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Review

Limitations of the primary events of CO_2 fixation in photosynthetic organisms: the structure and mechanism of rubisco

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Contents

I. Introduction

The realisation that in all photosynthetic organisms ribulose-P_2, the product and precursor of photosynthetic C-fixation is degraded by an oxygenation reaction, highlighted a severe limitation in the productivity of photosynthesis. Furthermore, the discovery that both carboxylation and oxygenation of ribulose-P_2 were catalysed by rubisco [1], provided a rational explanation for the competitive effects of CO_2 and O_2 on plant productivity (see Ref. 2). That one enzyme may dictate the ultimate yield of many agronomically important plants has been the reason for the intense interest in understanding the mechanism of the activity of rubisco in considerable detail.

Abbreviations: 2CABP, 2′-carboxyarabinitolbisphosphate; L subunit, large subunit; S subunit, small subunit.

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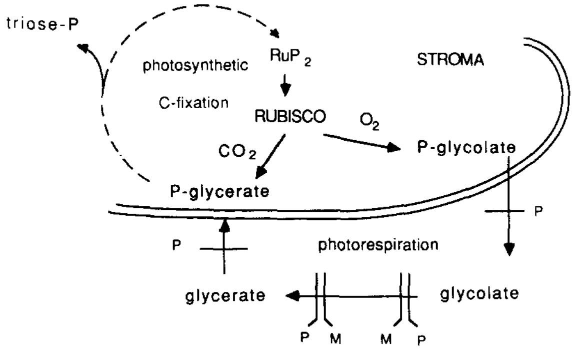

Fig. 1. The partitioning of ribulose-P_2 rubisco into photosynthetic carbon fixation or oxygenation and subsequent photorespiration. The products of carboxylation are utilised to produce triose-P and more ribulose-P_2 within the stroma of the chloroplast. The product of oxygenation, 2-P-glycolate has to be rescued by a complicated photorespiratory cycle that involves the metabolic processes of the peroxisome (P), mitochondria (M) and peroxisome (P) again. One of the steps in the mitochondria converting glycolate to glycerate results in the loss of carbon as CO_2.

Rubisco isolated and purified from all photosynthetic organisms catalyses to a greater or lesser extent, both carboxylation and oxygenation. All rubisco species isolated so far, require activation by carbamylation with CO_2 and co-ordination of Mg^{2+} [3] before either catalytic reaction can proceed. Based on the kinetic parameters of the fully activated higher plant rubisco and in normal atmospheric concentrations of the two gases, about 80% of the substrate ribulose-P_2 is committed to carboxylation initiating photosynthetic carbon-fixation (Fig. 1) and the generation of two molecules of 3-P-glycerate. The fraction that is oxygenated produces one molecule of 3-P-glycerate and 2-P-glycolate with no net gain of carbon. In reality, two processes add to the limitation. First, 25% of the carbon of 2-P-glycolate that is rescued through photorespiratory processes is lost as CO_2 [4]. Secondly, in the plant diffusion limitations of CO_2 transport ensures that a more realistic partitioning is about 70% and 30% between the two reactions. There may also be further diversion of substrate through oxygenation due to locally high concentrations of O_2 from Photosystem II complex during active photosynthesis.

That the oxygenase reaction causes a severe drain of carbon in the plant has been well demonstrated by the positive growth response of many C3 plants to increased atmospheric CO_2 (see, e.g., Ref. 5). The relief of this limitation wrought by increased CO_2 is based on the pure competition of the gaseous substrates at the active site of rubisco [6] diverting less of the ribulose-P_2 that normally partitions through the oxygenase reaction. Furthermore, the ability of photosynthetic organisms to survive in present atmospheres of CO_2 and O_2 is directly dependent on the rates of the competing reactions of carboxylation and oxygenation of ribulose-P_2. Too much oxygenation relative to carboxylation means that not enough C-fixation is possible to even maintain adequate amounts of the substrate ribulose-P_2.

At the present concentrations of atmospheric CO_2 and O_2 (0.03% and 21%, respectively), for there to be any net gain of fixed carbon, the ratio of the two activities (expressed as the ratio of the specificity factors for carboxylation relative to oxygenation) must be about 20 (see subsection IIC.) assuming diffusional limitations on the availability of CO_2. A survey of the relative specificities of the enzyme from a wide range of photosynthetic organisms from bacteria to higher plants [7–9] indicates that some of the procaryotic organisms, e.g., *Rhodospirillum rubrum*, are restricted to existing in anaerobic conditions or where CO_2 is unusually high. Nevertheless, photosynthetic organisms have contrived means of surviving atmospheres that have become progressively higher in O_2 because of dramatic changes to the structure of rubisco. How these changes have increased the relative specificity of the enzyme and how the protein might be modified in vitro to obtain an enzyme with minor oxygenating ability are the subjects of this review.

II. The structure and specificity of different forms of rubisco

IIA. The large subunit

Two forms of rubisco have been isolated from photosynthetic organisms. Structurally the simplest or form II obtained from some photosynthetic bacteria is composed of two identical subunits [10]. The subunit has a molecular weight of about 52000 and is designated the large (L) subunit. The most studied source of this form of the enzyme is *Rhodospirillum rubrum*, M_r 52435, and composed of 466 amino acids, although no longer routinely obtained from the host organism. The gene coding for the L-subunit has been isolated [11,12] and cloned into high copy number plasmids that are expressed efficiently in *E. coli*. Gramme quantities of this rubisco can be obtained easily from the bacterium [13] and was the source of the enzyme utilised to determine the first crystallographic structure of rubisco [14].

The amino acid sequence for one other form II rubisco has been determined for the enzyme from *Rhodobacter sphaeroides* [15], a carboxylase with a lower carboxylation efficiency than *R. rubrum* rubisco. The sequence of the *Rb. sphaeroides* L-subunit is about 77% homologous with the *R. rubrum* enzyme. The size of the functional enzyme is variable depending on pH, ranging from L_2 to an aggregate of at least L_8 [16]. *Rh. sphaeroides* is unlike *R. rubrum* in that it does not have to rely on this form of rubisco as the sole source of fixed carbon. If growth conditions change, e.g., limited availability of CO_2, the organism responds by synthesising the more complicated and more efficient form I

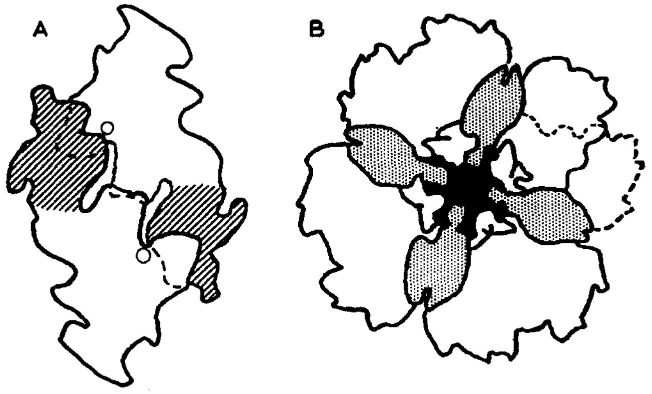

Fig. 2. The organisation of the subunits of the two forms of rubisco. (A) The form II enzyme of *R. rubrum* is composed of only one subunit, the L-subunit that exists as a dimer. Amino acids of the N-terminal domain (shaded) contribute to the structure of the active site and the C-terminal domain of the other partner, thus the dimer is the minimum functioning unit [14]. The position of the active site is marked by the circle. A similar organisation of the L-subunits can be recognised in the form I enzyme from spinach [18] where the L_8 core is essentially a tetramer of L dimers around a four-fold axis of symmetry, capped at either end by S-subunits (shaded). Looking from above the 4-fold axis, (B) shows the organisation of one set of S-subunits relative to the L_8 core. Each S-subunit has contact with two L-subunits and a second S-subunit and extends over and down into the prominent central depression of the L_8 core. The dashed line shows the relative position of a L-subunit within one of the dimers.

carboxylase that is composed of 8 L and 8 S subunits [17]. The ability of this organism to choose between rubisco forms depending on growth conditions supports the notion that for photosynthetic organisms to survive in an environment of increasing O_2, then the simpler form II rubisco is inadequate and may be unable to evolve into an enzyme with the catalytic properties required for fixing adequate amounts of CO_2.

The enzyme that emerged with the appropriate catalytic characteristics was dramatically different. The form I species is composed of a L subunit that resembles the form II subunit over only 35% of its sequence and aggregates into an L_8 core. A second protein, the S subunit has been conscripted to complete the transition to an enzyme that could be adopted by nearly all photosynthetic organisms. The advantages that accrued not only included increased carboxylation efficiency, but also the ability of the enzymes, at least those in higher plants to activate at relatively low concentrations of CO_2. This is a necessary requirement of rubisco of, for example C3 plants, that are unable to concentrate CO_2 internally near the site of CO_2 fixation.

The present view of the structure of the form I and II enzymes (see Fig. 2) has advanced recently by the determination of the crystal structure of spinach [18], tobacco [19] and *R. rubrum* rubisco [14]. The 8 L subunits of the form I enzyme are organised around a 4-fold symmetry axis capped at either end by 4 S subunits. The resolution of the crystal data for the more complicated enzyme, very much aided by the solutiuon of the structure of the simpler *R. rubrum* enzymes, indicates that the L_8 core might be viewed as a tetramer of L_2 dimers with the S subunits covering and interposed between the C-terminal ends of each dimer [18].

IIB. The small subunit

Assessing which particular change to the structure of rubisco has affected carboxylation efficiency can be investigated if the heterologous complex of L and S subunits of the form I enzyme were dissociable. Rubisco from *Synechococcus* PCC6301 has just these characteristics. In moderately acid conditions approaching the p*I* for the enzyme, *Synechococcus* rubisco dissociates to an L_8 core and individual S subunits with a concomitant loss of activity [21–23]. The isolated L_8 core is still receptive to the presence of S subunit, because with the addition of the latter the L_8S_8 complex reforms at the usual pH values and the activity recovers.

Although the majority of the S subunit can be removed from the aggregate with this treatment, it cannot be demonstrated that the residual activity associated with the L_8 core might be due to trace S subunit remaining in the core preparations. However, the genes coding for the L and S subunit of *Synechococcus* rubisco have been isolated and expressed separately in *E. coli* to investigate this further.

The *Synechococcus* rubisco gene is dicistronic coding for both the L and the S subunit [24] (see subsection IV B-1). The L subunit is the first gene in the operon downstream of the promoter with the S subunit gene 3′ to the end of the L subunit and separated by 94 bases. Incorporation of the structural genes behind the *lac* promotor in a high copy number plasmid such as pUC18 allows acceptable quantities of the intact L_8S_8 enzyme to be synthesised constitutively and isolated from transformed *E. coli* [25,26]. Derivatives of this construct that are missing either the S or L subunit gene offer a means of synthesising the two subunits independently of one another. The L subunit thus expressed adopts the L_8 core structure that can indeed be reactivated with isolated S subunit (Ref. 27 and Gutteridge, S., unpublished data). It can be concluded from studies of the subunits in isolation that the L_8 core does have both carboxylase and oxygenase activity at about 0.6% of the rate of the holoenzyme. The specificity factor of the L_8 core is almost identical with that determined for the intact enzyme. The S subunits that are relatively insoluble in the absence of the L_8 core, impart some conformational change on the core that affects both ribulose-P_2 consumption and binding but does not affect partitioning of the substrate between the two reactions (Ref. 27, see also Ref. 23).

TABLE I

Species	Form	τ	V/K [a]
C3 plants	I	80–100	15–20
C4 plants	I	70– 90	14–18
Green algae	I	60– 70	
Photosynthetic	I	50– 60	6– 8
bacteria	II	10– 20	6– 8

[a] V/K values are for the carboxylase activity in units $\times 10^4$ $M^{-1} \cdot s^{-1}$.

IIC. *Differences in relative specificity*

The advantage of the more complicated form I enzyme and thus its wide occurrence amongst photosynthetic species has been the huge increase in CO_2 fixing ability it afforded. A comparison of the specificities of carboxylation relative to oxygenation, obtained as the ratio

$$\frac{V_c}{K_c} \cdot \frac{K_o}{V_o}$$ (28)

and providing a measure of the 'relative specificity' of the enzyme, readily indicates the superiority of the higher plant enzyme (see Table I). However, the surveys of the kinetic characteristics of various rubisco species [7–9] indicates that although relative specificity has changed, the improvement may have been acquired at some cost. There is a factor of 10 difference comparing the worst example with the best in relative specificity. However, those enzymes that exhibit the best specificity have the worst turnover rates. For example, *Synechococcus* rubisco has a lower specificity than the spinach enzyme but catalyses the reaction about four times faster in saturating concentrations of substrate. The structural basis for these differences, particularly whether they are linked, is still unknown and is the present stimulus for in vitro manipulation of the protein. If the determinants of these variations can be defined then there is the possibility of altering rubisco specificity.

As a prelude to achieving these aims some considerable effort has been applied to understanding the details of the reactions catalysed by rubisco, particularly the carboxylase reaction.

III. The mechanism of rubisco activity

The isolation and cloning of the gene for the L subunit of the form II rubisco of *R. rubrum* finally settled a question concerning the bifunctionality of the enzyme, namely that *R. rubrum* rubisco isolated from *E. coli* catalysed both carboxylation and oxygenation of ribulose-P_2 with the same relative specificity as the wild-type enzyme [13]. Indeed, it is now clear that both reactions occur at a common active site involving a common intermediate of the bisphosphate substrate. The equilibria leading to the formation of active enzyme and the reactions that generate product are shown in Fig. 3.

IIIA. *Activation reactions*

To attain catalytic competency the enzyme has first to react with a molecule of CO_2 that carbamylates a specific lysine residue of the L subunit [30–32]. The second step of this activation process is the coordination of the essential magnesium ion (Fig. 3A) to the carbamate and other residues at the active site. All species of rubisco that have been isolated require activation with CO_2 and metal prior to catalysing either carboxylation or oxygenation. Unlike catalysis, many metals can substitute for the Mg^{2+} and stabilise the carbamate, a phenomenon readily exploited for spectroscopic probing of the active site [33,34,46] and localisation of the metal in crystals of the enzyme [35]. Although all species of rubisco conform to the above processes of activation some differ in detail. For example many higher plant rubisco species undergo a conformational change in addition to carbamylation that is required before catalysis can proceed [36–38]. In these cases binding studies with the tight binding intermediate analogue 2′-carboxyarabinitolbisphosphate (2CABP) indicate that the structural changes involve the formation of the bisphosphate binding site [39].

IIIB. *The partial reactions of catalysis*

Spectroscopic studies (Refs. 40 and 41 see also Ref. 42) have revealed that the first sep of catalysis is the binding of ribulose-P_2 to the activated enzyme. Abstraction of the C3 proton at the active site converts the bisphosphate to the 2,3-enediol in the absence of either O_2 or CO_2 substrates (Fig. 3B). Molecular oxygen or CO_2 then compete for the enediol at the active site without the apparent requirement to form a Michaelis intermediate with the enzyme. Once the gaseous substrates have reacted with the enediolate intermediate to form the 3-*keto*-2′-carboxy [20] or -hydroperoxyarabinitolbisphosphate intermediate [43], the enzyme is committed to forming a product. A further three steps, hydration, cleavage and protonation, generates the two molecules of 3-*P*-glycerate of carboxylation [44]. Hydration of the 3-*keto*-2′-hydroperoxy derivative and cleavage produce 2-*P*-glycolate and 3-*P*-glycerate of oxygenation.

Unlike activation, few metal ions can support catalysis. Only Mg ions are the physiological choice, although Ca^{2+} [45], Mn^{2+} [46,48] and Ni^{2+} [47] produce significant rates of carboxylation and oxygenation with the higher plant enzyme. In those conditions that allow

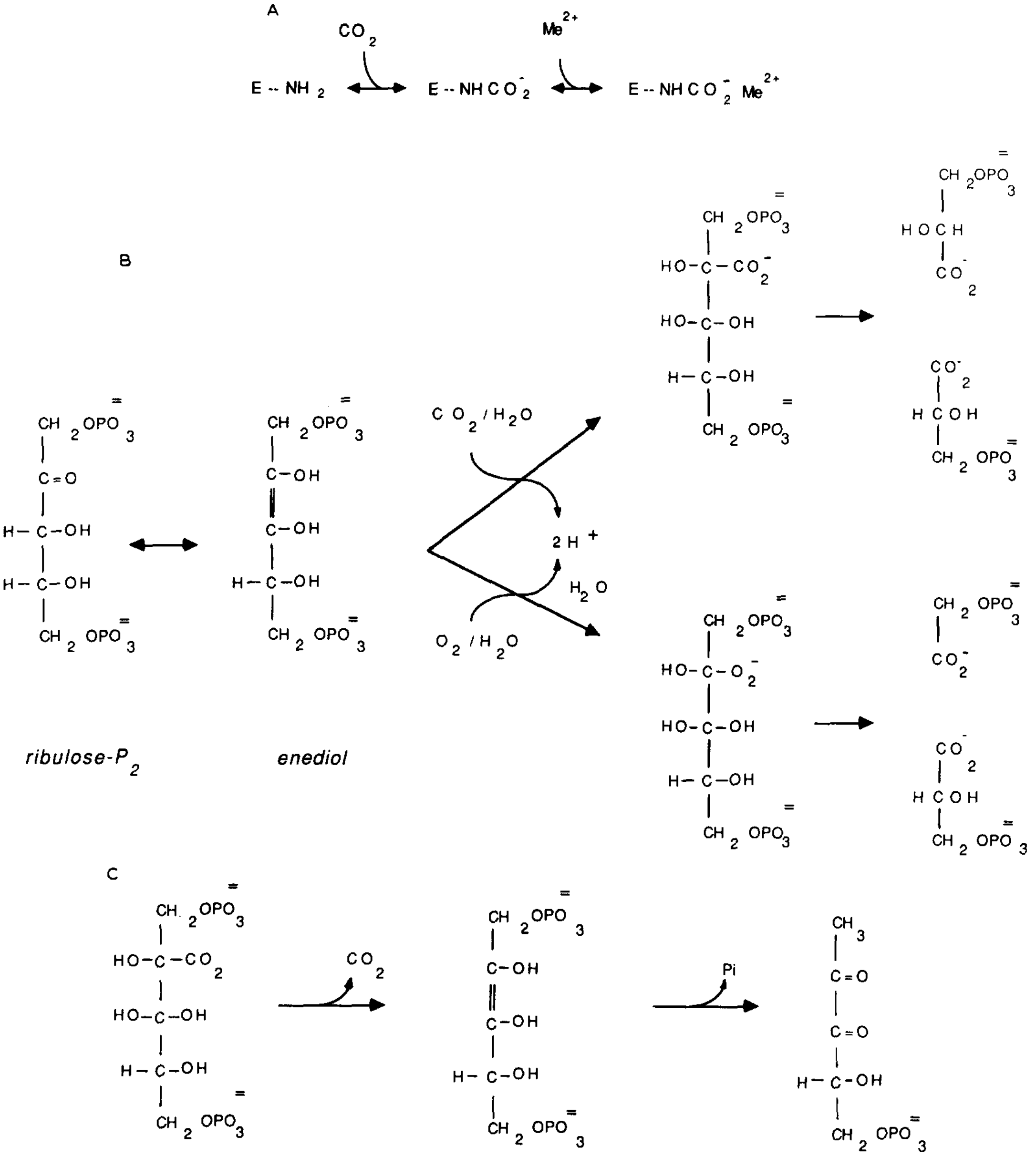

Fig. 3. The activation and catalytic reactions of rubisco. In (A) the amino group of a lysine residue at the active site is carbamylated by CO_2 before association of the essential metal forming the activated enzyme ternary complex. (B) shows the nature of the substrate and products of the two competing catalytic reactions. Carboxylation fixes CO_2 (not HCO_3^-) and consumes water to produce initially the hydrated form of 3-*keto*-2CABP and then two molecules of 3-*P*-glycerate. Oxygenation involves molecular O_2 and water to form 2-*P*-glycolate and 3-*P*-glycerate, but also generates a water from one atom of the oxygen molecule. (C) The deactivated form of rubisco is unable to hydrolyse 3-*keto*-2CABP to 3-*P*-glycerate, instead catalysing the decarboxylation of the intermediate to enediol and then inorganic phosphate (P_i) elimination rather than reversion to ribulose-P_2.

Ca^{2+} to substitute there is no difference in the partitioning of the bisphosphate through both reactions [2]. In the case of Mn^{2+} the oxygenase reaction is very much favoured in both the bacterial and plant enzymes. Indeed, the only example of the modification of the relative specificity of any species of rubisco in vitro has been through the substitution of the Mg^{2+} by transition metal ions (but see subsection IVC). Other metals that support some activity include Co^{2+} [47] and Cu^{2+} [34,50] that again favour oxygenation over carboxylation.

Both the form I and II enzymes conform to the ordered reaction mechanism described above and so the basis for the species variation in relative specificity described in Table I is not clear. However, other differences between rubisco species exist apart from specificity. Both the affinity for ribulose-P_2 and the second substrate CO_2 is highest for the plant enzyme whereas turnover is much less. Similarly the affinity for tight-binding intermediate analogues such as 2CABP conforms to this trend. With spinach rubisco, for example, 2CABP is essentially irreversibly bound ($K_d = 10^{-11}$) whereas *R. rubrum* rubisco is less by almost three orders of magnitude. Thus the variation may lie in the stability of the six carbon intermediate, that in the higher plant enzyme is preferred over the hydroperoxy intermediate of the oxygenase reaction.

The fate of the 3-*keto*-2CABP intermediate at the active site of the enzyme has been investigated separately [51]. This intermediate can be generated by arresting the turnover of ribulose-P_2 by rubisco in saturating concentrations of substrate CO_2. Two reactions (Fig. 3B and C) can be studies with the isolated intermediate [51,52] if labelled in the 2′-position. The first is the hydrolysis to the usual product 3-*P*-glycerate by the activated enzyme. The second is the decarboxylation and subsequent dephosphorylation of the 3-keto compound that occurs with the deactivated enzyme.

Unfortunately, the hydrolysis of isolated 3-*keto*-2CABP by rubisco is not a true simulation of the normal enzymic forward reaction because the binding of the intermediate to the active site is a two-step process similar to that found for 2CABP [53]. The first step involves reversible encounter typical of any competitive inhibitor and followed by a conformational change into the configuration that results in hydrolysis of the intermediate. Normally, it is the activated enzyme-enediol complex that is the natural precursor for the reception of the gaseous substrates. Nevertheless, two points are worth noting, once 3-*keto*-2CABP is bound to the activated enzyme it is all hydrolysed to product, there is no evidence that the intermediate reverts to enediol and CO_2. Secondly, the stability of the hydroperoxy intermediate of the oxygenase reaction is unknown, which if able to revert back to enediol and O_2 might provide CO_2 with a second chance of reacting with enediol. Mechanistically, the differences in relative specificities between different species of rubisco remains an open question, accepting that there is no evidence of selective binding of the gaseous substrates to the enzyme.

The decarboxylation reaction of synthesised 3-*keto*-2CABP occurs in free solution ($2 \cdot 10^{-4}$ s^{-1}) at ambient temperatures [51] and neutral pH. The catalysis of the decarboxylation is a characteristic of the deactivated form of rubisco and proceeds by a mechanism that has yet to be determined. Crystallographic data for the binary complex of deactivated *R. rubrum* rubisco and 2CABP, indicate that the inhibitor is bound the 'wrong' way around at the active site [54]. Presumably, 3-*keto*-2CABP adopts the same conformation, thus interacting with active site amino acids that are not normally involved in the forward reactions. Thus decarboxylation of the intermediate is not a true reversal of the steps leading to its formation. Nevertheless, it adds a further partial reaction that can be used to analyse the various mutants of rubisco, as the role and reactivity of individual active site amino acids is discovered.

IV. The structure of the active site of rubisco

With the solution of the structure of the simplest *R. rubrum* L_2 rubisco [14], an avalanche of information has been obtained not least the preliminary structures for the more complicated spinach and tobacco enzymes. The spinach data will ultimately provide the structure of the stable quaternary complex between activated enzyme and 2CABP [18]. The tobacco data have already indicated the particular disposition of the S subunit relative to the L subunits in the L_8S_8 aggregate [19].

At the level of secondary and tertiary structure the most striking feature is an eight-stranded β barrel surrounded and stabilised by eight helices. This α/β barrel accounts for some two-thirds of the amino acids at the C-terminal end of the L subunit. Common to those other enzymes with this same domain, e.g., triose phosphate isomerase, glycolate oxidase [55,56] the active site is located at the surface of the barrel described by the C-terminal end of the β strands. Many of the amino acids that compose the loop regions that connect each β strand with each helix are implicated in the active site structure and thus catalytic process.

The remaining one-third of amino acids of the L subunit form a domain characterised by antiparallel β strands surrounded by helices. The contact between the two domains of any one subunit is not extensive, but between the subunits there are multiple contacts. Each active site is not an independent functioning unit but requires the presence of the neighbouring subunit to complete the structure. Although the dimer has two sites, the minimum functioning unit is still L_2 because amino acids of the N-terminal domain of one subunit contribute to the active site located within the barrel of the partner of the dimer.

IVA. A dynamic model of the reactions of rubisco

Until the structure of rubisco was solved, the only amino acid of known function was Lys-191 of the L-subunit of *R. rubrum*, or its equivalent Lys-201 in the form I enzyme of spinach, that becomes carbamylated during activation with CO_2 and Mg^{2+} [31,32]. A number of other amino acids had been identified based on site-directed affinity labels, but their role in either catalysis or activation has remained speculative. A combination of mutagenesis and crystallographic data is now being used to ascertain the critical nature of specific amino acids and divulge details of their contribution to the enzymic reactions.

The necessary preamble that eventually leads to the co-ordination of the essential magnesium ion and the formation of a competent active site involves the carbamylation of the ϵ-amino group of Lys-191. The loop where Lys-191 resides is composed of acidic residues Asp-193 and Glu-194 also involved in metal co-ordination, that might be expected to stabilise a protonated lysine side chain preventing carbamate formation. Since these carboxylic amino acids are part of a flexible loop region, they may interact with the side

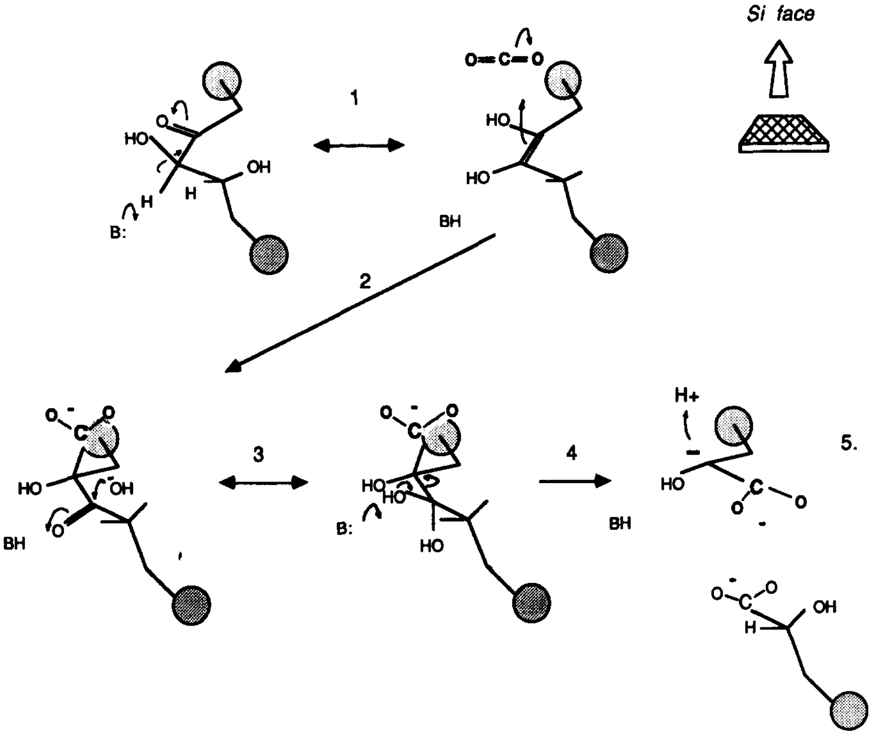

Fig. 4. The partial reactions and stereochemical direction of carboxylation of ribulose-P_2. Step (1) is the abstraction of the C3 proton of ribulose-P_2 to form the enediol by a group (B) at the active site. In (2) B may deprotonate to initiate then carboxylation (or oxygenation) at the *Si* face of the enediol by CO_2 (or molecular O_2) to form 3-*keto*-2CABP. (3) is the hydration of this 6-carbon intermediate and (4) subsequent cleavage to form the first molecule of 3-*P*-glycerate. The *aci*-acid is stereospecifically protonated again at the *Si* face to generate the second molecule of 3-*P*-D-glycerate (step 5). The particular orientation of the bisphosphate is shown as if occupying the active site of rubisco.

chains of other amino acids or Mg^{2+} until the carbamate is formed. Once Mg^{2+} has become co-ordinated to the carbamate, 193 and 194 swing in to complete the metal site. One other ligand of the metal is His-287 based on its distance from the metal in the crystallographic structure. The metal therefore resides at the surface of the barrel slightly offset towards loops 2 and 4 (see Ref. 35).

The encounter between activated rubisco and ribulose-P_2 in step 1 of catalysis is in a directed way (see Fig. 4). Potentially there are four orientations of the substrate at the active site. Looking down at the active site bound bisphosphate with the Si face of the C2 carbonyl 'up' toward the solution, the 1-phosphate could be at the phosphate binding site closest to the metal or the one furthest from the metal ion [41]. A similar situation applies if the Re face is 'up'. A combination of spectroscopic and rapid reaction data [57] have indicated that only one orientation is assumed by the bisphosphate with activated rubisco. The co-ordinated metal orients the C2 carbonyl group so that the *Si* face of the carbonyl is presented to the solution away from the surface of the barrel. The 1-phosphate occupies one of the two helix dipoles available for phosphate binding, the one closest to the metal. The combined effect of the metal and an, as yet unidentified, amino acid that acts as a base (B in Fig. 4) initiate the reaction by abstracting the C3 proton of ribulose-P_2 and generating the 2,3-enediol. The protein that is abstracted by B is readily exchangeable with solvent protons [40].

The competing gaseous substrates, CO_2 and O_2 approach the enediol from the solution toward the exposed *Si* face. If B became deprotonated then it may act to aid the formation of, in the case of carboxylation, the 3-*keto*-2CABP intermediate (step 2). Unlike the C2 carbonyl of ribulose-P_2, the C3 carbonyl of this intermediate is not reducible by borohydride. Apparently, 3-*keto*-2CABP, unlike its free solution form, resides primarily at the active site as the hydrate.

The next step in the reaction pathway (step 4) is the cleavage of the C2–C3 bond that assuming the intermediate is already hydrated, requires deprotonation of the hydrate by a base on the enzyme. The lower 3-P-glycerate so formed is the correct stereoisomer, the deprotonated upper *aci*-acid molecule at the active site requires protonation either from the solution or via the enzyme in a stereochemically directed way. Consistent with reaction involving the 2,3-enediol, it is the *Si* face of the *aci*-acid that is protonated to generate only D-3-*P*-glycerate as the other product molecule. It has yet to be established if the lower 3-*P*-glycerate is released from the active site before protonation of the upper carbanion occurs. It is possible a quinternary complex may exist between the activated enzyme and the two

3-P-glycerate molecules at the terminal stage of the reaction.

IVB. Site-specific mutations at the active site

Understanding the function of individual amino acids has been greatly enhanced by the ability to generate site specific mutations in the protein and to analyse the partial reactions of the catalytic process; at least those leading to carboxylation. Thus specific mutations of the enzyme that are lethal to overall catalysis can be subjected to a number of assays. The affinity of activated rubisco for 2CABP and the stability of the resulting E · CO_2 · Mg · 2CABP quaternary complex is exploited to determine if mutations have affected either activation or bisphosphate binding. Mutants with lesions in the catalytic process are investigated with ribulose-P_2 tritiated at the C3 position to investigate disruption of enediol formation. Isolated 3-*keto*-2CABP labelled at the 2′ position with ^{14}C allows the hydrolysis step to 3-P-glycerate product or decarboxylation by the deactivated enzyme to be investigated. The particular plasmids that have been manipulated to generate mutants of both form I and II rubisco species are shown in Fig. 5. In both cases exploitation of unique restriction sites that have been engineered into the structural gene for the L subunit has ensured production of mutants with high efficiency [44,59]. The dissection of the L and S subunit genes of *Synechococcus* rubisco (Fig. 5C and D) has also allowed the investigation of the two subunits in complete isolation of one another for the first time (see subsection IIB).

IVB-1. Probing the metal binding site

A role for the metal ion can be proposed in many of the reactions of rubisco. A series of mutations of some of the amino acids that co-ordinate the essential metal have been generated in the *R. rubrum* L subunit (Fig. 5A) to investigate these proposals. One conserved amino acid that was the target of the first site specific mutation was Asp-188 that was converted to a glutamic acid [58]. The extra methylene was expected to perturb the metal and modify activity. Both expectations were realised as determined by EPR spectroscopy and activity characteristics. However, the changes were slight because the residue is situated, as is now clear from the X-ray data at the N-terminal surface of the barrel, not the active site C-terminal surface.

The crystallographic data of the *R. rubrum* enzyme indicate that three amino acids, Asp-193, Glu-194 and His-287 in addition to the carbamate are implicated in metal binding. Table II shows that mutations at Asp-193 and Glu-194 destroy overall catalysis but do not prevent some reaction of CO_2 with Lys-191 to generate the active site carbamate. Although both residues are coordinated to the metal ion, changes at these positions produce quite distinct phenotypes. The Asn-193 mutant has the ability to hydrolyse 3-*keto*-2CABP intermediate to product but is unable to catalyse the exchange of the

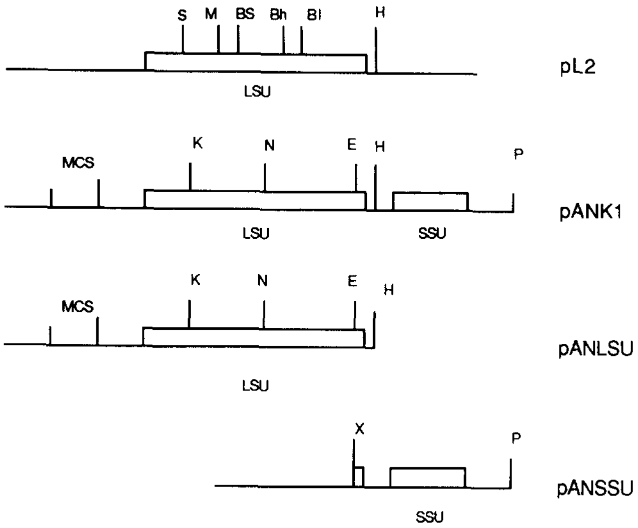

Fig. 5. The organisation of the various plasmid constructs used to generate mutants of *R. rubrum* (L_2) and *Synechococcus* PCC6301 (L_8S_8) rubisco. The plasmid pL2 is a construct utilising the L-subunit gene of *R. rubrum* rubisco from pRR 2119 under *lac* control but with the β-galactosidase amino acids removed from the N-terminus [11–13,44]. The restriction sites have been so organised by site-specific mutagenesis to be unique around specific regions of the subunit so that cassettes can be used to generate the various active site mutants [44]. S, Sma 1; M, Mlu 1; BS, BstEII; Bh, BssHII; Bl, Ball; H, HindIII. pANK1, the high expression plasmid utilised to generate large quantities of recombinant L_8S_8 rubisco has also been constructed with some unique sites, in this case for generation of chimaeric L-subunit genes [59]. Two derivatives of pANK1 have been used to synthesise the L-subunit (pANLSU) and S-subunit (pANSSU) independently of one another. All of these constructs are based on high copy number plasmids (pUC18) under *lac* promotor control. The sites are K, *Kpn*I; N, *Nsi*I; E, *EcoR*I; H, *Hin*dIII; P, *Pst*I; X, destroyed *EcoR*1 site and MCS is the multiple cloning site of pUC18.

TABLE II

The partial reactions catalysed by the site specific mutants of R. rubrum rubisco described in the text

n.d., not determined.

Mutants	Relative activity [a]	^3H-ribulose exchange	3K2CABP [b] hyd.	3K2CABP [b] dec.	2CABP binding	Refs.
D-N193	-	−	+	±	+	44
E-Q194	-	−	−	±	+	44
K-C191	-	−	+	−	+	93
K-E191	-	−	−	+	+	44,92
H-N287	1%	+	+	+	+	44
K-G166	-	−	+		+	61
E-Q48	-	+	n.d.	n.d.	+	65

[a] Overall catalysis is relative to wild type at 100%; a − sign means less than 0.2% activity detected.

[b] A ±-sign indicates that the rate of decarboxylation of 3keto2CABP declines with increasing Mg^{2+} concentrations. Thus with N193 increasing Mg^{2+} ensures more of the intermediate partitions into hydrolysis relative to decarboxylation.

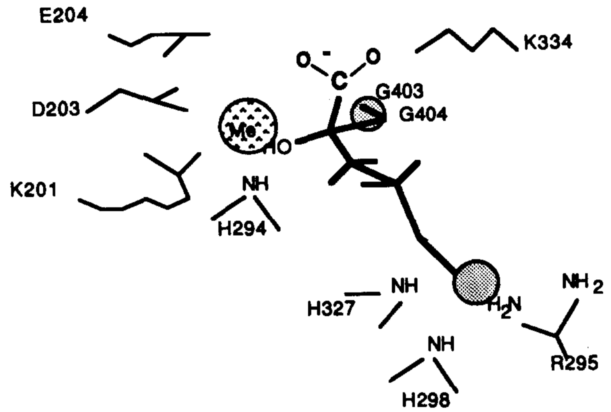

Fig. 6. The identity of some of the amino acids that compose the active site of rubisco. The structure of the stable quaternary complex of activated spinach rubisco and 2CABP is shown with the amino acids that are involved in binding the essential metal ion (Me) and the 1- (smaller shaded sphere) and 5-phosphates of the intermediate analogue. Apart from His-327 (H327) which is part of a sheet of the barrel structure, all of the residues reside in the loops that extend above the surface of the eight stranded barrel (adapted from Ref. 18). The amino acids are numbered according to the spinach sequence and thus the carbamate resides on lysine-201 (K201).

C3 proton of ribulose-P_2 to generate enediol. The table also shows that the amount of intermediate that is hydrolysed relative to the fraction decarboxylated is dependent on the concentration of the metal ion. Thus with Asn-193, more hydrolysis is detected at elevated Mg^{2+} concentrations. This suggests that the hydrolysis step of the reactions that leads to cleavage between C2 and C3 of 3-*keto*-2CABP is discrete from the proton abstraction step that generates the enediol intermediate. Furthermore, Asp-193 is an essential part of the structure that generates enediol, but is not absolutely required for hydrolysis. This is consistent with the proposal that ligand identity might change around the metal at different stages of the reaction.

In the case of the Gln-194 mutant the fate of the intermediate is also dependent on the concentration of the metal ion. However, unlike the 193 mutant no hydrolysis of the intermediate can be detected even though decarboxylation is progressively inhibited at elevated Mg^{2+} concentrations. Thus Glu-194 in the wild-type enzyme is critical for both enediol formation and hydrolysis of the six-carbon intermediate.

Spectroscopic studies of the stable 2CABP quaternary complex with activated spinach rubisco indicate that both the 2′-carboxyl and the 2-OH group of the inhibitor are co-ordinated to the metal [50,60]. In addition to the carbamate oxygen, Asp-193, Glu-194 and His-287 should complete the co-ordination of the Mg^{2+}. However, both an amino acid from the N-terminal domain and a water molecule might also occupy positions around the metal at some stage of the reaction chemistry. Therefore it is not unreasonable to postulate that ligand identity changes during catalysis. That His-287 can be mutated without completely destroying catalysis, e.g., an Asn-287 mutation is about 1% active [44], suggests that this might be one position around the metal that can change.

IVB-2. Searching for the essential base

Overall catalysis requires the movement of three protons to and from reaction intermediates, at least two of which must involve one or more amino acids that reside in the loops above the active site. It has been speculated that only one group might function as both proton donor and abstractor [88]. However, site-specific mutagenesis studies indicate that the two processes of enediol formation and hydrolytic cleavage of 3-*keto*-2CABP are distinct events. Therefore, if only one group is involved it requires adjustment to the metal co-ordination sphere to attain the appropriate geometry and/or reactivity to satisfy both roles.

Two amino acids that have been identified as potential candidates responsible for the abstraction of the C3 proton of ribulose-P_2 are Lys-166 and His-291. Lys-166 is a conserved amino acid that is part of the active site and has an unusually low pK_a [61]. Three changes at this position with amino acids that might substitute for lysine produced mutants Arg-166, His-166 and Ser-166 [63]. All retained a small but measurable amount of carboxylase activity compared to Gly-166 which was inactive, supporting the proposal that Lys-166 might be the basic group involved in initiating catalysis.

Histidine 291 had been implicated because reaction of His-298, the equivalent residue in higher plant rubisco, with diethylpyrocarbonate inactivates the enzyme [90,91]. Conversion of the His to Ala-291 in *R. rubrum* produced an enzyme that was still about 50% active [61]. Clearly the role of this residue is not critical to catalysis.

The structure shown in Fig. 6 is an attempt to combine the available data into a single scheme that satisfies the results of spectroscopic, mutagenic and crystallographic studies. The metal co-ordinated to the carbamate at the active site directs much of the subsequent catalytic events between ribulose-P_2 and the gaseous substrates. Clearly, other amino acids are involved in binding and reacting with ribulose-P_2 that have yet to be fully analysed. The identity of the base involved in enediol formation is not shown as Lys-166 because if the orientation implied from the NMR, EPR and crystallography of the stable quaternary complex with 2CABP also applies to ribulose-P_2 then Lys-166 which resides in loop 1 may be too distant to function as the protein abstracting group.

Other amino acids that might be strong contenders very much depend on the orientation that the C3 group of ribulose-P_2 assumes on binding to the active site. One candidate that has yet to be explored is the carbamino nitrogen group itself. Aside from being a potential ligand of the metal, it is also in close proximity to the bound substrate. The presence of three other negatively charged ligands, carbamate oxygen, Asp-193 and Glu-194 around the Mg^{2+} might screen the metals positive charge. The carbamino N group might then acquire a pK_a that varies depending on the ligand identity during each stage of catalysis, thus not only mediating the abstraction of the C3 proton of the substrate but also involved in other protonmotive events.

Unfortunately, mutations at Lys-191 although lethal to activity [92] do not provide a ready means of investigating the chemistry of the carbamate. That mutant Glu-191 is inactive might simply be due to the shorter dimensions of the acid side chain. Of greater potential is the replacement of Lys-191 with cysteine and then reaction with appropriately designed thiol reagents, a method elegantly used to restore about 4% of the activity to the Cys-191 mutant with ethyleneimine [93]. Interestingly, this mutant is also able to catalyse a small but detectable amount of the hydrolysis of 3-*keto*-2CABP.

IVC. Prospects for altering carboxylation

Two aspects of rubisco function are being addressed as implied by these in vitro mutagenesis studies. The first is to determine the amino acids critical to functionality, i.e., the groups involved in binding metal or substrate and mediating proton movements. The second is to identify those amino acids that may not be directly involved in catalytic events themselves but do modulate the encounter of substrates or stabilise intermediates that may influence the specificity of the reactions. With the availability of X-ray crystallographic data, the former has moved ahead rapidly. The other strategy may require comparative X-ray analysis before a rational investigation can be attempted. The analysis is presently in progress with the recombinant enzymes of *R. rubrum* and *Synechococcus* for comparison with the higher plant enzymes from spinach and tobacco.

Site-specific mutations of critical amino acids of the L subunit allow some conclusions to be determined about the function of these residues. At present the function of amino acids that contribute to metal binding and possible interactions with the biphosphate substrate have been probed in some detail. However, the active site is shared between the two subunits and the function, or indeed the number of those amino acids that are donated by the N-terminal domains is unclear. One residue invariant amongst rubisco species that resides within the N-terminal domain of the L subunit shown by cross-linking [64], mutagenesis [65] and crystallographic studies [14] to be in close proximity to active site amino acids of the C-terminal domain of the other subunit is Glu-48. The function of the amino acid is unknown but in the mutant Gln-48 the enzyme still retains some activity. Other conserved residues of the N-terminal domain require similar investigations to determine their potential contribution. It might be that the amino acids of the active site of the C-terminal domain contributes those elements that bind and orient correctly the bisphosphate substrate for catalysis. The N-terminal domain may contribute the amino acids that direct the interaction of the gaseous substrates with the enediol intermediate during catalysis.

Increasing the relative specificity of rubisco might not be as simple as introducing a single mutation into the L subunit but may require multiple alterations. For example, Met-330 adjacent to a conserved lysine is present as a leucine residue in the form IL subunit. Conversin of Met-330 to leucine [66] alters substrate binding but does not destroy activity nor does it impart any form I characteristics on the form II enzyme. Nevertheless, mutations around this region of the L subunit have been reported to affect relative specificity [96]. Conversion of a conserved valine at position 331 to alanine in the L subunit of the enzyme from *Chlamydomonas reinhardtii*, generated in vivo with mutagens, produced an enzyme with about 7% turnover of the wild type and a relative specificity reduced by about 40% Interestingly, a threonine–isoleucine revertant at position 342 allowed significant recovery of some of the lost potential.

One other approach to determining the function of subdomains or regions of subunit structure in vitro is in progress using genes for *Synechococcus* rubisco [26,59]. It involves constructing chimaeric genes composed of parts of the higher plant L subunit structure to determine if differences in function can be localised to small regions of the L-subunit. A comparison of the amino acid sequences for form I rubisco L subunit for plants and bacteria indicate that some regions have been more susceptible to mutation than others. These regions may therefore be the source of the variations in specificity. Thus the strategy is to investigate those regions of low homology, i.e., the opposite of that applied to site-specific changes.

The catalytic characteristics of *Synechococcus* rubisco compared with higher plant rubisco, are quite distinct, e.g., a factor of 2 or less in relative specificity and yet a factor of 4 faster in turnover. Activation of the *Synechococcus* enzymes also requires about 5 times more CO_2 than the spinach enzyme. These differences are measurable experimentally and thus the conversion of the bacterial enzyme to one resembling the higher plant should be readily detected. Two regions of the L subunit have been manipulated to date based on the low homology between the two species. The first region was the N-terminus that is of low homology but critical for function. Limited proteolysis of this region of the subunit with both *Synechococcus* and spinach, produces an enzyme that loses at least 80% of its catalytic activity without disturbing activation of 2CABP binding [67]. Replacement of the first 36 bases of the *Synechococcus* L subunit with the same region from a cloned wheat L subunit generated a chimaera that was able to be expressed in *E. coli*, assemble into holoenzyme and had activity but did not show altered kinetic characteristics.

A second region of low homology surrounds the interconnection between the N- and C-terminal domains of the L subunit. Existing restriction sites in the wheat and *Synechococcus* L subunit genes can be exploited to replace some 114 amino acids including 16 mutations across this region and into the first three segments of the α/β barrel. The resulting chimaera generates only small quantities of the L subunit that is insoluble and unable to assembly correctly [59]. Four of the mutations cover both the interdomain region and the first two segments of the barrel. Constructing a chimaeric gene that carries just these four mutations results in active holoenzyme with almost identical activity as the wild type. Therefore the particular region of the L subunit that apparently causes the assembly obstacle must be one or more of the remaining mutations localised around loop 3 of the barrel. Indeed crystallographic data indicates that this region is implicated in interactions between the two L subunits of each dimer. Conversely, those chimaeric constructs that retain activity but do not show a significant change in partitioning of the substrate, reduces the number of mutations that impart functional differences between species of rubisco.

V. In vivo regulation and assembly

VA. Assembly of the holoenzyme

The manipulation of the enzyme structure by mutagenesis has provided details of the contribution of amino acids in catalysis. However, during this period there has also been progress in defining mechanisms that regulate rubisco in vivo, both assembly of the holoenzyme and catalytic turnover.

Assuming that a more desirable functional trait might be engineered, there still remains the obstacle of reintroducing the altered protein into the host plant. The arrangement of plant genetic systems with regard to rubisco synthesis is not totally amenable to present molecular manipulation techniques. The two subunits of the enzyme are coded for in two separate compartments of the cell. The L subunit is synthesised in the chloroplast and the S subunit in the cytosol as a precursor protein. The presence of a signal peptide of about 56 amino acids directs the translocation of the precursor across the chloroplast envelope into the stroma. By the time the protein appears in the stroma it has been processed to the mature form that associates with L subunit to form the holoenzyme [68,69].

The mechanism of assembly of the hexadecameric aggregate has been investigated for some time and is thought to involve other proteins that assist the folding and presumably the association of the L subunits into the L_8 core. The assembly proteins or 'chaparonins' of the stroma can be isolated as a 600 kDa aggregate of about 12 assembly proteins subunits, that has newly synthesised L subunit associated with it. Exploiting the ability of isolated chloroplasts to synthesise L subunit, the time-course of the loss of L subunit from the aggregate and its appearance in the holoenzyme was determined using radiolabelled methionine as a marker. The results suggested that the large protein complex was an intermediate on the pathway of assembly of the L_8S_8 holoenzyme [70,71]. We know from the studies of the *Synechococcus* enzyme that the S subunit can be reversibly removed from the complex, indicating that the association between native L and S subunits requires no protein mediation. Therefore, it is likely that the role of chaparonins is in directing the correct folding of the L subunit prior to the formation of the L_8 core.

There is still some question concerning the functional size of the assembly aggregate and also its function in protein folding. For some time the proposal that a multisubunit enzyme might require other proteins to catalyse aggregation was not well established. However, the sequence of the plant assembly protein is about 50% homologous with a groE gene product of bacteria, a protein that is conscripted for phage head assembly in *E. coli* [73]. When recombinant bacterial rubisco genes are expressed in *E. coli* coinfected with groE bearing plasmids, the quantities of the active enzyme are stimulated, suggesting a role for the groE proteins (groEL and groES) in the stabilisation or assembly of rubisco subunits [72,74]. It remains to be seen if the groE proteins may assist in the aggregation of other foreign proteins expressed in *E. coli*.

If it is necessary to introduce mutations into the L subunit to generate a more favourable trait, it is likely that the L subunit genes might need to be nuclear encoded, producing a precursor protein that will translocate to the chloroplast stroma. Can such a protein be constructed and use the existing mechanisms of transport and assembly and appear as holoenzyme? Gatenby et al. [75], were able to show that a *Synechococcus* L subunit fused to a S subunit signal sequence is transported into isolated chloroplasts and assembled into holoenzyme with pre-existing S subunit. The next step is to determine if that heterologous aggregate has the appropriate activity to support CO_2 fixation.

VB. Regulation of rubisco activity and activation in vivo

The requirement of rubisco for CO_2 and Mg^{2+} for activation provides a potential locus for regulation that is responsive to light. In the dark, Mg^{2+} flows from the stroma as protons move in, causing a decline in pH. At lower pH and lower magnesium ion concentration rubisco is less likely to be carbamylated. However, it has never been fully established that this is a significant regulatory process of the enzyme, or that it might be a means of fine tuning the enzyme during changes in light intensity throughout a normal day. It is to be expected that there are a number of phosphorylated compounds that abound in the chloroplast that can interfere both in the activation process and in dissociation of the activating cofactors as concentrations decline below suboptimal quantities [36,82,94].

A survey of the state of activation of the enzyme isolated from plants after various light regimes has shown that from some species, rubisco has variable activity. One of the best examples is rubisco from *Phaseolus* where in complete darkness the specific activity is lower by 80% compared with the enzyme released from the plant after some time in bright light [77]. This diurnal variation of the activity of the enzyme is not due to the state of activation because release into saturating concentrations of the cofactors does not restore turnover [76–78]. The reason is that a tight binding inhibitor 2CA1P, that resembles 3-*keto*-2CABP the transition state intermediate of carboxylation, is synthesised during darkness and binds preferentially to activate rubisco. The binding of the inhibitor was tight enough that co-purification with rubisco could be exploited for its isolation and identification [79,80]. 2CA1P is similar to the irreversible inhibitor 2CABP but without the 5-phosphate which reduces the affinity for the enzyme by about three orders of magnitude. Thus the inhibitor dissociates from the active site with $t_{1/2}$ of about 10 min which is less than the time of reactivation (approx. 60 min) of the inhibited enzyme in vivo [81].

The processes involved in the synthesis and degradation of the compound are not well established, although in those plants that exhibit significant inhibition of rubisco, the concentrations attained must be at least 1–2 mM. One enzyme has been isolated from tobacco chloroplast stroma that is able to utilise 2CA1P as a substrate [97,98], that dephosphorylates the molecule to 2CA. The activity that is isolated from stroma would account for that required to consume all the 2CA1P inhibiting rubisco with appropriate rates of catalysis [97].

The enzyme shows some specificity for branch chain phosphorylated acids such as 2CA1P, but does not hydrolyse 3-*P*-glycerate or fructose-P_2 at significant rates. For the process to be truly the same as that observed in vivo, then the enzyme must be responsive to a light-driven mechanism. The particular phosphatase isolated from these chloroplasts requires the presence of DTT to remain active, suggesting that in vivo it is associated with the thioredoxin activation process utilised by other light-stimulated enzymes of the stroma.

Not all plants utilise 2CA1P as the sole means of regulation. Those that do not synthesise significant quantities of the inhibitor still exhibit variation in rubisco specific activity depending on the state of illumination of the plant. The identity of other phosphate esters involved in this type of inhibition have yet to be determined, although a somewhat controversial proposal is that the substrate ribulose-P_2 might also play a role in this capacity [83]. There are a number of reports that ribulose-P_2 binds to the deactivated form of rubisco blocking the active site and interfering with the carbamylation reaction (see, e.g., Refs. 36 and 82). Unlike 2CA1P that binds most tightly to activated rubisco and inhibits substrate turnover, ribulose-P_2 inhibits through binding to the deactivated enzyme. An enzyme has been identified in *Arabodpsis thaliana* that is required for ensuring that rubisco becomes activated even in the presence of ribulose-P_2 [84]. The enzyme has been identified in a number of plant species [99] and requires ATP for activity. The mechanism of action of activase is obscure but clearly affects the equilibria between rubisco and activating cofactors or effectors (see also Ref. 85). Activase has been demonstrated to relieve the inhibition of rubisco by 2CA1P in vitro [100] and thus may be an alternative and more sensitive form of regulation by 2CA1P than its consumption by 2CA1P phosphatase.

One additional limitation of the rubisco mechanism is the inhibition of the enzyme that accompanies substrate turnover. With all higher plant rubisco, initial rate determinations for carboxylation or oxygenation of rubulose-P_2 are complicated by a progressive loss of enzyme activity to a final lower rate as much as 30–50% of the initial rate [83,86,87,95]. It is not certain whether this inactivation is due to a further partitioning of the substrate bisphosphate generating a potent inhibitor, or a pre-existing inhibitor in the substrate solution. Nevertheless, the inactivation is relieved when rubisco turns over substrate in the presence of activase and ATP [101]. Therefore, there are a number of mechanisms that have been identified that may regulate rubisco activity in vivo that have yet to be fully defined [88,89]. From the point of view of plant productivity, these mechanisms need to be characterised to determine exactly the limitations that they superimpose over and above the intrinsic faults of rubisco.

VI. Conclusion

Irrespective of the perceived complications of the regulation of rubisco turnover in vivo, this does not alter the potential advantages that a modified enzyme would impart on a plant. The most obvious one is that less ribulose-P_2 would be wasted through oxygenation, as more became committed to carboxylation. With less product cycling through photorespiration, then less carbon would be evolved as CO_2 and the energy cost of recapturing the rest of the carbon of phosphoglycolate would also be reduced. The increased efficiency of CO_2 use in this hypothetical plant might yet lead to two further desirable traits. Firstly, the increased specificity for CO_2 would mean that the enzyme could function at lower intercellular concentrations of the substrate. Water efficiency should therefore be significantly better as the plant would spend less time acquiring the substrate through open stomates. Finally, the amount of nitrogen invested in the form of rubisco is considerable. In C3 plants the enzyme accounts for at least 50% of the cell contents. By comparison the amount is less by at least one half in the case of C4 plants such as maize that do not require as much rubisco because of their CO_2 concentrating ability. Clearly, nitrogen use of our ideal plant would be one more trait that should improve considerably along with the others described above, assuming, of course, that the plant did not find some means of negatively compensating for the hard won improvements.

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