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STRUCTURE, FUNCTION, AND REGULATION OF RIBULOSE-1, 5-BISPHOSPHATE CARBOXYLASE/OXYGENASE

ARAN INCHAROENSAKDI[a,b] AND TETSUKO TAKABE[a]

[a]*Research Institute for Biochemical Regulation, School of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan.*

[b]*Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10500, Thailand.*

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Abstract

Ribulose-1, 5-bisphosphate carboxylase/oxygenase is a key enzyme in photosynthesis. The structure of the enzyme in both prokaryotes and eukaryotes consists of eight pairs each of large subunits (A) and small subunits (B) with the quaternary structure A_8B_8. *The only exception is that found in a photosynthetic bacterium* Rhodospirillum rubrum *which has the structure* A_2. *Subunit A is the site of catalytic activity for both carboxylase and oxygenase. Subunit B is also essential for the manifestation of both activities and its partial role is to stabilize the activated form of the enzyme. The biosynthesis of the enzyme in higher plants requires the participation of both chloroplast and nucleus. Subunit A is encoded by chloroplast DNA whereas subunit B is encoded by nuclear DNA. The assembly process of the two subunits is not well understood at present. Regulation of the enzyme activity is mainly mediated by light which causes an increase in pH in the chloroplast, optimal for enzyme activity, as well as an increase in magnesium concentration. Changes in the level of a phosphate ester inhibitor, 2-carboxyarabinitol-1-phosphate, as affected by light can also regulate the enzyme activity. Recent studies have shown the presence of a new protein, RuBisCO activase, which is responsible for the regulation of the enzyme* in vivo. *Light also regulates the synthesis of the enzyme. The synthesis of subunit A is light-regulated at the post-transcriptional level, whereas the synthesis of subunit B is regulated at the level of transcription. With recent developments in recombinant DNA technology, the possibility exists of altering the reactivity of the enzyme towards either substrate,* CO_2 *or* O_2. *However, so far such attempts by using site-specific mutagenesis have not proved successful yet.*

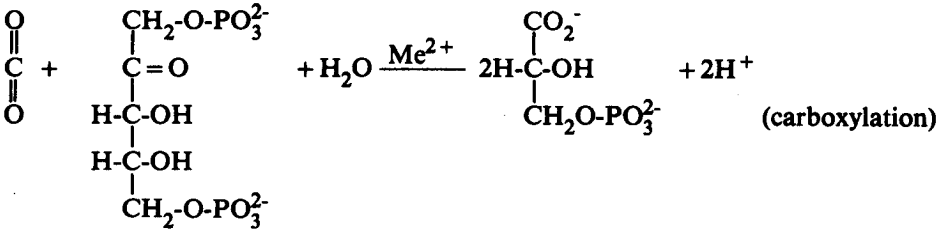
Abbreviations used: CABP, carboxyarabinitol-1, 5-bisphosphate; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; RuBisCO, ribulose-1, 5-bisphosphate carboxylase/oxygenase; RuBP, ribulose-1, 5-bisphosphate; SDS, sodium dodecyl sulfate.

Introduction

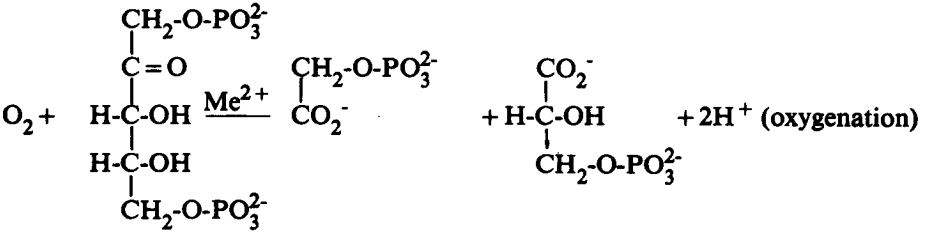
Life on earth would be impossible without the photosynthetic $C0_2$ assimilation process. The pathway of CO_2 assimilation in plants was formulated in the early 1950's by Calvin, Benson and Bassham. This pathway, the Calvin cycle or photosynthetic carbon reduction cycle, consists of a total of thirteen enzyme-catalyzed steps of which three actually use cofactors (ATP and NADPH) generated by the light reaction by means of light-driven electron transport coupled to phosphorylation.

Ribulose-1, 5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (RuBisCO) catalyzes the initial step of the Calvin cycle, i.e. the addition of CO_2 to the C-2 of ribulose-1, 5-bisphosphate (RuBP). The capacity of plants to photosynthesize is largely dependent on this enzyme, when other environmental factors are not limiting. The product of the carboxylation reaction, 3-phosphoglycerate, is phosphorylated to 1,3-diphosphoglycerate, which is then reduced to 3-phosphoglyceraldehyde. This triose phosphate and its isomer, dihydroxyacetone phosphate, can be translocated across the chloroplast envelope into the cytoplasm, where the actual synthesis of sucrose takes place.

In 1973, RuBisCO was also shown to catalyze the oxygenation of RuBP[1]. The carboxylation and oxygenation of RuBP are shown below.



(carboxylation)



(oxygenation)

One product of the oxygenation reaction, 2-phosphoglycolate, is the precursor of glycolate which in turn is a substrate for photorespiration. Since photorespiration is a process which opposes photosynthesis in plants in that a fraction of fixed CO_2 is released, it is an enigma that RuBisCO can contribute to both photosynthesis and photorespiration.

RuBisCO is said to be the most abundant protein in the biosphere[2]. In the first biochemical studies on leaf protein, RuBisCO was designated as Fraction-1-protein due to its major component and electrophoretic homogeneity[3]. Later studies by comparing their

physicochemical properties have shown that RuBisCO and Fraction-1-protein are indeed the same protein[4]. RuBisCO is ubiquitously found in autotrophic eukaryotes such as green plant leaves and green algae, accounting for as much as 80 % of total soluble protein. It is also a major protein in autotrophic prokaryotes, though in most cases its content is lower than that in autotrophic eukaryotes. For example, it represents about 50 and 17 % of total soluble protein in *Rhodospirillum rubrum*[5] and *Thiobacillus neapolytanus*[6], respectively. In this review, we have attempted to describe the recent advances in our understanding of the structure-function relationships and the regulation of the enzyme. The application of the recombinant DNA technology to the study of the structure and function of RuBisCO at a molecular level is also presented.

Molecular and Structural Aspects

Owing to high cellular levels of the enzyme, it is relatively easy to isolate and purify the enzyme in large quantities. This, coupled with the stability of the enzyme *in vitro*, makes RuBisCO one of the most extensively studied enzymes with respect to its molecular and structural properties. The usual steps employed in the purification of the enzyme involve ammonium sulfate precipitation, ion exchange column chromatography, followed by either gel filtration or isoelectric focusing column chromatography. However, a much easier technique involving one-step sucrose density-gradient centrifugation has been successfully used for the purification of RuBisCO from a variety of plants as well as microbial organisms[7]. Recently, the availability of the vertical rotor has made it possible to accomplish the purification of RuBisCO by sucrose gradient centrifugation within less than one day[8]. Also the use of fast protein liquid chromatography (FPLC) has been shown to be efficient in purifying RuBisCO from spinach leaves[9].

Subunit Composition and Quaternary Structure

It is now established that RuBisCO of both prokaryotes and eukaryotes is composed of two different subunit types, large (A or L) and small (B or S) subunits. Subunit A has M_r 50,000-58,000 daltons whereas M_r for subunit B is 12,000 - 18,000 daltons. Table 1 shows a partial list of the quaternary structure of RuBisCO isolated from various sources. The enzyme molecule in general has a symmetric oligomeric structure, designated as A_8B_8, based on several lines of experimental evidence. However, there are a few exceptions to this general rule. For example, *R. rubrum* enzyme has been reported to lack subunit B and to have the quaternary structure A_2[10]. The enzyme from *Thiobacillus intermedius* was previously found to have the A_8 structure[11] but was later shown to be A_8B_8[12]. Some contradictory observations were reported for the structure of RuBisCO from cyanobacteria. It is worth mentioning that, except only in a few cases, the quaternary structure models have not been based on firm physical or chemical data. They are mostly derived from molecular weight determinations of the native holoenzyme as well as the two individual subunits. In this regard, Andrews and co-workers have recently shown that the methods used for the determination of molecular weight may give quite different results. For example, by using gel filtration and pore gradient electrophoresis, a molecular weight of

TABLE 1 QUATERNARY STRUCTURE OF RUBISCO ISOLATED FROM VARIOUS SOURCES

Enzyme source	M_r	Quaternary structure
Bacteria		
Alcaligenes eutrophus	5.05×10^5	A_8B_8
Chromatium vinosum	5.2×10^5	A_8B_8
Rhodopseudomonas spheroides	3.6×10^5 (5.5×10^5)	A_6 (A_8B_8)
Rhodospirillum rubrum	1.14×10^5	A_2
Thiobacillus intermedius	4.6×10^5	A_8
	5.5×10^5	A_8B_8
Thiobacillus neapolytanus	5.0×10^5	A_8B_8
Cyanobacteria (Blue-green algae)		
Anabaena cylindrica	4.5×10^5	A_8
	5.0×10^5	A_8B_8
Anabaena variabilis	5.0×10^5	A_8B_8
Agmenellum quadruplicatum	4.5×10^5	A_8
Aphanocapsa	5.25×10^5	A_8B_8
Aphanothece halophytica	4.6×10^5	A_8B_8
Plectonema boryanum	5.0×10^5	A_8B_8
Synechococcus ACMM 323	5.3×10^5	A_8B_8
Green algae		
Chlamydomonas reinhardtii	5.3×10^5	A_8B_8
Chlorella ellipsoidea	5.0×10^5	A_8B_8
Euglena gracilis	5.25×10^5	A_8B_8
Higher plants		
Barley	5.1×10^5	A_8B_8
Spinach	5.15×10^5	A_8B_8
Tobacco	5.25×10^5	A_8B_8

430,000 was obtained indicating an A_6B_6 structure for the enzyme isolated from a marine cyanobacterium *Synechococcus* ACMM 323[13]. However, by using a more reliable technique, equilibrium density centrifugation, a value of 530,000 indicating an A_8B_8 structure was obtained[14]. Another factor that should be taken into account when judging whether the enzyme structure deviates from the A_8B_8 type is the methods or conditions employed for the purification of the enzyme. The acid treatment of the enzyme from fresh water cyanobacterium *Anabaena cylindrica* results in a removal of subunit B from the enzyme with a reported structure being A_8[15], which in fact was later shown to be of A_8B_8 structure[16]. The enzyme from some species may also dissociate into subunits under certain conditions, resulting in the report of incorrect structures. An example is found in RuBisCO from a halophilic cyanobacterium *Aphanothece halophytica,* in which the structure A_4 was reported in studies under conditions of low ionic strength[17], whereas the A_8B_8 structure was found when care was taken to isolate the enzyme under conditions of high ionic strength[18].

The initial model structure of the enzyme protomer, containing eight pairs each of subunits A and B, was proposed from experiments in spinach RuBisCO, which showed eight binding sites for RuBP and eight binding sites for the reaction intermediate analog, carboxyarabinitol bisphosphate (CABP), per mole spinach enzyme[19,20]. The quaternary structure of RuBisCO has been extensively studied in *Alcaligenes eutrophus* and *Nicotiana tabacum,* which are representatives of the enzymes from prokaryotes and eukaryotes respectively.

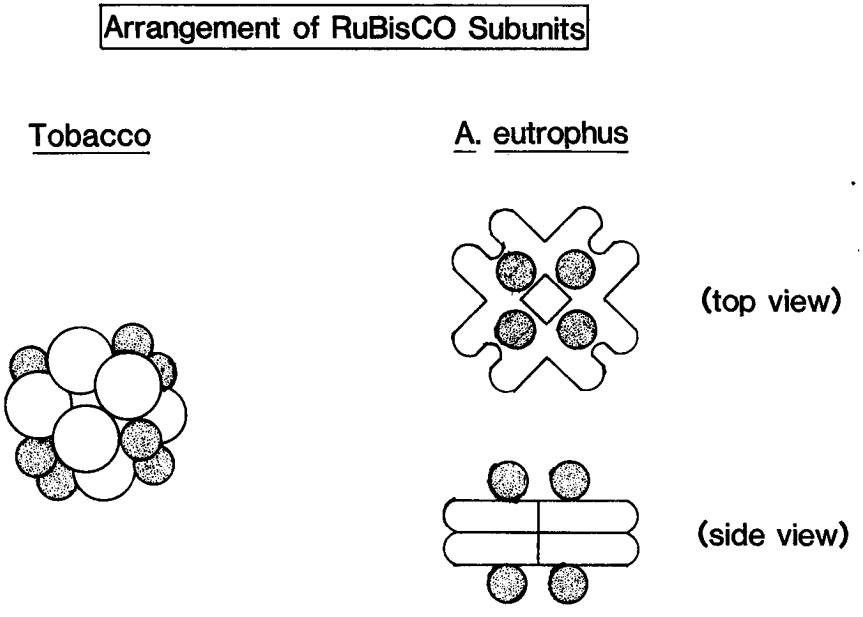


Fig. 1 *Arrangement of subunits forming the quaternary structure of RuBisCO from tobacco (left) and A. eutrophus (right).* The model of tobacco enzyme is viewed obliquely, whereas for *A. eutrophus* enzyme the top view (top) and the side view (bottom) are shown.

The proposed structures, shown in Fig. 1, are based on a combination of techniques including X-ray diffraction, electron microscopy and optical diffraction analyses. In the tobacco protein, the two subunits are arranged as a bilayer, in which the eight A subunits are organized as a cube and pairs of B subunits are positioned onto four out of the six faces of the cube of A subunits[21,22]. This results in a 6-fold symmetry with a well defined central hole in one axis. The bacterial protein, on the other hand, consists of four bilayers of subunits comprising of two central eclipsed layers, each of which contains four U-shaped A subunits arranged perpendicularly to the 4-fold axis of symmetry[23,24]. The other two outer layers each consists of four B subunits in eclipsed positions to the A subunits. The arrangement of bacterial protein results in the contact of one subunit B with one subunit A, as opposed to one subunit B in contact with two A subunits in the tobacco protein. Experiments using cross-linking reagent demonstrated that B subunits of pea leaf RuBisCO are closely paired[25]. It remains to be seen whether the same technique can be applied to study the arrangement of B subunits in the bacterial protein. It is noted that the crystal of tobacco protein used for structural determination was the deactivated form, while that of the bacterial protein was the activated form. Recently, studies of the shape of the enzyme from spinach, using small angle neutron scattering measurements, have shown that the structure of the enzyme, in both activated and deactivated forms, resembles that determined for the deactivated enzyme from tobacco[26]. The estimated dimensions of the model structure of the spinach enzyme also correspond strikingly well to those predicted for adjacent pairs of A subunits in the tobacco protein. The previous suggestion that differences in structural arrangements between the plant and bacterial enzymes may result from the different states of the enzyme is thus unlikely[27]. Velocity sedimentation experiments, showing that the average $S_{20,w}$ was unchanged for both activated and deactivated enzymes from either spinach or *R. rubrum* enzyme, and the lack of differences in scattering data for both forms indicate that no major conformational changes occur upon activation of the spinach enzyme[26]. These recent data are at variance with previous spectroscopic data supporting a change in the conformation of the enzyme upon activation[28,29], including reported differences in sedimentation coefficients between activated and deactivated *A. eutrophus* enzyme[30]. Bowien *et al* suggested that structural differences between higher plant and bacterial enzymes may be related to the differences in the evolution of the two enzymes[24]. More recently, however, Anderson *et al* have cloned the genes of RuBisCO from *A. eutrophus* and found their sequences to be remarkably similar to those of *Anacystis nidulans* and tobacco[31]. Thus the possibility that the genes from *A. eutrophus* evolve separately can be ruled out. The observations that the pattern of residue hydrophobicities is similar along the two polypeptides of activated enzyme of *R. rubrum* and activated tobacco subunit A suggest similar three-dimensional folding of subunit A in bacterial and plant enzymes[32]. Up until now, most of the available data tend to favor the structural arrangement proposed for RuBisCO from tobacco. It is the authors' view that the discrepancies concerning the structural differences between higher plant and bacterial enzymes, the question of and whether the activation process is really accompanied by a conformational change, can be

resolved by more comparative studies on RuBisCO from other sources, especially from bacterial RuBisCO having the A_8B_8 structure. Recently, the crystal structure of RuBisCO (A_8B_8) from a purple sulfur bacterium *Chromatium vinosum* has been reported[33].

Amino Acid Composition

The A subunits from various divergent organisms have been shown to be strikingly homologous[27]. The evolutionary conservation of the subunit A polypeptide is consistent with the fact that subunit A is the site containing the catalytic entities. With the recent advances in recombinant DNA methodologies, the genes coding for subunit A from a number of higher plants, as well as some algae and bacteria, have been cloned and sequenced. The amino acid sequences deduced from such cloned genes may then be compared. For example, striking sequence homology (greater than 85 %) is observed between green alga *Chlamydomonas* and higher plant *Zea mays*. More detailed analysis of the conservation of the amino acid residues will be described in later section. Subunit A of *R. rubrum* enzyme contains a very different amino acid composition from the plant enzymes. This is initially based on the S $\triangle$ Q analysis of relatedness of the structural homologies for subunit A[34]. Recent amino acid sequence of *R. rubrum* enzyme, determined by classical methods[35] and by deduction from the cloned gene[36], has shown only about 25 % homology to subunit A from other sources. However, there are short stretches of *R. rubrum* polypeptide, including those implicated as activator and catalytic sites, with greater than 80 % homology to other subunit A polypeptides. It can then be assumed that throughout evolution, subunit A has been genetically well preserved. In this connection, it is worth mentioning the controversies about the heterogeneity of subunit A. Heterogeneity of subunit A has been basically demonstrated by the variation in the isoelectric focusing patterns of carboxylated subunits[37]. Later evidence pointed to the conclusion that subunit A is homogeneous. For example, the heterogeneity of subunit A was reported to arise as an artifact during the alkylation procedure that preceded the isoelectric focusing[38], and that the presence or absence of heterogeneity depends on the age of the tissue used and also on the procedure of the extraction of the enzyme, indicating that the heterogeneity observed can be a product of a limited modification rather than the real existence of more than one subunit A pool[39].

On the other hand, B subunits are quite dissimilar in amino acid compositions. These dissimilarities have been observed among different species, and also among different genera of higher plants. The amino acid sequence of subunit B appears to be species-specific. The rate of amino acid substitutions in subunit B has been calculated to be significantly greater than that for subunit A[40]. Comparative studies of various higher plant enzymes indicate that subunit B is much more variable in the 40 amino acids of its N-terminal sequence than is subunit A[41].

Biosynthesis

RuBisCO, as well as many chloroplast proteins, requires the activities of both the chloroplast and nuclear genetic systems for its biosynthesis. Subunit A is encoded by chloroplast DNA, whereas subunit B is encoded by nuclear DNA. The site of subunit B synthesis is in the cytoplasm. It is now established that subunit B is synthesized as a larger precursor, which is then processed to the mature size during or shortly after transport across the chloroplast envelope to assemble with subunit A[42-44]. For example, subunit B of RuBisCO from pea was shown to be synthesized as a polypeptide with M_r 20,000 (P 20). This P 20 binds to the surface of the chloroplast and enters the chloroplast in an ATP – dependent manner[45]. The precursor is then processed by soluble chloroplast protease activity in two steps to the mature size of M_r 14,000 via an intermediate of M_r 18,000[46,47]. Similar studies on the processing of subunit B from a green alga *Chlamydomonas reinhardtii* have recently shown that subunit B is also processed to the mature form M_r 16,500 in two steps via an intermediate of M_r 18,500[48]. It is noted that for prokaryotic RuBisCO where there is no need for subunit B to be transported across the membrane, subunit B is synthesized as a mature form[49].

The other constituent of RuBisCO, subunit A, is synthesized probably as a precursor form[50] by stroma-associated ribosomes[51]. Recently, however, thylakoid-bound polyribosomes from mature spinach chloroplast have been shown to synthesize subunit A of RuBisCO[52]. By immunoprecipitation analysis of the translation products of thylakoid-bound polyribosomes using a wheat germ cell-free system, Hattori and Margulies could detect in the thylakoid fraction mRNA for subunit A accounting for at least 44 % of total translatable chloroplast mRNA, thus suggesting that thylakoids may be an important site of subunit A biosynthesis. Although the role of thylakoid-bound polyribosomes for the synthesis of thylakoid proteins seems to be established, the localization of mRNA for subunit A is still controversial.

To be functional, the synthesized A and B subunits must assemble to form the A_8B_8 molecule. The mechanism of the assembly is still not very well understood. It seems that the newly synthesized subunit A is bound to the subunit A binding protein before assembling with subunit B[53]. Recent developments in the study of subunit A binding protein have shown that the binding protein in pea has an M_r of 720,000 and is composed of equal numbers of two types of subunits of M_r 61,000 and 60,000, respectively[54]. The structure of this protein is thus $\alpha_{16}\beta_6$. It is not synthesized by isolated chloroplasts but it can be precipitated from products of pea poly A^+ mRNA translated in a wheat germ extract. These results indicate that the binding protein is a product of cytoplasmic ribosomes and that its two subunits are probably encoded in nuclear genes. It is thought that one of the roles of this binding protein is to maintain subunit A in a solubilized form suitable for assembly with subunit B[55]. When maize chloroplast subunit A gene is expressed in *E. coli in vivo,* the resulting gene product is insoluble, probably due to the absence of the binding protein in *E. coli.* It is not certain whether the combination of subunit A with the binding protein

is an obligatory step in the assembly of the holoenzyme in the higher plants. Although little is known of the mechanism of subunit assembly at present, there have been signs that efforts to unveil this mechanism have been increasing. Recent studies on three cyanobacteria[56-58] and one bacterium[59] have demonstrated the successful assembly of the two subunits to make the holoenzyme. All methods employed recombinant DNA procedures in which the genes for A and B subunits were cloned and expressed in *E. coli*. These achievements may open up ways in which site-specific mutagenesis may be used to study the site(s) in subunit B involved in combining with subunit A. In analogy to the work done using recombinant DNA techniques, the study of subunit assembly can also be approached by utilizing the reversible reconstitution of the catalytically active enzyme molecules as reported in various organisms[60,61]. It might be possible to locate the site(s) on subunit B involved in recombining with subunit A by employing site-specific affinity labels in conjunction with the use of cross-linking reagents.

Functional Aspects

Activation of the Enzyme

The indication that there may be two forms of RuBisCO, namely activated and nonactivated forms, comes from the observations that the enzyme can exhibit low and high affinity for substrate CO_2 and that the activity of RuBisCO can be enhanced by preincubation with CO_2 and magnesium ion[62-64]. Kinetics as well as physical studies have been intensively investigated since the mid 1970's. The enzyme having high affinity for CO_2 is, in fact, the activated form of the enzyme[65,66].

It is now well established that the activation applies to both carboxylase and oxygenase activities. The kinetics of activation and inactivation are the same for both activities. CO_2, rather than HCO_3^- is the species involved in the activation process[66]. Activation is initiated by the addition of activator CO_2 ($^ACO_2$) to the ε-amino group of lysine to form a carbamate[66]. The carbamate formation is a rate-limiting step which is then followed by a rapid addition of a divalent metal ion (usually Mg^{2+}) to form the activated ternary enzyme.$^ACO_2$.Mg complex. The evidence that $^ACO_2$ is different from substrate CO_2 ($^SCO_2$) has been obtained by two independent experiments. Miziorko utilized the competitive binding of CABP with $^ACO_2$ at the activator site[67]. He showed that CABP, which contains the element of $^SCO_2$, was not able to displace $^ACO_2$ from the enzyme. This indicated that the carboxyl moiety of CABP and $^ACO_2$ bind at different sites. Lorimer[68] also confirmed this by using kinetic turnover experiments in which he showed that the ^{14}C bound to the enzyme.$^ACO_2$.Mg complex, after kinetic turnover, was not isotopically equilibrated with the CO_2 in the medium. This is the case when $^ACO_2$ and $^SCO_2$ are bound at different sites. The carbamate formation was shown to occur exclusively in subunit A[69]. Many studies on the active site of RuBisCO have employed CABP, an analog of the reaction intermediate, as a probe. The usefulness of CABP is based on the

observation that the addition of CABP to the activated ternary enzyme.$^{A}CO_2$.Mg complex leads to the formation of an exceptionally stable quaternary enzyme.$^{A}CO_2$.Mg.CABP complex with a stoichiometry of 1:1:1:1[67,70]. The stability of the complex is demonstrated by the inability of either $^{A}CO_2$ or Mg^{2+} to exchange freely with the free ligand. This is a useful property because one can specifically trap either $^{A}CO_2$ or metal ion, and their kinetics as well as localization can be followed if either $^{A}CO_2$ or metal ion is made radioactive. The utility of CABP has been exploited in showing the $^{A}CO_2$ to be different from $^{S}CO_2$[66], in studying the binding domain of activator site[69,71], in examining the effect of various effectors on enzyme activity[72,73], as well as in the quantitation of the amount of RuBisCO[74,75]. Recent experiments employing CABP have indicated the importance of carbamate formation as a prerequisite to catalytic competence in *R. rubrum* enzyme[76]. Site-specific mutagenesis in which lysine 191, the residue involved in carbamate formation[77], is replaced with glutamate produces enzyme that lacks enzyme activity under standard assay conditions. The proof that the lysine 191 → glutamate 191 mutant enzyme does not form a carbamate comes from the demonstration that the binding of CABP is not stabilized by incubating the mutant enzyme with divalent metal and CO_2. On the other hand, the mutant enzyme is capable of binding CABP suggesting that the amino acid substitution does not cause a major change in tertiary structure and that carbamate is not specifically involved in the binding of the substrate RuBP. From this study, it is suggested that the creation of an ionic site by carbamate may reflect a possible regulatory value in that, without CO_2 as a substrate for carboxylation, the enzyme remains in an inactive state. Although the metal binding in the mutant is not directly shown it is implied that the mutant enzyme is unable to bind Mg^{2+}. This is consistent with the body of evidence suggesting the participation of metal ion in catalysis. The role of metal ion in catalysis will be described later. An attractive proposed role of metal ion in activation as a stabilizer through interaction with a negatively charged carbamate still lacks direct experimental proof to support it.

Catalysis

Both carboxylation and oxygenation are catalyzed by the same enzyme. Some supporting lines of evidence include the fact that both activities are activated and inhibited in parallel[78-80], and the fact that both activities have co-evolved[81]. The mechanism of the carboxylation reaction first involves the formation of two intermediates, the enediol of RuBP[82,83] and the carboxylated 6-carbon intermediate[84]. The latter intermediate is stabilized via interaction with metal ion[85] and is subsequently hydrolyzed at the C2-C3 bond of the 6-carbon intermediate[86] to yield two molecules of 3-phosphoglycerate. Recent NMR measurements and isotope trapping experiments have indicated that the carboxylation of RuBP proceeds via the ordered addition of RuBP followed by reaction with the gaseous substrate[87]. The mechanism of oxygenation of RuBP is much less understood compared with the carboxylation reaction. However, a similar mechanism to carboxylation has been proposed in which the oxygenation of RuBP results in the formation of 5-carbon

intermediate which is finally hydrolyzed to yield one molecule each of 3-phosphoglycerate and 2-phosphoglycolate. A more recent experiment has shown that once the carboxylated 6-carbon intermediate is formed it is committed to product formation[87a]. By analogy, the corresponding 5-carbon intermediate in the oxygenation reaction was suggested to be similarly committed for product formation. Therefore, the crucial step in the enzyme reaction resides at the step where either CO_2 or O_2 substrate is added to the enediol of RuBP.

Biochemical Studies at the Active Site

The active site of RuBisCO resides in subunit A. Many investigations to identify the residues involved in catalysis have made use of group-specific or active site-directed reagents. The rationale of the use of group-specific reagents is based on the selectivity of the reagent for a specific residue resulting in the inactivation of the enzyme. The residues implicated as essential for catalysis are lysine[88,89], arginine[90-92], tyrosine and histidine[93-94], cysteine[95,96], and the carboxyl group[97]. As has been pointed out by Miziorko and Lorimer[27], the usefulness of group-specific reagents is limited by their inherent non-selectivity. To circumvent this problem, active site-directed affinity labels have been extensively employed by Hartman's group. They have identified the essentiality of lys-175, lys-334 of spinach subunit A[98] through the use of such affinity labels. By using affinity labels and sequencing of the labelled tryptic peptide in *R. rubrum* enzyme, in comparison with the sequence of spinach enzyme, Hartman *et al*[35] could assign at least three active site peptides and one activator site peptide as shown in Fig. 2. The first active site peptide encompasses

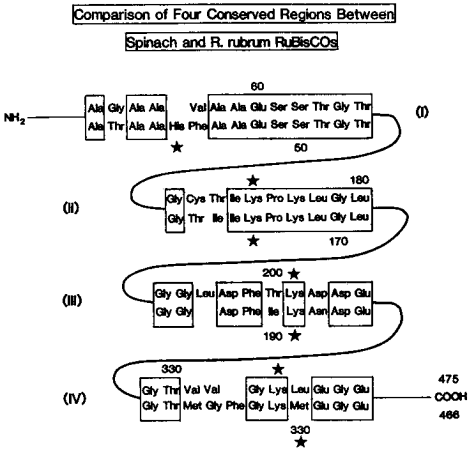


Fig. 2 *Comparison of four conserved regions between spinach and R. rubrum RuBisCO.* Amino acid sequences of spinach (upper) and *R. rubrum* (lower) RuBisCOs identified as three active site peptides (I, III, and IV) and one activator site peptide (II) are shown. Residues identical in both enzymes are boxed. Residues marked with star symbols indicate those identified by selective chemical labelling.

lys-175 in the spinach enzyme and the corresponding lys-166 in the *R. rubrum* enzyme. There are seven identical residues encompassing the reactive lysine residue. A second active site region contains lys-334 in the spinach enzyme and the corresponding met-330 in the *R. rubrum* enzyme. Although met-330 is not essential for enzyme catalysis because it is replaced by leu-335 in the spinach enzyme, the conserved amino acids around lys-334 provide evidence for the involvement of this peptide in catalysis. The last active site peptide is a region encompassing his-44 in the *R. rubrum* enzyme, which is implicated by the selective labelling of histidine with 2-(4-bromoacetamido) anilino-2-deoxypentitol-1,5-bisphosphate[99]. Recently, experiments with trinitrobenzene sulfonate (TNBS) inactivation of RuBisCO suggested that lys-334 of the spinach enzyme and lys-166 of the *R. rubrum* enzyme are important to catalysis rather than to substrate binding[100]. This is based on the observations that the spinach enzyme, after selective reaction of lys-334 with the inhibitor, can still exhibit tight binding with CABP, and that the enzyme of *R. rubrum* exhibits an unusually low pK_a (7.9) of lys-166 as determined by titrating with TNBS. That lys-166 of *R. rubrum* enzyme is not required for CO_2/Mg^{2+}-dependent activation or for substrate binding has been recently confirmed by replacing lys-166 with glycine-166 using site-directed mutagenesis. The mutant enzyme lacked enzyme activity but was able to form a complex with CO_2,Mg^{2+} and CABP[100a].

Possible Role of Metal in Catalysis

The first strong evidence to suggest the involvement of metal in catalysis came from ^{13}C-NMR data showing that the metal (Mn^{2+}) and the substrate CO_2 are closely situated[101]. Further evidence includes the ability of different cations in changing the ratio of carboxylase : oxygenase reactions[102,103], as well as the inhibitory effect of F^- on the enzyme activity[104]. Other metals that were shown to coordinate directly with substrates are Cu^{2+} and Co^{2+}. Coordination of Cu^{2+} to either [^{17}O]-water or [2 – ^{17}O] -RuBP was established by paramagnetic resonance spectroscopy[105]. There are many steps in catalysis that may involve participation of metal. For example, since Cu^{2+} coordinates to one oxygen atom in the carboxyl group of CABP and to one water molecule, it is possible that Cu^{2+} may facilitate the hydrolytic cleavage of the 6-carbon reaction intermediate by delivering water to the cleavage site. Metal can also stabilize the intermediate of the reaction by serving as an electron sink[27], or it may be involved in the initiation of the first step, i.e., formation of an enediol intermediate[106].

The consequence of the close proximity of a metal ion to the substrate and its coordination to the carbamate at the activator site is that the metal ion can help bring about the close proximity between the catalytic site and the activator site. This is supported by the fact that only one metal binds per active site[101,107]. The close proximity between catalytic and activator sites has also recently been confirmed by experiments using NMR techniques[108]. The quaternary complexes enzyme.$^{A}CO_2$.Me.CABP, were prepared with various metals. ^{31}P and ^{13}C NMR studies of these complexes demonstrated that the

activator CO_2 site, the metal binding site, and the substrate binding site are contiguous, i.e., both activator site and catalytic site share a common domain.

Functional Importance of Subunits

Subunit A: There are at least four lines of evidence strongly implicating subunit A as the site of carboxylase reaction. First, the *R. rubrum* enzyme does not contain subunit B, therefore the catalytic activity resides in subunit A. Second, activator CO_2 and metal in the enzyme.$^{A}CO_2$.Me complex bind directly to amino acid residues in subunit A[71,107]. Third, by the use of group-specific and affinity-labelling reagents that act as active site-directed inhibitors, several essential amino acid residues are found to locate on subunit A[95,109,110]. Fourth, antibody directed against subunit A of spinach enzyme inhibits the enzyme activity[111].

Kinetic and immunochemical studies indicate that oxygenase function is also located in subunit A[111,112]. Moreover, both carboxylation and oxygenation occur at the same site[113]. This is substantiated by kinetic analysis of competitive inhibition of the carboxylase reaction by O_2 and of the oxygenase reaction by CO_2, i.e., K_m (CO_2) of carboxylase = K_i (CO_2) of oxygenase and that K_m (O_2) of oxygenase = K_i (O_2) of carboxylase[114].

Subunit B: At present it is not exactly known what role subunit B plays in the catalysis of RuBisCO, despite considerable efforts by many investigators. Subunit B was suggested to play a regulatory role in enzyme catalysis[115]. This is based on the observed Mg-induced optimal pH shift for the enzyme with the presence of subunit B but not for enzyme without subunit B or enzyme treated with antiserum against subunit B. The fact that the *R. rubrum* enzyme lacks subunit B, and yet can be activated by CO_2 and Mg^{2+}, and can catalyze both carboxylase and oxygenase reactions, indicates that activation and catalysis are not absolutely dependent on subunit B. Most studies on the function of subunit B have been done by comparing various properties of the enzyme containing subunit B with those of the enzyme lacking subunit B. By this way, Gibson and Tabita concluded that subunit B accelerated the rate of activation of the enzyme by the observation that form I of *Rhodopseudomonas spheroides* enzyme (A_8B_8) was activated more rapidly than form II (A_6) enzyme[116]. However, this conclusion did not take into account the possibility that the different activation rate might arise from a difference in the structure of subunit A, which in fact was the case as proven by the lack of immunological cross reactivity of subunit A between form I and form II[117]. It is thus more appropriate to say that the presence of subunit B may help to maintain the favorable structure needed for activation. More recently, work on *Aphanothece* and *Chromatium* RuBisCO has demonstrated the involvement of subunit B in the activation process by stabilizing the ternary activated enzyme[118]. Subunit B does not participate in an essential manner in the activation process *per se*, i.e., its presence is not absolutely required for the reaction of the enzyme

with $^{A}CO_2$ and Mg^{2+}, but it stabilizes the ternary enzyme.$^{A}CO_2$.Mg^{2+} complex.

The function of subunit B has just recently been described by two independent groups, namely Andrews' and Akazawa's. The two groups have made use of the reversible dissociation of the enzyme by relatively mild treatments, i.e., mild acidic pH for *Synechococcus* enzyme[119], and low ionic strength condition and slightly alkaline pH for *Aphanothece*[18,120] and *Chromatium*[60] enzyme, respectively. Both groups almost simultaneously demonstrated the essentiality of subunit B for both carboxylase and oxygenase activities. Andrews' group reported that the enzyme activity corresponded to the amount of subunit B associated with the separated catalytic core (A_8) of *Synechococcus* enzyme[119], whereas Akazawa's group showed that the increase of enzyme activity corresponded to the amount of subunit B added to the catalytic core of *Aphanothece* enzyme[18,121]. The two groups further showed that subunit B is not essential in the binding of substrate, based on the results that formation of the quaternary enzyme.$^{A}CO_2$.Mg.CABP complex with a stoichiometry almost equal to one is independent of subunit B[122,123].

There have been suggestions that the role of subunit B may be to modulate the affinity of the enzyme for substrate CO_2. These suggestions arise from the different K_m (CO_2) values between *R. rubrum* and plant enzymes, where about 10-fold lower affinity for CO_2 is observed with *R. rubrum* enzyme[124]. However, recent experiments utilizing the hybrid enzyme in which both A and B subunits were derived from different organisms did not appear to support the role of subunit B in modulating the affinity for substrate CO_2[118]. Although the increase of K_m (CO_2) of the heterologous hybrid between *Synechococcus* subunit A and spinach subunit B compared to the homologous *Synechococcus* enzyme was observed, this was the consequence of qualitative changes at the active site induced by the reassembly process, i.e., it was not due to the inherent property of subunit B *per se*[125]. Recently, Andrews, *et al* reported that subunit B is also required in all three partial reactions of the carboxylation reactions[126].

Apart from the studies on the function of subunit B using reversible dissociation system, it is also possible to study the interaction between subunits A and B. Such studies have been demonstrated in a series of experiments by Incharoensakdi *et al*[60,61] showing the successful reconstruction of catalytically active RuBisCO molecules in which subunit A and B are derived from different species of divert origins. Furthermore, by using reversible dissociation of *Aphanothece* RuBisCO they also showed that the nature of subunit interactions is hydrophobic and that the binding of substrate RuBP to the enzyme can influence the association between the two subunits[121]. More detailed studies are required to locate the residues involved in subunit interactions. Techniques such as fluorescent spectroscopy in conjunction with the use of either affinity labelling or cross-linking reagents can be employed to facilitate such studies. The results obtained may help to better understand the assembly process of RuBisCO molecule.

Regulatory Aspects

Regulation at the Level of Enzyme Activity

The regulatory mechanism of RuBP carboxylase *in vivo* is very complicated. Most studies on enzyme regulation have dealt with the effect of the light-dark transition. The activity of RuBP carboxylase is high in light and is low in the dark. The suggestion that the activity is low in the dark comes from early experiments showing that the pool of RuBP is not completely used up by the enzyme during light to dark transitions[127]. The effect of light has been shown to increase the pH of the chloroplast stroma to about 8.1, which is the optimal pH for CO_2 fixation[128,129]. Increased Mg^{2+} level due to light is also observed[130]. However, from the low concentrations of CO_2 and Mg^{2+} *in vivo* compared to those required in the activation process[64], the amount of enzyme in the active ternary form would be only about 20 % of the total enzyme at physiological conditions of pH and CO_2 concentration. To increase the activation of the enzyme, various chloroplast metabolites have been shown to stimulate enzyme activities at suboptimal CO_2 and Mg^{2+} concentrations[131-133]. These chloroplast metabolites, the so-called effectors, including NADPH, 6-phosphogluconate, and fructose bisphosphate, were found to exert their influence by stabilizing the active ternary form of the enzyme[73]. More recently, Jordan *et al*[134] have demonstrated that activation promoted by such effectors is mediated by the alteration in the relative rates of activation and deactivation to favor the active enzyme, rather than the preferential binding of effectors to the activated enzyme.

Another aspect of light-modulated regulation of pea RuBisCO has recently been reported to be due to changes in the concentration of an inhibitor, a phosphate ester, capable of tight binding with the eyzyme catalytic site[135]. This phosphate ester was later identified as 2-carboxyarabinitol-1-phosphate[136]. It is claimed that the levels of the inhibitor *in vitro*, which vary with light intensity, could account for light-dependent changes in RuBisCO activities. A phosphorylated inhibitor in tobacco leaves was also independently reported by Servaites[137] and again the same compound as that in pea was shown to be present in tobacco leaves[137a].

The activity of RuBisCO can also be regulated by changes in the structure of the enzyme caused by other environmental factors apart from light. Conformational changes of the enzyme without the dissociation of subunits were shown to be closely associated with the cold-induced inactivation of tobacco enzyme[138,139]. On the other hand, RuBisCO from *Aphanothece halophytica* is inactivated at low temperature as a result of a partial dissociation of subunits[121]. More recent experiments have demonstrated the importance of a quaternary ammonium compound, glycinebetaine which is present *in vivo*, in stabilizing RuBisCO molecule of *Aphanothece halophytica*[140]. Glycinebetaine is able to protect RuBisCO against heat and cold inactivation as well as to reduce the extent of the dissociation of subunits. It is not known whether glycinebetaine can also stabilize RuBisCO from other sources although the compound has been reported to accumulate inside a number of plants[141].

Another facet of the regulation of RuBisCO has recently been unveiled. A new enzyme, RuBisCO activase, has been reported to be responsible for the activation of RuBisCO *in vivo*[142]. This is based on comparisons of the properties of the wild type and the mutant (incapable of activating RuBisCO upon illumination) RuBisCO from the stromal extracts of *Arabidopsis* leaves. In the mutant, the absence of RuBisCO activation was correlated with the absence of the chloroplast polypeptides. Moreover, a soluble chloroplast extract could stimulate RuBisCO activation in an illuminated reconstituted system containing chloroplast thylakoid membranes and RuBisCO. The observation that RuBisCO activation in illuminated leaves is insensitive to changes in the external CO_2 concentrations also supports the concept that RuBisCO activation *in vivo* is not a spontaneous process but is catalyzed by a specific protein, RuBisCO activase[142a]. More recently this protein has been partially purified[142b]. In the illuminated reconstituted system, this protein was able to catalyze RuBisCO activation at the physiological concentrations of 10 μ M CO_2 and 2 to 4mM RuBP found in intact leaves. RuBisCO activase is also present in the chloroplasts of many plants as revealed by Western blot analysis[143]. The 1.6 kb cDNA clone for RuBisCO activase from spinach leaf has recently been obtained[144]. This clone, after subcloning into pUC 8 and testing for expression, produced three polypeptides cross-reacting with anti-activase antibodies. The translation products of mRNA, hybrid-selected with the 1.6 kb cDNA, also comigrated with spinach leaf activase on SDS-polyacrylamide gel electrophoresis. It is quite certain that the discovery of activase and the availability of its cDNA clone will form a new foundation for further study on the activation of RuBisCO.

Regulation at the Level of Gene Expression

The most important environmental factor controlling the synthesis of RuBisCO is light. In pea, RuBisCO is barely detectable in dark-grown plants and accumulates after illumination[145]. In barley, RuBisCO is present in the dark and additional accumulation occurs upon illumination[146]. Light is required for both the initiation and continued increase in net synthesis of *Euglena* RuBisCO over the dark level[147]. The expressions of the chloroplast subunit A gene and nuclear subunit B gene are controlled by light and these effects are mediated by phytochromes[148-150].

It now appears that light affects the regulation of subunit A synthesis at the post-transcriptional level[151]. This is based on the observations that 1)although dark-grown pea chloroplasts show low levels of subunit A synthesis, the organelle contains a significant level of mRNA for subunit A and 2) dark-grown plants or dark-grown plants subjected to light are found to contain similar amounts of translatable mRNA for subunit A per unit weight of plastid RNA, i.e., the increase in mRNA for subunit A as affected by light is related largely to the increase in chloroplast genome copy number and not due to a significant increase in transcription *per se*. On the other hand, effect of light on mRNA for subunit B of pea seedlings appears to be due to a specific increase in transcriptional activity, since

the copy number of subunit B gene in the nucleus is not significantly affected by light[149,152]. Recent work by Sasaki demonstrating the inhibition of the induction of subunit B mRNA by α-amanitin supports the notion that subunit B synthesis is under transcriptional control[153].

A series of studies concerning the light regulation of the subunit B gene has come from Chua's group. It now appears that subunit B is encoded by more than one gene, a multigene family, within the nuclear DNA. The use of petunia plant transformed by T_i plasmid containing subunit B gene from pea has shown that a pea subunit B gene (pPS 4.0) can be expressed after being transferred into petunia cells[154]. Messenger RNA transcripts from the transferred pea gene are processed correctly and translated to yield mature subunit B polypeptide which is localized in chloroplasts of petunia cells. The region of conserved 33-base pair sequence around the *TATA* box is identified as being able to confer light inducibility of subunit B gene[155]. These experiments were based on the use of deletion mutants at the 5′-flanking regions of *rbc*S-E9, which enabled the 5′ boundary of the light responsive sequence to be identified at -35 position (minus sign indicates upstream direction from the transcription initiation site). The 3′ limit of the light responsive sequence was established by using the chimeric gene in which the -1,052 to -2 sequence of *rbc*S-E9 was fused with bacterial chloramphenicol acetyltransferase (cat) gene. This chimeric gene could be expressed in the petunia cell and its expression was light-regulated.

Regulation through Manipulation by Site-Specific Mutagenesis

Structure-function relationships are known to exist in many enzymes. The function of an enzyme can be modified through the modification of its structure. RuBisCO has been targeted as a promising enzyme for such modification by the techniques of recombinant DNA. This is not surprising since the dual function of the enzyme as carboxylase and oxygenase reduces greatly the efficiency of photosynthetic process. It has been estimated that complete suppression of oxygenase reaction could increase net photosynthetic productivity by 45 %[156]. Experiments designed to test whether the carboxylase and the oxygenase can be changed independent of one another have yielded mixed results. For example, Laing *et al*[157] found that temperature gives different responses to carboxylase and oxygenase. On the other hand, Chollet and Anderson[158] could not detect any differential regulation of carboxylase and oxygenase of tobacco RuBisCO using temperature pretreatments and chloroplast metabolites. From detailed studies of the reaction mechanism and structure of RuBisCO, it has been concluded that carboxylase and oxygenase reactions are an inevitable consequence of the chemistry of reactions involving RuBP[159]. However, Jordan and Ogren[160] have reported that the specificity factor of the two activities, i.e., the ratio of carboxylase to oxygenase, varies among different organisms, a difference of as much as one order of magnitude is observed between some photosynthetic bacteria and higher plants. It is possible then to change the

partition coefficient through the manipulation of the structure of the enzyme by site-specific mutagenesis. Until recently, only the enzyme from *R. rubrum* has been employed for such mutagenesis. This is partly due to the simplicity of the enzyme structure that does not require two different subunits for activity. Two recent attempts to effect the partition coefficient of the *R. rubrum* enzyme have been reported. Replacing asp-198 with glu-198 does not alter the carboxylase : oxygenase ratio[161]. However, by EPR spectroscopy analysis of the quaternary enzyme.$^{A}CO_2$.Mn.CABP complex of the wild type and mutant enzymes, further support that asp-198 contributes to the formation of the metal binding site is obtained. Similarly, a change at met-330 to leu-330 is not able to affect the carboxylase : oxygenase ratio[162]. The insensitivity of the carboxylase : oxygenase ratio might be due to the lack of subunit B. However, it has been shown that subunit B does not influence the partition coefficient of *Synechococcus* enzyme[125]. The hope to modulate the partition coefficient must await more detailed data on X-ray crystallographic studies of the enzyme so that the putative residues essential for enzyme catalysis can be located.

Concluding Remarks

RuBisCO catalyzes reactions of opposing consequences. The carboxylase reaction leads to CO_2 assimilation and photosynthesis, whereas oxygenation opposes photosynthesis by initiating photorespiration. One of the ultimate aims of the study of RuBisCO is to manipulate the enzyme so that the ratio of carboxylase to oxygenase can be increased. The primary structure of subunit A has been mapped out and at least four regions have been identified as essential for enzyme catalysis. Recent advances in recombinant DNA technology make it possible to alter the structure of one of these four regions by means of site-specific mutagenesis. The obvious obstacle is how to select an appropriate residue for the manipulation. Until the data on the X-ray crystallography of the enzyme become available, what can be done is to select the residues that reside in the four regions for mutagenesis. The effects of these alterations can be assessed by isolating the mutated subunit A, which is expressed in *E. coli*, and recombining it with the isolated subunit B *in vitro*. To be on safe ground, the source of the enzyme for this purpose should be that from cyanobacteria or bacteria as the successful reassembly of the two subunits *in vitro* has only been shown in these two sources, not yet in higher plants.

Figure 3 illustrates the current knowledge of the biosynthesis and the regulation of RuBisCO. The synthesis of RuBisCO requires the cooperation of both chloroplast and nucleus. Light is the major regulatory element in many steps. The crucial step in producing the functional enzyme is the assembly process. It is this assembly mechanism that we are partly ignorant of. Further basic research in this area, especially through the use of recombinant DNA techniques, is needed. The enzymes having A_8B_8 structure from some bacteria and cyanobacteria will prove to be good starting materials since the genes for A and B subunits are cotranscribed and some experiments have already shown the successful

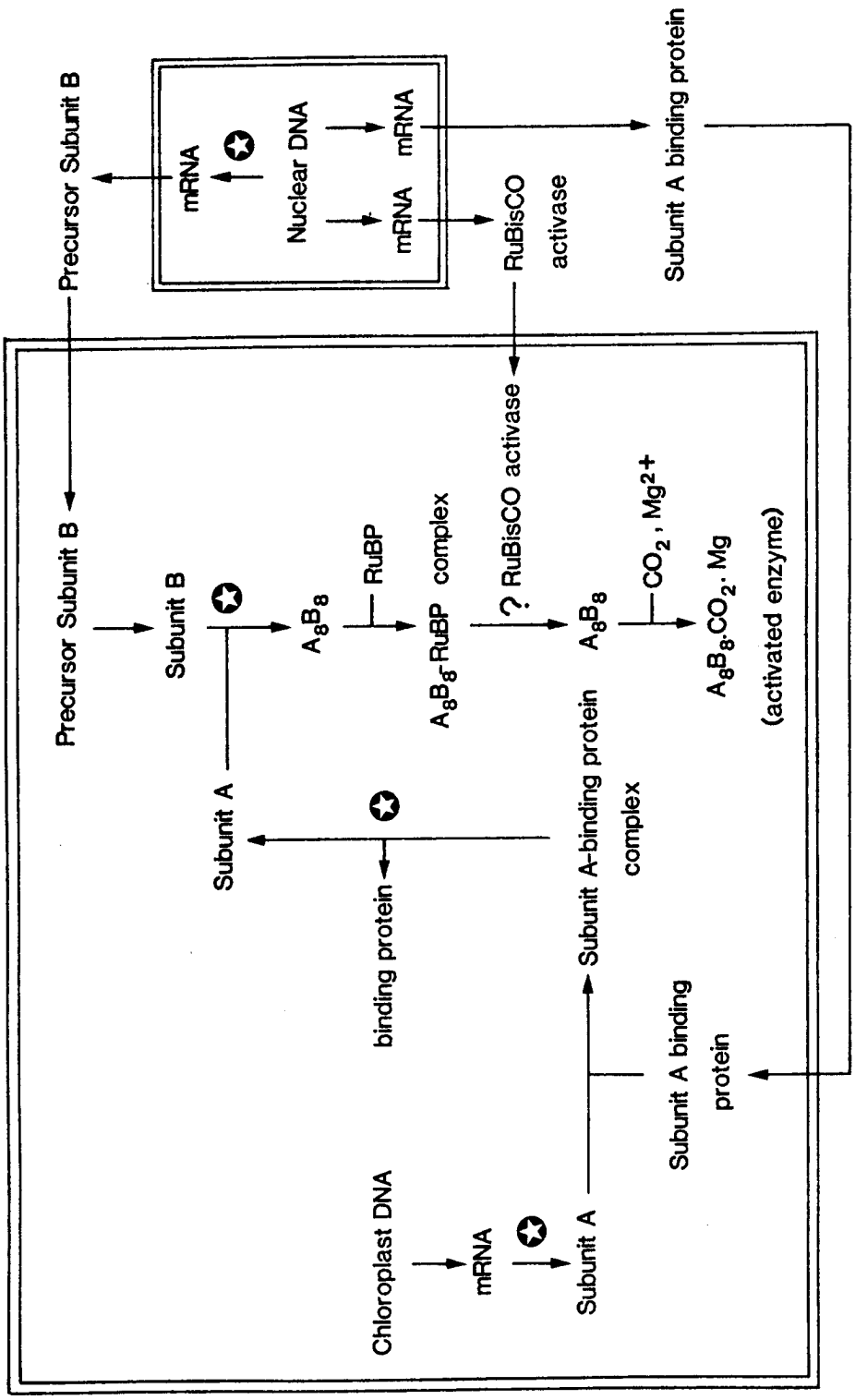


Fig. 3 *Diagrammatic presentation of current knowledge of biosynthesis and light-regulation of RuBisCO.* The cooperation between chloroplast and nuclear activities is shown. The steps regulated by light are marked by star symbols.

expression of the holoenzyme.

The discovery of a new enzyme, RuBisCO activase, which can regulate the activity of RuBisCO *in vivo* is a breakthrough in the study of RuBisCO activation. Some progress has already been made and it is predicted that more exciting results will emerge in regard to the role of activase in the activation of RuBisCO.

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บทคัดย่อ

ไรบูโลสบิสฟอสเฟต คาร์บ็อกซิเลส/ อ็อกซิเจนเนส เป็นเอ็นไซม์ตัวสำคัญในกระบวนการสังเคราะห์แสง โครงสร้างของเอ็นไซม์ประกอบด้วยหน่วยใหญ่ (A) และหน่วยเล็ก (B) อย่างละ 8 หน่วย โดยมีโครงสร้างจตุรภูมิเป็น $A_8 B_8$ อย่างไรก็ตามเอ็นไซม์ที่ประกอบด้วยหน่วยใหญ่ (A) แต่เพียงอย่างเดียว และมีโครงสร้างจตุรภูมิเป็น A_2 สามารถพบได้ในบักเตรีสังเคราะห์แสง *Rhodospirillum rubrum* ปฏิกิริยาการเร่งของเอ็นไซม์เกิดขึ้นกับหน่วยใหญ่ (A) โดยที่หน่วยเล็ก (B) ก็มีความสำคัญในการทำให้ปฏิกิริยาการเร่งของเอ็นไซม์ดำเนินไปได้ จากการทดลองพบว่า หน้าที่ส่วนหนึ่งของหน่วยเล็ก (B) ช่วยทำให้เอ็นไซม์ที่ตื่นตัวแล้ว (activated enzyme) มีความเสถียรเพิ่มขึ้น ในพืชชั้นสูง

การสร้างเอ็นไซม์ต้องอาศัยทั้งคลอโรพลาสต์ และนิวเคลียสโดยที่หน่วยใหญ่ (A) จะถูกสร้างโดย DNA ในคลอโรพลาสต์ และหน่วยเล็ก (B) ถูกสร้างโดย DNA ในนิวเคลียส กลไกที่หน่วยใหญ่ (A) และหน่วยเล็ก (B) มาจับตัวกัน เพื่อให้ได้โครงสร้าง A_8 B_8 ยังไม่เป็นที่กระจ่างชัด

การทำงานของเอ็นไซม์จะถูกกระตุ้นด้วยแสงสว่าง โดยแสงสว่างจะทำให้ค่าความเป็นกรด-ด่าง (pH) ภายในคลอโรพลาสต์สูงขึ้น อีกทั้งแสงสว่างยังทำให้ความเข้มข้นของแมกนีเซียมสูงขึ้นด้วย นอกจากนี้พบว่าแสงสว่างควบคุมการทำงานของเอ็นไซม์ โดยทำให้เกิดการเปลี่ยนแปลงของระดับตัวห้ามการทำงานของเอ็นไซม์ โปรตีนชนิดใหม่ที่มีชื่อว่า RuBisCO activase สามารถควบคุมการทำงานของเอ็นไซม์ *in vivo* แสงสว่างสามารถมีผลต่อการสร้างปริมาณเอ็นไซม์ ในการสร้างหน่วยใหญ่ (A) ของเอ็นไซม์ อิทธิพลของแสงสว่างจะเกิดที่ระดับหลังการถอดรหัสของยีน (post transcription) ในขณะที่อิทธิพลของแสงสว่างต่อการสร้างหน่วยเล็ก (B) จะเกิดที่ระดับการถอดรหัสของยีน (transcription) ล่าสุดนี้ได้พยายามนำเทคโนโลยีทางด้าน Recombinant DNA มาใช้ในการหาความเป็นไปได้ที่จะเปลี่ยนแปลงโครงสร้างของเอ็นไซม์เพื่อให้มีประสิทธิภาพในการจับตัวกับสับเสตรท CO_2 ดีกว่าสับเสตรท O_2 อย่างไรก็ดี การทดลองโดยใช้เทคนิค Site-specific mutagenesis เท่าที่ผ่านมายังไม่ประสบผลสำเร็จ