enzyme.

#### Role of the magnesium ion

One of the key players in the reaction catalysed by Rubisco is the magnesium ion. Apart from the carbamylated Lys201 which provides a monodentate ligand, the

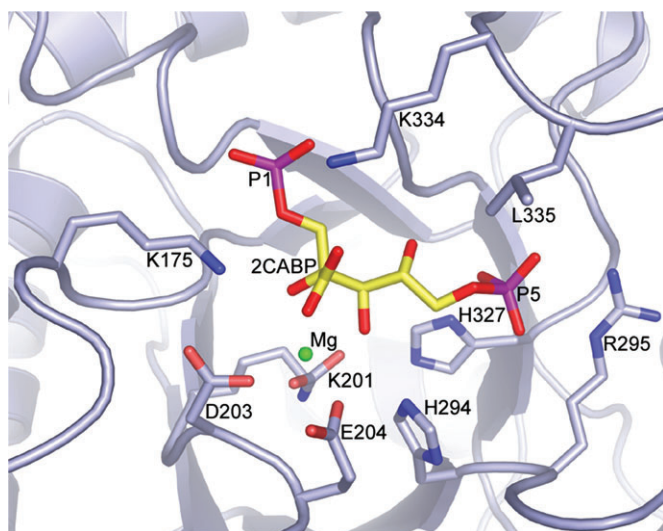
magnesium ion is liganded by two (monodentate) carboxylate ligands provided by Asp203 and Glu204 (Fig. 3) and three water molecules (Andersson *et al.*, 1989). RuBP replaces two of these water molecules. For the reaction to proceed, a tight control of the charge distribution around the metal ion is crucial (Taylor and Andersson, 1997a) and this presumably also includes residues outside the immediate co-ordination sphere. For instance, the metal ligands Asp203 and Glu204 interact with their free oxygen atom with the  $\epsilon$ -amino groups of Lys175 and Lys177, respectively. These interactions may help avoid bidentate co-ordination of the carboxylate groups to the metal ion, which, if it occurred, would block the binding of the gaseous substrates.

CO<sub>2</sub> replaces the last Mg<sup>2+</sup>-co-ordinated water molecule and adds to the enediol directly without forming a Michaelis complex (Pierce *et al.*, 1986; Mauser *et al.*, 2001). The resulting six-carbon compound, 3-keto-2'-carboxyarabinitol-1,5-bisphosphate is relatively stable and can be isolated (Lorimer *et al.*, 1986). The six-carbon carboxylated intermediate is closely mimicked by the inhibitor 2'-carboxyarabinitol-1,5-bisphosphate (2CABP; Pierce *et al.*, 1980), which forms an exchange-resistant complex ideal for crystallographic comparisons (Knight *et al.*, 1990; Newman and Gutteridge, 1993; Schreuder *et al.*, 1993; Andersson, 1996; Sugawara *et al.*, 1999; Taylor *et al.*, 2001; Mizohata *et al.*, 2002). The crystal structures show Lys334 positioned to facilitate the addition of the gaseous substrate (Fig. 3; Knight *et al.*, 1990; Andersson, 1996) in accord with site-directed mutagenesis and chemical modification (Lorimer *et al.*, 1993).

#### Conformational changes during catalysis

During catalysis, Rubisco undergoes a conformational change, which serves to close the active site and prevent access of water during the reaction. The closing mechanism involves movements of loop 6 (a loop connecting  $\beta$ -strand 6 with  $\alpha$ -helix 6 in the  $\alpha/\beta$ -barrel), the carboxy-terminal strand, and a loop from the amino-terminal domain of the adjacent large subunit of the L2 dimer (Fig. 1C; Schreuder *et al.*, 1993; Taylor and Andersson, 1996; Duff *et al.*, 2000). Rubisco structures can be divided into two states (Duff *et al.*, 2000); open with the active site unliganded or occupied by loosely bound substrates or products, or closed with substrates or inhibitors tightly bound and completely shielded from solvent. Apart from the movement of loop 6 (residues 331–338) to cover the opening of the  $\alpha/\beta$ -barrel, the transition between open and closed forms involves a rigid-body movement that brings the amino- and carboxy-terminal domains of adjacent subunits together, and the ordering of residues 63–69 of the amino-terminal domain. Packing of the carboxy-terminal strand (residues 463 to the carboxy-terminal end) against loop 6 completes the





**Fig. 3.** The active site in carbamylated Rubisco with a reaction intermediate analogue (2CABP) bound across the  $\alpha/\beta$ -barrel. Residues implicated in the reaction mechanism are highlighted.

closure (Schreuder *et al.*, 1993; Taylor and Andersson, 1996).

Two strictly conserved glycine residues, Gly333 and Gly337, maintain flexibility in the hinge of loop 6. The other strictly conserved residue, Lys334, is located at the tip of the loop and interacts with the incoming gaseous substrate during catalysis. The Lys334 side chain extends into the active site and hydrogen bonds to one of the two oxygen atoms of the inhibitor 2CABP that is equivalent to that of substrate  $\text{CO}_2$  (Knight *et al.*, 1990; Andersson, 1996). It also interacts with the  $\gamma$ -carboxylate of Glu60 and the hydroxyl group of Thr65 in the amino-terminal domain of the adjacent large subunit.

The exact timing of the closure of the active site is not known. The inhibitor 2CABP forms a tight-binding closed complex ideal for crystallization. Complexes with ligands that bind less tightly, such as the natural substrate RuBP or the product 3-PGA have been more difficult to crystallize (Lundqvist and Schneider, 1989, 1991; Taylor *et al.*, 1996; Taylor and Andersson, 1997*a, b*). A stable complex with RuBP could be obtained by the soaking of RuBP into crystals of the carbamylated spinach enzyme in the presence of  $\text{Ca}^{2+}$ , because this complex does not support turnover to an appreciable extent (Parry *et al.*, 1983). The resulting structure is open, with loop 6 partially retracted (Taylor *et al.*, 1997*a*). It appears that the presence of RuBP only is not enough to trigger the closing of the active site. The complex of 2CABP with  $\text{Ca}^{2+}$  is of the closed type with distances between the terminal phosphate groups that are compatible with the closed state (9.05 Å). Also, soaking 2CABP into crystals of calcium-activated Rubisco induces closing of the active site in the crystal (Karkehabadi *et al.*, 2003). This indicates that it is not the calcium ion per se that prevents the

conformational switch. It may be that the interaction of the substrate- $\text{CO}_2$  with Lys334 is required for the shortening of the interphosphate distance of the substrate (Duff *et al.*, 2000) that seems to be needed for proper loop closure.

#### *Mutations in loop 6 influence the $\text{CO}_2/\text{O}_2$ specificity*

The importance of loop 6 for catalysis and specificity has been demonstrated by genetic selection and site-directed mutagenesis (Chen and Spreitzer, 1989). Residue Val331 is part of the hinge on which the loop moves and is highly, but not strictly, conserved (Newman and Gutteridge, 1993). Replacement of Val331 by Ala in the green alga *Chlamydomonas reinhardtii* (Chen and Spreitzer, 1989) reduces specificity and carboxylation turnover. Genetic selection for restored photosynthesis identified second-site T342I and G344S substitutions with increased specificity (close to the wild-type level) and modest improvement in  $V_{\text{c}}/K_{\text{c}}$  (Chen and Spreitzer, 1989; Chen *et al.*, 1991). Similar results were obtained in *Synechococcus* Rubisco (Gutteridge *et al.*, 1993; Parry *et al.*, 1992).

The crystal structures of V331A, the V331A/T342I- and V331A/G344S-revertant enzymes and the T342I-suppressor enzyme (which contain wild-type Val331) illustrate how seemingly minor structural changes can have relatively dramatic effects on catalysis (Karkehabadi *et al.*, 2007). In general, the mutations resulted in local, but significant deviations close to the substituted residues, but left the C $\alpha$  backbone of the mutant enzymes unperturbed. The side chain of Val331 is located in a hydrophobic pocket and makes van der Waals interactions with residues Thr342, Ile393, and the main-chain C $\alpha$  atom of Arg339. Substitution of the Val331 side chain by the smaller Ala weakened these interactions considerably and created a small cavity that was partly filled by solvent. Van der Waals contact was restored between residues 331 and 342 in the V331A/T342I-revertant enzyme due to the somewhat bulkier Ile side chain at position 342. The main-chain conformation of the loop was not visibly altered by the mutation, but the presence of the additional Ile342 C $\gamma$  atom caused changes in local conformations and interactions of several residues, for example, Arg339, Glu338, and Ser328 close to I342. Val331 and Thr342 flank Lys334 located at the apex of loop 6, and it seems likely that the mutation could destabilize the loop or alter its flexibility in a way that could influence the catalytic properties of the mutant enzymes. Similar conclusions were drawn from the observation of altered catalysis in the tobacco enzyme induced by a L335V mutation, which caused a reduction in specificity and altered sensitivity to inhibitors (Whitney *et al.*, 1999; Pearce and Andrews, 2003). In the V331A/G344S-revertant enzyme, the substitution of Gly344 by Ser caused a displacement of the entire large-subunit  $\alpha$ -helix 6 (residues 338–347), shifting the backbone atoms of these residues approximately 1 Å (Karkehabadi *et al.*, 2007). This displacement also brings

Thr342 closer to Ala331. It thus appears that specificity is restored by two fundamentally different mechanisms in the two revertant enzymes. The presence of Val331 in the G344S single mutant may limit the displacement of  $\alpha$ -helix 6, and may account for the different catalytic properties of the V331A/G344S-revertant and G344S-suppressor enzymes.

#### *The carboxy-terminus of the large subunit is important for proper loop closure*

The interaction of the carboxy-terminus with loop 6 seems to be intimately involved in the transition from the open to the closed state of the Rubisco active site (Schreuder *et al.*, 1993; Taylor and Andersson, 1996; Duff *et al.*, 2000). As shown by site-directed mutagenesis, the carboxy-terminus is not absolutely required for catalysis, but is needed for maximal activity and stability (Morell *et al.*, 1990; Ranty *et al.*, 1990; Gutteridge *et al.*, 1993; Esquivel *et al.*, 2002). Residue Asp473 was proposed to serve as a latch responsible for placing the large-subunit carboxy-terminus over loop 6 and stabilizing the closed conformation required for catalysis (Duff *et al.*, 2000). Directed mutagenesis and chloroplast transformation in *C. reinhardtii* showed that although Asp473 is not essential for catalysis, mutations D473A and D473E caused substantial decreases in catalytic efficiency and specificity (Satagopan and Spreitzer, 2004). The crystal structure of D473E (Karkehabadi *et al.*, 2007) showed that the relatively modest substitution of Asp473 by Glu causes disorder of the last six carboxy-terminal residues. It appears that the mutations disrupt contacts of residue 473 with Arg134 and His310. This may cause a destabilization of the underlying loop 6 with consequences for catalysis and specificity.

#### *Another role for Lys175?*

Hydration at C3 may occur either simultaneously with carboxylation, or as a separate step. The crystal structure of the activated spinach enzyme in complex with RuBP has a water molecule close to C3 of RuBP (Taylor and Andersson, 1997a). A similar situation is observed in the enzyme from *Rhodospirillum rubrum* (Lundqvist and Schneider, 1991). No further details are known for this step, but it is noted that the carbamate group of Lys201 and the side chain of His327 are within hydrogen bonding distance to the water molecule in the spinach structure and thus well placed to assist the hydration step. It is possible that key active site residues, such as the carbamylated Lys201, may assist several of the partial reactions. One example is the final stereospecific protonation of the carboxylate ion of upper 3PGA. Structurally, protonated Lys175 is ideally positioned to donate this proton (Taylor and Andersson, 1997a) and experimental evidence indicates that deletion of the side chain interferes with this step (Harpel and Hartman, 1996). If Lys175 assists in the

delivery of a proton to C2 in the enolization reaction as suggested (King *et al.*, 1998) the residue must be reprotonated before it can act as a proton donor in the last step. Such proton relocations could presumably occur as internal protonation steps within the active site.

## Regulation

The requirement of CO<sub>2</sub> and Mg<sup>2+</sup> for activation provides a potential for regulation by light. In the dark, Mg<sup>2+</sup> flows from the stroma at the same time as the pH declines, reducing the pool of activated Rubisco. The enzyme is also subject to control by the redox status of the chloroplast (Moreno *et al.*, 2008). In addition, a number of other metabolites (mainly phosphorylated compounds) also present in the chloroplast may interfere or interact with the activating process (reviewed in Parry *et al.*, 2008).

### *Effector-mediated regulation*

The interaction of Rubisco with anions and phosphorylated sugar compounds is complex. It has been estimated that a large fraction of the phosphorylated sugar compounds in the chloroplast is bound to Rubisco (Pettersson and Ryde Pettersson, 1988). Modulation of the activity of Rubisco has been observed by the binding of various effectors such as anions, sulphate, inorganic phosphate or phosphorylated sugars (such as pyridoxal 5-phosphate, phosphogluconate, and NADPH). At limiting concentrations of CO<sub>2</sub>, effectors may act positively or negatively by either promoting or inhibiting activation (Whitman *et al.*, 1979; Badger and Lorimer, 1981; McCurry *et al.*, 1981; Jordan *et al.*, 1983; Parry and Gutteridge, 1984; Parry *et al.*, 1985, 1997; Anwaruzzaman *et al.*, 1995; Marcus and Gurevitz, 2000; Marcus *et al.*, 2005). Some of these effectors, but not all, act as competitive inhibitors of the enzyme with respect to the substrate RuBP. As a further complication, some of the effectors give rise to biphasic kinetics, suggesting negative co-operativity between active sites (Whitman *et al.*, 1979; Parry and Gutteridge, 1984; Johal *et al.*, 1985; Parry *et al.*, 1985; Belknap and Portis, 1986).

The purified enzyme from higher plants becomes progressively inhibited during *in vitro* catalysis by strongly inhibitory phosphorylated compounds that bind in the active site, a process termed *fallover* (Edmondson *et al.*, 1990a, b, c, d). Some compounds, notably xylulose-1, 5-bisphosphate (XuBP), are formed by the incorrect stereochemical reprotonation of the enediolate bound at the catalytic site and often bind to the non-carbamylated enzyme with high affinity. This leads to a tight complex that lacks the metal ion and is catalytically inactive (McCurry and Tolbert, 1977; Zhu and Jensen, 1991; Newman and Gutteridge, 1994; Taylor *et al.*, 1996). An inactive complex with the natural substrate, RuBP, appears to play an important role in the light-dependent regulation



of Rubisco activity *in vivo*. As part of this mechanism RuBP binding to decarbamylated enzyme locks the enzyme in an inactivated form (Jordan and Chollet, 1983). Release of inhibitory RuBP from Rubisco is facilitated by an ancillary chloroplast protein, Rubisco activase in an ATP-dependent manner (reviewed in Portis, 2003).

#### *A nocturnal inhibitor*

Light-dependent regulation may also be mediated by a naturally occurring tight-binding inhibitor, the monophosphate 2'-carboxyarabinitol 1-phosphate (CA1P). CA1P resembles 3-keto-2CABP and accumulates in plants in the dark and in low-light conditions, often to amounts exceeding Rubisco active site concentrations (5 mM). It has an affinity for the activated form of the enzyme in the nanomolar range (Gutteridge *et al.*, 1986). In binding to the carbamylated enzyme, 2CA1P effectively prevents its interaction with RuBP and inactivates the enzyme. Release of CA1P from the active site is facilitated by Rubisco activase (Robinson and Portis, 1988) followed by degradation by a specific phosphatase (Gutteridge and Julien, 1989; Holbrook *et al.*, 1989). The level to which CA1P can accumulate varies considerably in plant species. *Phaseolus vulgaris* has a very large capacity for CA1P accumulation whereas in wheat, *Arabidopsis*, and spinach low levels of accumulation are observed (Moore *et al.*, 1991). It may be that species that show low accumulation of CA1P primarily use changes in the extent of carbamylation and binding of decarbamylated enzyme to RuBP as a means for light regulation. Both mechanisms (CA1P metabolism and carbamylation/decarbamylation and binding to RuBP) may thus operate in parallel. In this model, the release of the inhibitor both from decarbamylated and carbamylated sites is facilitated by Rubisco activase which works as light/ATP driven switch between the two systems (Seemann *et al.*, 1990).

The biphasic kinetics can be interpreted in more than one way. Biphasic inhibition may be caused by isomerization of a loose and reversible complex to a much tighter complex (Edmondsson *et al.*, 1990d). Negative cooperativity is difficult to prove or disprove and the behaviour can often be mimicked in a sample contaminated by a less active or slightly denatured form of the protein, or it can be linked to ageing processes in the protein. For instance, preincubation with CO<sub>2</sub> and Mg<sup>2+</sup> exposes Rubisco to processes that may reduce its activity, especially if activation is performed at prolonged times and at elevated temperatures.

The kinetic behaviour may also be influenced by simultaneous binding to an effector site outside the active site. The first crystal structures gave no information on such a site (Schneider *et al.*, 1986). Therefore, an important clue was the observation of the binding of inorganic sulphate or phosphate at a surface site in the

structure of non-carbamylated Rubisco from tobacco (Curmi *et al.*, 1992; Duff *et al.*, 2000). Because of its location at a latch site that holds down the carboxy-terminus in the closed complexes, it could not be detected in the ligand-bound complexes (Knight *et al.*, 1990; Schreuder *et al.*, 1993; Andersson, 1996). Inorganic phosphate binds to three sites in the structure from tobacco Rubisco (Duff *et al.*, 2000). Two of the sites are within the active site and coincide with the positions occupied by the P1 and P5 phosphate groups of RuBP. Because of the shift of the position of the phosphates when the active site closes, these sites consist of two distinct subsites; a distal and a proximal site for the P1 group, and a lower and an upper site for the P5 group in the nomenclature of Duff *et al.* (2000). Closing of the active site causes a shift of the phosphate groups of the substrate between these sites and shortening of the inter-phosphate distance resulting in tight binding of the ligand (Duff *et al.*, 2000). The third phosphate ion binds at a surface site where it takes the place occupied by the side chain of the carboxy-terminal Asp473 in the closed complexes. The binding of inorganic phosphate to the two types of sites, the latch site and the P5 phosphate site, has been investigated by mutagenesis in Rubisco from *Synechocystis* sp. strain PCC6803 (Marcus *et al.*, 2005). It was found that mutations at both sites abolished the phosphate-stimulated activation, whereas mutation of the P5 site interfered with the inhibition of Rubisco catalysis by phosphate.

Although several questions remain to be answered, it may be possible to interpret the dual role of phosphate ions and phosphorylated compounds as activators of carbamylation and as competitive inhibitors to the sugar substrate by invoking two types of binding sites with different affinities for the effector molecules (Duff *et al.*, 2000; Marcus and Gurevitz, 2000). When binding at the two sites within the active site, these compounds interfere with substrate binding and behave as competitive inhibitors. When binding at the latch site, the effectors compete with the closing mechanism and again act as inhibitors. However, at the same time they stabilize the carbamylated enzyme in a manner that appears similar to how Rubisco activase works. One might speculate that the binding of effectors at the latch site destabilizes the (closed) inactivated complex with RuBP and helps the enzyme to open, rid itself of the ligand, and reactivate. If this were the case, binding at the latch site would stabilize the carbamylated form of the enzyme. Also, binding of these compounds at the two types of sites with different affinities might explain the biphasic kinetics.

#### *The elusive Rubisco activase*

Rubisco activase is intimately involved in the light-dependent regulation of Rubisco by relieving the

inhibition of phosphorylated compounds that bind to both carbamylated and non-carbamylated Rubisco active sites (Portis, 2003; Portis *et al.*, 2008). The three-dimensional structure of activase is not known, nevertheless, evidence is slowly gathering towards its function. Based on multiple sequence alignment, activase has been suggested to be a member of an extended AAA<sup>+</sup> family of ATPases (Neuwald *et al.*, 1999). Members of the family typically form large ring-structures and interact with other proteins, in a way characteristic for molecular chaperones.

Site-directed mutagenesis studies of Rubisco from *C. reinhardtii* have provided evidence for a region on the enzyme, the  $\beta$ C- $\beta$ D loop (Fig. 1D), that when mutated causes a reversal in Rubisco/activase specificity (Larson *et al.*, 1997). Unlike the wild type *Chlamydomonas* enzyme, P89R, P89A and D94K mutant enzymes could no longer be activated by the spinach enzyme (Larsson *et al.*, 1997; Ott *et al.*, 2000). Instead, the mutant enzymes now could be activated by the tobacco enzyme. The  $\beta$ C- $\beta$ D loop containing residues 89 and 94 is located on the surface of the enzyme (Fig. 1D) close to loop 6 and the carboxy-terminus that shield the active site from solvent in the closed complex. Thus the binding of activase at this site is likely to interfere with the closing mechanism. Portis (2003) has presented a mechanism in which activase interacts with Rubisco and opens the active site by a reversal of the closing mechanism (discussed above). Binding of activase at or around residue 89 on Rubisco initiates movement of the amino-terminal domain towards the open conformation leading to the opening of loop 6 and the carboxy-terminus. The location of the  $\beta$ C- $\beta$ D loop at a central position on the surface of Rubisco could allow for the oligomerization of activase in a girdle around the centre of the enzyme (see Fig. 4 in Portis, 2003). Residues in the carboxy-terminal region of activase (311 and 314) have been identified that may directly interact with residues on the loop in Rubisco (Li *et al.*, 2005).

### Concluding remarks

It is a sobering thought that our life depends on an enzyme that is so incomplete. Rubisco, or rather the Rubisco catalytic large subunit, emerges as a molecule in need of its helpers. Due to the extraordinary large hydrophobic surface presented by the newly synthesized large subunit, proper folding requires the interaction with groEL/ES type chaperonins, such as the cpn60/cpn10 chaperonins in plants. It is not yet known what additional chloroplast factors are needed for proper assembly in plants, but it is reasonable to assume that some additional helper protein, similar to the RbcX assembly chaperone found in cyanobacteria (discussed above), is needed in order to form the large subunit (L8) core. In analogy with

what is known about the assembly process in cyanobacteria (Saschenbrecker *et al.*, 2007), the small subunit will step in at some stage to assist the process to assemble and concentrate the large catalytic subunits and to complete the assembly of L8S8 holoenzyme (in plants the small subunit itself depends on a transport system to enter the chloroplast following synthesis on cytosolic ribosomes). Once a functional enzyme has been assembled, catalysis (carboxylation or oxygenation) depends on proper activation by formation of a lysyl-carbamate that also plays a crucial role during several stages of catalysis. The activation process requires the help of Rubisco activase and several effector molecules. Information on the molecular level are still lacking, but as discussed above, several investigations point to a role for the carboxy-terminus of the large subunit in several of these processes.

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