The Plant Cell, Vol. 7, 809–819, July 1995 © 1995 American Society of Plant Physiologists

Rubisco Synthesis, Assembly, Mechanism, and Regulation

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INTRODUCTION

The great abundance of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in plants has afforded molecular biologists and biochemists many opportunities to study and attempt to understand various cellular processes. High levels of expression from nuclear *rbcS* and chloroplast *rbcL* genes have facilitated work ranging from gene isolation to studies on light regulation. The different cellular compartments used for Rubisco gene transcription and translation, together with import of the small (S-) subunits into chloroplasts for assembly with large (L-) subunits, enable the problems of cellular coordination of complex processes to be examined. The assembly of Rubisco holoenzyme itself has presented us with a new problem concerning the role of molecular chaperones in protein folding, and the enzyme has become a substrate for examining the mechanism of action of chaperonins and cochaperonins from mammals, fungi, viruses, and plants. Genetic engineering approaches are being used in an attempt to improve Rubisco activity, and site-specific mutagenesis, together with structural analyses, is giving us a fuller understanding of how the enzyme functions.

RUBISCO GENE ORGANIZATION AND EXPRESSION

The *rbcS* genes of higher plants are located in the nucleus and constitute a small multigene family (Berry-Lowe et al., 1982; Broglie et al., 1983; Dean et al., 1985) ranging from two to 12 members. Many of these genes are closely linked (Dean et al., 1985) and may have arisen from multiple gene duplications. *rbcS* genes contain one to three introns, encode mature proteins of ~120 amino acids, and are more divergent than the chloroplast-encoded *rbcL* genes. There are often substantial increases in *rbcS* expression after induction with light (Tobin and Silverthorne, 1985), and this is mediated by both phytochrome and blue light photoreceptors (Fluhr and Chua, 1986). Both positive and negative regulatory sequences are located in *cis*-acting transcriptional control regions, and these elements exhibit the types of complex interaction and sequence arrangement that are commonly found in the regulatory regions of eukaryotic genes (Okamuro and Goldberg, 1989). Light-responsive regulatory elements are positioned to the 5′ side of the transcriptional initiation site and possess at least two autonomous control sequences. The highest levels of mRNA are found in leaves, with transcription of *rbcS* also occurring in the photosynthetic tissues of stems, petals, and pods; mRNA is virtually undetectable in roots (Coruzzi et al., 1984). S-subunits are synthesized on free cytoplasmic polysomes as precursor molecules with an N-terminal transit peptide (Dobberstein et al., 1977). These precursors are post-translationally imported into chloroplasts in an ATP-dependent process, and the transit peptide is removed (Chua and Schmidt, 1978; Smith and Ellis, 1979).

The *rbcL* gene in higher plants is present as a single copy per chloroplast genome, but because many copies of the genome are present in each plastid, the actual *rbcL* copy number per chloroplast can be high. With rare exceptions, *rbcL* does not contain introns and encodes ~475 amino acids. For many chloroplast genes, including *rbcL*, the transcriptional (Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985) and translational (Gatenby et al., 1989) recognition sequences resemble those found in prokaryotic organisms to the extent that chloroplast *rbcL* genes can be readily expressed in *Escherichia coli* (Gatenby et al., 1981). Transcriptional initiation rates from the *rbcL* promoter are not only influenced by the promoter sequence but also modified by the nearby *atpB* promoter. The two promoters are positioned ~100 bp apart in opposite orientations, resulting in divergent transcription. They do not function independently, because RNA polymerase binding at the *rbcL* promoter interferes with binding and transcription from the *atpB* promoter, presumably by steric hindrance at the two RNA polymerase binding sites. Deletion of one promoter or increasing the distance between them eliminates this mutual interference (Bradley and Gatenby, 1985; Hanley-Bowdoin and Chua, 1987), which may be a control mechanism to regulate different levels of expression in chloroplasts.

ASSEMBLY OF RUBISCO SUBUNITS IN CHLOROPLASTS

After import into chloroplasts and processing, the mature S-subunits are assembled with plastid-synthesized L-subunits into the L_8S_8 Rubisco holoenzyme (Chua and Schmidt, 1978; Smith and Ellis, 1979). Although Rubisco L-subunits synthesized

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in isolated chloroplasts can assemble into holoenzyme, a significant proportion of L-subunits are also stably associated with a large oligomeric protein (Barraclough and Ellis, 1980). This oligomeric protein is >600 kD in size, contains subunits of 60 kD (Barraclough and Ellis, 1980; Hemmingsen and Ellis, 1986), and was later named chaperonin 60 (cpn60; Lubben et al., 1989). It was observed that as newly synthesized L-subunits assemble into Rubisco, the pool of L-subunits associated with cpn60 declines. This observation raised the possibility that nascent Rubisco L-subunits are specifically associated with cpn60 before assembly into holoenzyme and that the cpn60• L-subunit binary complex is an obligatory intermediate in the assembly of Rubisco (Barraclough and Ellis, 1980).

Other experiments have demonstrated that Rubisco L-subunits synthesized in vivo or in organello can be recovered from intact chloroplasts in the form of two different sedimentation complexes of 7S and 29S (Roy et al., 1982). The 29S complex contains unassembled Rubisco L-subunits associated with cpn60, and the 7S complex may represent Rubisco dimers. When chloroplasts are incubated in the light, it was found that the pool of newly synthesized L-subunits present in both the 7S and 29S complexes diminishes, and these subunits instead accumulate in assembled 18S Rubisco holoenzyme (Roy et al., 1982). The assembly of Rubisco is accelerated by ATP, but the 29S cpn60 oligomer remains intact (Bloom et al., 1983). However, in the presence of Mg^{2+}, ATP causes dissociation of the 29S cpn60 molecule, whereas a nonhydrolyzable ATP analog has no effect (Bloom et al., 1983; Musgrove et al., 1987; Roy et al., 1988). A complex set of reactions was proposed by Bloom et al. (1983) that requires nucleotides, Mg^{2+}, cpn60, and putative intermediates in the assembly of the Rubisco holoenzyme.

The initial examples of imported proteins associating with cpn60 were for Rubisco L- and S-subunits following their uptake into isolated pea chloroplasts (Ellis and van der Vies, 1988; Gatenby et al., 1988). The L-subunits of Rubisco from the cyanobacterium *Anacystis nidulans* were fused to soybean chloroplast transit peptides and, after import, were observed to interact with plastid cpn60 (Gatenby et al., 1988). This interaction is transient, and over time the number of Rubisco L-subunits associated with cpn60 decreases at a similar rate, as L-subunits become assembled into Rubisco holoenzyme. Similar kinetics are seen in the presence of chloramphenicol, indicating that active protein synthesis is not required for the interaction of imported L-subunits with cpn60 and their subsequent transfer into holoenzyme. These data are similar to results obtained by Barraclough and Ellis (1980), except that they studied L-subunits synthesized within chloroplasts. The two experimental approaches indicate that chaperonin interactions and Rubisco assembly follow similar kinetics, irrespective of whether L-subunits are in partially folded states after translocation through chloroplast membranes or are synthesized and released from ribosomes in the stromal compartment. Imported S-subunits of Rubisco are also associated with cpn60 and form stable complexes, although not to the same extent as observed for L-subunits (Ellis and van der Vies, 1988; Gatenby et al., 1988).

In addition to interacting with Rubisco subunits, chloroplast cpn60 can bind to many different imported proteins, and it clearly plays a general role in chloroplast biogenesis (Lubben et al., 1989). A cochaperonin protein has been identified and characterized in chloroplasts, where presumably it functions like other cochaperonins to effectively discharge target proteins bound to cpn60 (Bertsch et al., 1992; Baneyx et al., 1995). There have been no reports of imported or stromal synthesized Rubisco subunits binding to the chloroplast heat shock protein 70 chaperones, but this is clearly a possibility, given that other imported proteins can interact with heat shock protein 70 (Madueño et al., 1993; Tsugek and Nishimura, 1993).

CHAPERONIN-MEDIATED FOLDING AND ASSEMBLY OF RUBISCO IN VITRO

Experiments designed to examine the assembly of L_8S_8 Rubisco in isolated chloroplasts show that an initial binding step between cpn60 and newly synthesized or imported Rubisco subunits is detectable (Barraclough and Ellis, 1980; Roy et al., 1982; Bloom et al., 1983; Ellis and van der Vies, 1988; Gatenby et al., 1988). Chloroplast cpn60 is related to the GroEL protein from *E. coli* (Hemmingsen et al., 1988), and like chloroplast cpn60, the GroEL oligomer binds to newly synthesized L-subunits (Figure 1). When prokaryotic L_2 and L_8S_8 forms of Rubisco are synthesized in *E. coli*, successful assembly requires fully functional GroES and GroEL proteins (Goloubinoff et al., 1989a). The two GroE proteins are required for bacteriophage morphogenesis, and mutations in *groES* or *groEL* block virus assembly. These mutations also prevent the assembly of L_2 and L_8S_8 forms of prokaryotic Rubisco (Figure 2), an assembly block that can be suppressed by overexpression of the appropriate *groE* gene encoded on a compatible expression plasmid (Goloubinoff et al., 1989a).

To understand the mechanism of action of the GroE proteins on Rubisco folding, an in vitro refolding assay was developed that consists of purified GroES and GroEL proteins, MgATP, K^+ ions, and chemically denatured and unfolded dimeric Rubisco (Goloubinoff et al., 1989b). From many similar studies, and when using a range of unfolded target proteins, it is apparent that chaperonins regulate protein folding by stabilizing folding intermediates, thereby influencing the kinetic partitioning between aggregated (misfolded) and correctly folded proteins (Gatenby and Viitanen, 1994). The release of target proteins bound to cpn60, and subsequent progression to the native state, occurs through interactions with the cpn10 cochaperonin and MgATP. Spontaneous chaperonin-independent reconstitution of Rubisco at lower temperatures is inhibited by GroEL binding, which leads to the formation of a stable binary complex (Viitanen et al., 1990). Discharge of the cpn60•

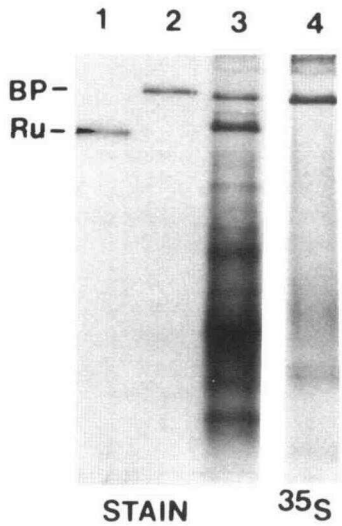

Figure 1. In Vitro Formation of a Stable Binary Complex between the Maize Rubisco L-Subunit and *E. coli* cpn60 (GroEL).

A cloned maize *rbcL* gene (Gatenby et al., 1981) was expressed in an *E. coli* cell-free coupled transcription/translation lysate in the presence of ^{35}S-methionine. Samples of the translation reaction mixture were analyzed by nondenaturing gel electrophoresis. Samples in lanes 1 to 3 were stained with Coomassie Brilliant Blue, and lane 4 is an autoradiograph of lane 3. A complex collection of stained protein bands can be seen in lane 3, as expected for an *E. coli* S30 lysate. In contrast, the autoradiograph (lane 4) of the stained sample shown in lane 3 reveals that most of the synthesized Rubisco L-subunits migrate as a discrete band near the top of the gel. The radioactive band in lane 4 does not have the same mobility as purified Rubisco (Ru; lane 1), but it does comigrate with pea Rubisco subunit binding protein (BP; lane 2), now referred to as cpn60. A band similar in mobility to cpn60 is present in the *E. coli* S30 lysate (lane 3), and subsequent experiments demonstrated that this band is the bacterial cpn60 (GroEL) to which newly synthesized Rubisco L-subunits have bound.

Rubisco complex, which then results in active Rubisco dimers, requires the cpn10 cochaperonin.

The requirement for a cpn10 cochaperonin in successful Rubisco folding depends on the folding environment. Two generic folding environments have been described: (1) a "permissive" environment, in which unassisted spontaneous folding can occur; and (2) a "nonpermissive" environment, in which spontaneous folding cannot occur (Schmidt et al., 1994). Spontaneous folding is generally achieved by one or more of the following variables: a reduction in temperature or protein concentration, an increase in salt concentration, or the presence of adjuncts. Under nonpermissive conditions, unassisted spontaneous folding of Rubisco cannot occur, and both cpn60 and cpn10 are required. However, under permissive conditions, cpn10 is not essential (Schmidt et al., 1994). Folding intermediates of Rubisco are highly prone to aggregation, and this off-pathway reaction is kinetically favored. To observe spontaneous refolding under permissive conditions, Rubisco concentrations are kept below the "critical aggregation concentration" (van der Vies et al., 1992). Chloroplast cpn21 effectively substitutes for bacterial cpn10 in the chaperonin-facilitated refolding of denatured bacterial Rubisco (Baneyx et al., 1995).

RUBISCO STRUCTURE

There are two structural forms of Rubisco based on subunit composition. The simplest Rubisco found in some photosynthetic bacteria is a dimer of L-subunits and designated form II. The organisms that rely on this form for CO_2 fixation, such

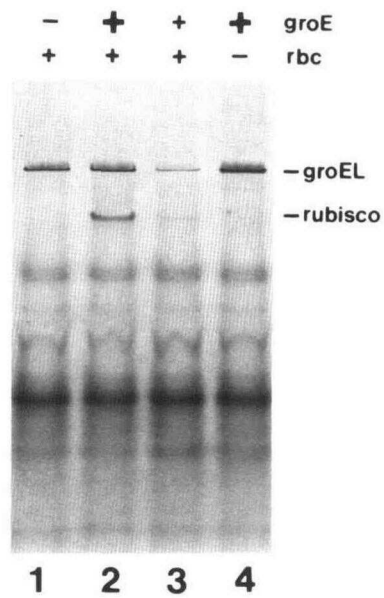

Figure 2. The Influence of Molecular Chaperones on in Vivo Folding and Assembly of *Synechococcus* L_8S_8 Rubisco in *E. coli.*

Bacterial cells transformed with various combinations of compatible *groE* or *rbc* expression plasmids were grown to log phase, disrupted by sonication, and analyzed by electrophoresis on a nondenaturing gel. Proteins were stained with Coomassie Brilliant Blue, and the positions of the GroEL tetradecamer and Rubisco hexadecamer are marked on the right border. The relative amounts of GroE and Rubisco proteins are indicated (+ ,+, or –) at the top of each lane. Lane 1 is from cells expressing *rbcL*, *rbcS*, and *groEL* from plasmids but not *groES*, and Rubisco fails to assemble. Lane 2 cells are expressing *groEL* and *groES* at high levels from a plasmid, and efficient assembly is therefore obtained from the expressed *rbc* operon. Lane 3 shows the result of lower levels of *groE* expression from the chromosomal operon, with a resulting reduction in Rubisco assembly. Lane 4 is a control that does not express either *rbcL* or *rbcS* but overexpresses the *groE* operon. Rubisco was synthesized using plasmid pANK1 (lanes 1 to 3), GroEL and GroES were from plasmid pGroESL (lanes 2 and 4), and GroEL alone (lane 1) was from plasmid pGroEL (Goloubinoff et al., 1989a).

as the nonpurple S bacteria, are unable to exist in present atmospheric concentrations of CO_2 because molecular oxygen inhibits the carboxylase reaction. Other bacteria that have a dimeric form of the enzyme have survived the increased levels of oxygen in the atmosphere because they are able to switch to a more efficient form I enzyme, particularly under conditions in which low CO_2 prevails. Form I Rubisco is composed of two different-sized subunits. In addition to eight L-subunits, the enzyme requires an equivalent number of S-subunits to be fully active. Although the quaternary structures of the two forms are apparently very distinct, they are nevertheless related by the domain structure of the larger subunit and the nature of the subunit interactions.

The first structural models of Rubisco based on diffraction data were of the dimer from *Rhodospirillum rubrum* (Schneider et al., 1986, 1990a). These provided information about the organization of domains that constitute the L-subunit and confirmed that the active site of the enzyme is shared between elements of both subunits of the dimer (for review, see Hartman and Harpel, 1993). Therefore, although there are two active sites per dimer, amino acids essential for the function of each site are located on both L-subunits, constraining the minimum functioning size to a dimer. Most of the active-site residues are located on loops of an eight-stranded barrel domain that is formed by the C-terminal two-thirds of the L-subunit sequence. The N-terminal third is folded into a second domain composed of strands surrounded by helical segments. Some of the amino acids essential for catalysis are located on loops of this domain.

In form I Rubisco, the L-subunits come together as an eight-subunit core organized as a tetramer of dimers distributed around a fourfold axis of symmetry. Four S-subunits are located close to each pole of this axis, situated between and making extensive interactions with the L-subunit dimers to stabilize the L_8 core (Chapman et al., 1988; Andersson et al., 1989; Knight et al., 1990). The S-subunits do not contribute to the structure of the active site directly, but, through contacts to elements that form the site, they do influence substrate affinities and turnover (Andrews, 1988; Schneider et al., 1990b; Gutteridge, 1991).

THE RUBISCO ACTIVE SITE

The active site is formed from elements of the C-terminal barrel domain of one L-subunit of the dimer and the N-terminal domain of the second L-subunit of the dimer. In the inactive enzyme, the site is open and accessible to activating cofactors and bisphosphate substrate (Curmi et al., 1992). After formation of the essential carbamate and coordination of the Mg^{2+} (see following discussion), ribulose-1,5-bisphosphate substrate binds and a series of loops close over the site to enfold and capture the bisphosphate (Knight et al., 1990; Newman and Gutteridge, 1993). Closure of the loops brings together amino acids that are critical for catalysis and determine the fate of the substrate. Figure 3 shows the loops where these amino acids reside.

The region of the barrel that comprises the active site is at the C-terminal end of the eight β-strands that form the core of this domain. The loops that connect the strand and helical elements of the barrel extend above and over the surface of the domain, contributing the amino acids that form the 1- and 5-phosphate binding sites and an extensive hydrogen bonding network with the sugar backbone of ribulose-bisphosphate.

REACTIONS CATALYZED BY RUBISCO

An obligatory first step before catalysis can proceed is activation of the enzyme involving carbamylation of an active site Lys (Lys-201 in spinach Rubisco) residue by CO_2 (Lorimer and Miziorko, 1980; Lorimer, 1981). The presence of the carbamino group in close proximity to two adjacent acidic residues, Asp-203 and Glu-204, provides a site for the essential Mg^{2+} ion

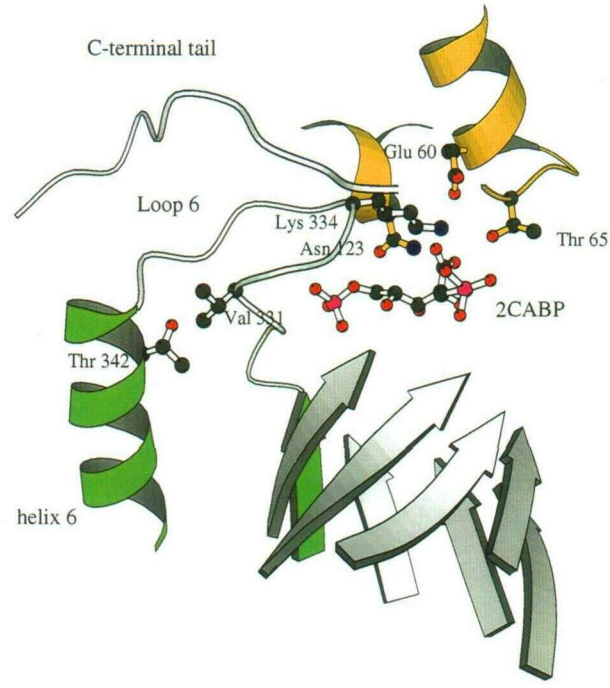

Figure 3. Loops of the C-Terminal and N-Terminal Domains of the L-Subunit of Rubisco That Close over the Active Site after Binding Bisphosphate.

The amino acids that affect the partitioning of ribulose-bisphosphate between carboxylation and oxygenation are shown. The active site is occupied with the intermediate analog 2CABP (Newman and Gutteridge, 1993). This view of the *Synechococcus* Rubisco quaternary complex is illustrated using Molscript (Kraulis, 1991). Green has been used to highlight the strand, loop, and helix of element 6 of the C-terminal barrel, and yellow highlights the N-terminal elements.

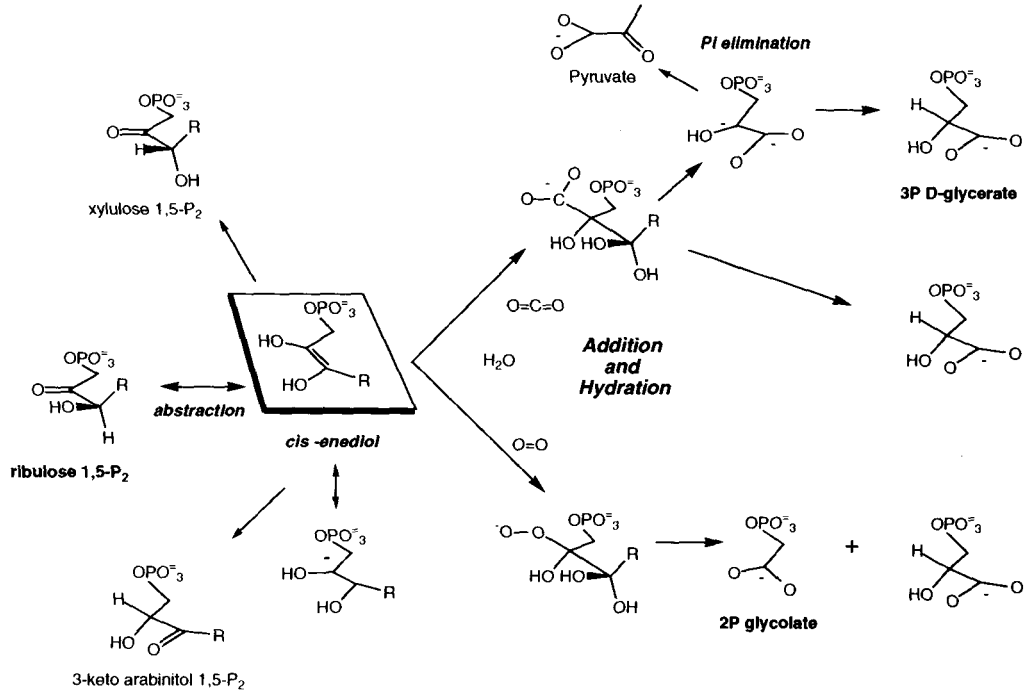

Figure 4. The Reactions Catalyzed by Rubisco.

The first intermediate of catalysis is the C2,C3 *cis*-enediol form of ribulose-bisphosphate (ribulose 1,5-P_2) after abstraction of the C3 proton. The enediol can partition a number of ways, the majority into the products of carboxylation (upper reactions) or oxygenation (lower reactions). However, a number of misprotonated isomers of ribulose-bisphosphate, for example, xylulose-bisphosphate, have been detected with the wild-type enzyme that are produced in quantity by mutations of specific amino acids involved in proton transfer. Phosphate elimination of the carbanion forms of intermediates are also produced by some mutants (see Morrell et al., 1994). R, -CHOH-$CH_2OPO_3^=$; 3P D-glycerate, 3-phosphoglycerate; 2P glycolate, 2-phosphoglycolate.

to bind. Coordination of the metal does not complete the active site but simply positions the carbamate and Mg^{2+} relative to those centers of the bisphosphate substrate, namely, the C2 carbonyl oxygen and C3 hydroxyl, that are involved in the catalytic events. With substrate bound in the correct orientation (Lorimer et al., 1989), the C2 and C3 oxygen atoms complete the coordination sphere (Gutteridge and Lundqvist, 1994).

The catalytic mechanism of the carboxylation of ribulose-bisphosphate can be depicted as five discrete partial reactions (Andrews and Lorimer, 1987). The first step has been established as the formation of an enediol intermediate of the bisphosphate substrate that is catalyzed by the enzyme in the absence of the second substrates, CO_2 or O_2 (see Figure 4; Gutteridge et al., 1984a; Pierce et al., 1986). During carboxylation, the C2,C3-enediol reacts with CO_2 at the C2 position, forming a six-carbon intermediate (Schloss and Lorimer, 1982) that is hydrolytically cleaved to two molecules of 3-phosphoglycerate (3P-glycerate). The reaction thus results in a net gain of carbon, which ultimately, via the other reactions of photosynthesis, flows into usable sugars. The enediol intermediate, however, is also susceptible to reaction with molecular oxygen, forming a hydroperoxy derivative that breaks down to 2-phosphoglycolate (2P-glycolate) and 3P-glycerate (Lorimer, 1981). This process not only consumes the bisphosphate and results in no net gain of carbon but also requires the plant to consume energy recycling the lost carbon back into useful metabolites. The intense interest in the structure and mechanism of the enzyme has been stimulated by the fact that the enzyme might be altered in ways that favor carboxylation and reduce, if not remove, the oxygenase reaction. The proposal is strengthened by surveys of the enzyme from a number of different photosynthetic organisms, which indicate that partitioning of the substrate between the two reactions can vary by an order of magnitude (Jordan and Ogren, 1981; Parry et al., 1989).

STRUCTURAL ELEMENTS INVOLVED IN THE REACTION MECHANISM

The structures of Rubisco now available have provided an opportunity to understand the chemistry of the various reactions

in terms of the groups that catalyze the partial reactions (see Gutteridge and Lundqvist, 1994). Previous speculation suggested not only that the metal plays an essential role at each step of the cycle but also that a group acting as a base is involved in many of the proton movements between substrate and intermediates (Gutteridge and Gatenby, 1987). Based on the structure of the *Synechococcus* Rubisco with the intermediate analog 2′-carboxy arabinitol 1,5-bisphosphate (2CABP) at the active site, Newman and Gutteridge (1993) proposed that a carbamino oxygen atom of the active site Lys acts as the base that is the acceptor/donor of protons and therefore leads to the formation of the various intermediates of the partial reactions.

Binding of the bisphosphate in the correct orientation induces specific elements of the N-terminal domain of the second L-subunit of the dimer to close over the substrate (Figure 3). At least three amino acids of this domain, Glu-60, Thr-65, and Asn-123, form part of the active site and are involved in these events. Closure of the loops positions the reactive centers of the ribulose-bisphosphate, that is, C2 and C3, so that the hydroxyls are ligated to the metal. Solvent molecules are expelled from the site, except for those water molecules associated with the 1- and 5-phosphate binding sites and a substrate water poised close to the C3 center of the bisphosphate required for the hydration reaction of the cycle. In this configuration and in the absence of CO_2 or O_2 substrate, the enzyme is still capable of performing the first partial reaction of the catalytic cycle, that is, abstraction of the C3 proton to form an enediol intermediate. With both C2 carbonyl oxygen and C3 hydroxyl oxygen coordinated to Mg^{2+}, the C3 proton is in close proximity to the carbamino oxygen that is not ligated to the metal. The combined effect of the Mg^{2+} polarizing the C2 carbonyl and the proximity of a carbamino oxygen positioned to accept the proton favors the formation of the C2–C3 enediolate. The intermediate is most likely further stabilized by protonation of the C2 oxygen, possibly with the proton from C3 that is on the carbamino group. If the carbamino group loses the proton to the C2 oxygen, it is now available for the next step of catalysis.

The ability of one of the oxygens of the carbamino group to be ligated to Mg^{2+} and the ability of the other to mediate proton movements to and from the substrate are reasonable because a resonance form of the carbamate can exist with positive density on the nitrogen and negative density distributed over both oxygens (Jabri et al., 1995; W.W. Cleland, personal communication). For example, the carbamate required at the binuclear Ni^{2+} center of urease (Park and Hausinger, 1995) acts as a bridging ligand between both metal ions, with each oxygen atom of the carbamino group ligated to a Ni^{2+}. As shown in Figure 5, a similar resonance form in Rubisco would be stabilized by hydrogen bonding of the carbamino N to the main chain carbonyl of Asp-202 that is in close proximity (2.9 Å).

The reaction involving the addition of CO_2 to the C2 position of the enediolate is not fully understood. What is clear is that with the loops in the closed position over the intermediate, only small molecules such as CO_2 and O_2 can gain access

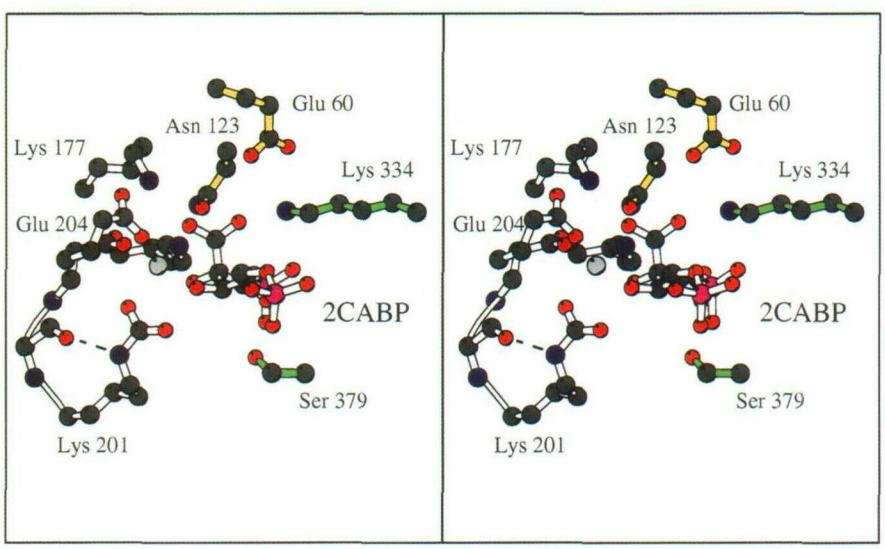

Figure 5. A Stereo View of the Active Site of *Synechococcus* Rubisco.

The amino acids that constitute the active site of Rubisco have different roles. Asp-203, Glu-204, and carbamate of Lys-201 are involved in binding Mg^{2+} (gray sphere). Others, such as Ser-379, make hydrogen bonds to hydroxyls of the backbone of bisphosphate; a third set, which includes Lys-334, Glu-60, and Asn-123, is essential for CO_2 or O_2 addition; and yet others, such as Lys-177, stabilize this framework through an extensive hydrogen bonding network. This view is from the 5-phosphate end of 2CABP into the plane of the paper. Lys-175, another residue involved in enediol formation, and Asp-203 are toward the back of the active site. Green denotes those residues of element 6 of the C-terminal domain, and yellow denotes the N-terminal residues (see Figure 3).

to the enediolate. The organization of the loops is such that they form a funnel that "points down" into the active site directly above the position occupied by the C2 carbon and the Mg^{2+} ion (Knight et al., 1990; Newman and Gutteridge, 1993). The ε-amino group of Lys-334 is well positioned relative to the metal for both to polarize the two oxygens of the CO_2 molecule. The electrophilic carbon of CO_2 is now poised just above the C2 center of the enediolate. The resulting addition reaction produces the hydrated form of the six-carbon intermediate, 2′-carboxy, 3-keto arabinitol 1,5-bisphosphate (see, for example, Cleland, 1990).

The penultimate step to product formation is cleavage of the carboxylated intermediate. This is accompanied by a number of events. The first molecule of 3P-glycerate originating from the lower half of the intermediate can now be released from the site. Presumably, breaking the C2–C3 bond is the signal for the active site loops to begin to open. However, there is still chemistry to be completed; the upper three carbons are in the form of a carbanion that must be stereospecifically protonated to generate the second and correct isomer of 3P-glycerate. This final step cannot involve the carbamino group because of distance constraints, and therefore a second base is required to complete this last protonation. Release of the second molecule leaves the active site loops in the more open conformation and ready to accept another molecule of ribulose-bisphosphate.

MUTANT FORMS OF RUBISCO

Site-specific mutations of the dimeric enzyme from *R. rubrum* and the hexadecamer from *Synechococcus* have been generated to determine the function of specific amino acids. Without the benefit of a crystallographic structure, some of these endeavors were more successful than others (Gutteridge et al., 1984b; Lorimer et al., 1988). However, even with excellent structural models, the multistep nature of the reaction cycle requires that the same critical residues be involved in some capacity at each step. This has complicated the interpretation of mutagenesis studies. It is now clear that the active site is organized with essential residues not only within the active site but also at some distance from the action. For example, those closest to the C2 and C3 centers of the bisphosphate substrate are critical for overall activity, and alterations at these positions ensure that one or more of the partial reactions are inhibited. Because nearly all the steps involve the movement of protons to and from substrate and intermediates, the effects of mutations are best revealed by analysis of the misprotonated products that are produced (see, for example, Morrell et al., 1994).

Other site-specific mutants have confirmed the multistep nature of catalysis through dissection of the cycle into its individual steps. Thus, a second set of amino acids, such as Lys-334, has been identified that is essential for interaction with the gas substrates. The role of the ε-amino group of this residue is to act in concert with the metal to activate CO_2 and stabilize the carboxylated addition product once formed. It might be expected that replacement with other residues would influence the partitioning of ribulose-bisphosphate into carboxylation or oxygenation processes. This was confirmed when Lys-334 was replaced with Arg to retain the basic nature of the residue but alter side-chain size. The mutant proved to have little carboxylase activity, partitioning the bisphosphate into oxygenation with a turnover rate similar to that of the wild-type enzyme (Gutteridge et al., 1993).

More distant amino acids are of two general types. The first are those that directly interact with the residues that compose the Mg^{2+} binding site, that is, Asp-203 and Glu-204. The side chains of these acidic residues interact with the side chains of basic residues Lys-175 and Lys-177, respectively (see Figure 5). Lys-334, the residue critical for addition of CO_2, interacts directly with an acidic residue, Glu-60 of the N-terminal domain. Changes at this residue and the two others of the N-terminal domain that form the active site, Thr-65 and Asn-123, also affect partitioning of the enediol (Smith et al., 1990; Chene et al., 1992; Morell et al., 1994). Each of the residues in this "secondary sphere" affects metal binding and its interaction with the C2 carbonyl of the substrate, is involved in shuttling protons at specific steps of catalysis, or interacts with residues that stabilize reaction intermediates. Finally, those amino acids that are critical for the correct movement of loops that close over the bisphosphate to form the active site (see, for example, Spreitzer, 1993) also influence all aspects of the reaction cycle. Most interestingly, one of the obvious differences between the structures of spinach and *Synechococcus* Rubisco is the location of loop 6 of the barrel domain that positions Lys-334 into the site. Replacement of the residues that influence the movement of this loop in *Synechococcus* Rubisco with those of the higher plant altered the partitioning of ribulose-bisphosphate, in some cases positively (that is, toward carboxylation) (Parry et al., 1992; Gutteridge et al., 1993).

REGULATION OF RUBISCO ACTIVITY

From investigations of Rubisco structure, particularly of the nature of the interactions with substrate and intermediate analog, the influence that natural effectors may have on enzyme actvity in vivo has become more understandable. 2′-carboxy arabinitol 1-phosphate (2CA1P) is a naturally occurring inhibitor that resembles 2CABP. It accumulates in the dark and in low-light conditions, binding to the activated form of the enzyme (Gutteridge et al., 1986; Berry et al., 1987). Presumably in the plant, as photosynthesis slows in shade or dark conditions, this intermediate analog stabilizes the activated state of the enzyme. The absence of the 5-phosphate reduces the affinity of the analog by some four orders of magnitude compared with 2CABP, ensuring that it dissociates from the active site long enough to be degraded by a specific phosphatase

(Gutteridge and Julien, 1989; Holbrook et al., 1989). Presumably, the process of release is assisted by interaction of the inhibited form of Rubisco with activase (Portis, 1990).

A second natural inhibitor is a substrate analog, xylulose-bisphosphate, which differs from ribulose-bisphosphate only in the organization of the hydroxyl and proton around C3. This is a misprotonated product of the enediol that occurs during turnover approximately once every 400 cycles. In the higher plant enzyme, the affinity for this analog is so high that catalysis is irreversibly inhibited. A structure of the *Synechococcus* enzyme with the inhibitor bound at the active site confirmed that a binary complex with all the loops closed over the active site is the preferred state (Newman and Gutteridge, 1994; see also Zhu and Jensen, 1991). Furthermore, the loops are stabilized in the closed position by contacts to the hydrated form of the analog. In the case of the higher plant enzyme, photosynthesis is not affected adversely by the inhibition because activase also influences the affinity for this bisphosphate so that it is released from the active site. After inhibitor release, the enzyme must reactivate with CO_2 and Mg^{2+} before catalysis can proceed.

In the inactivated state, the enzyme is also susceptible to inhibition by the natural substrate, ribulose-bisphosphate, which often accumulates to high levels. Presumably, it is the hydrated form of the substrate that also inhibits both activation and catalysis by closing off access to the site by activating cofactors. One positive consequence of these naturally occurring stable binary states might be the protection of flexible elements from proteolysis during periods of low photosynthetic activity.

FUTURE DIRECTIONS

Although we now know many of the details of chaperonin interactions with folding intermediates and how these interactions facilitate protein folding (Gatenby and Viitanen, 1994), the folding and assembly of the L_8S_8 form of Rubisco from higher plants are still elusive (Gatenby et al., 1987; Gatenby and Ellis, 1990). This failure reflects our incomplete understanding of how this major component of the photosynthetic pathway in chloroplasts is assembled; it is a puzzle because the analogous L_8S_8 Rubisco from cyanobacteria is assembled in *E. coli* (Gatenby et al., 1985) using the GroE chaperonins (Goloubinoff et al., 1989b). Even with the knowledge that chaperonins facilitate cyanobacterial Rubisco assembly in *E. coli*, reconstruction of the folding pathway in vitro in the presence of chaperonins has failed to yield a functional hexadecamer (A.A. Gatenby, unpublished data). The problem appears to reside at the stage of L-subunit folding and assembly into a soluble core octomer; indeed, the insolubility of plant Rubisco L-subunits is well known (Gatenby, 1984). Once a stable L_8 core is formed from plant subunits, the binding of eight S-subunits to the core should proceed relatively well because it is known that plant S-subunits remain soluble when expressed in *E. coli* (Gatenby et al., 1987), and they also possess the ability to bind to a cyanobacterial L_8 core in vivo to give a functional hybrid Rubisco enzyme (van der Vies et al., 1986). Clearly, many problems remain to be solved in our understanding of the biogenesis of proteins in photosynthetic organisms and of the ways this process is influenced by molecular chaperones.

Since a previous review by Gutteridge and Gatenby (1987) about the assembly and function of Rubisco appeared, our understanding of the mechanism has grown enormously as a result of the structures that are now available. The chemistry underlying the partial reactions of catalysis and the fate of intermediates can now be described in terms of that most important participant—the enzyme. Ultimately, the basis for variation in the catalytic events shown by different forms of Rubisco will be understood, and the most desirable features, such as improved carboxylation and turnover, will be introduced into the enzyme of crop plants (see Gutteridge, 1990). To ensure the success of these structural manipulations, some means of altering the L-subunit gene of the chloroplast will be required. Recent experiments have shown that the L-subunit is an attainable target for specific mutagenesis in vivo (I. Kanevski, P. Maliga, D.F. Rhoades, and S. Gutteridge, unpublished data).

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