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Review

Rubisco activase

Archie R. Portis, Jr.

Agricultural Research Service, United States Department of Agriculture and Department of Agronomy, University of Illinois, Urbana, IL (U.S.A.)

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Contents

1. Introduction

Rubisco activase is a protein that catalyzes the activation of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco *). Rubisco is a prime site for the regulation of photosynthesis because it initiates both photosynthetic carbon reduction and photorespiratory carbon oxidation. The discovery and present knowledge of the rubisco activase system have already resolved several enigmatic features of rubisco activation as it occurs in vivo. Rubisco activase provides a means to regulate photosynthetic activity such that the activation level of rubisco is coordinately regulated with the rate of ribulose bisphosphate (RuBP) synthesis in the stroma and the rates of the energy-capturing reactions in the thylakoid membranes. This article reviews our current knowledge of the properties of rubisco activase, what we know about the mechanism of activation by this protein and its regulation, and aspects of the molecular biology of the protein. Some of the major gaps in our

* The acronym rubisco first appeared [35] in a running title (as RuBisCO), used again in a review [45] as Rubisco, and now often appears in the literature.

Abbreviations: RuBP, ribulose bisphosphate; CA1P, carboxyarabinitol 1-phosphate; P_i, inorganic phosphate.

Correspondence: A.R. Portis, Jr., Agricultural Research Service, United States Department of Agriculture and Department of Agronomy, University of Illinois, Urbana, IL 61801, U.S.A.

current understanding of the rubisco activase system also will be pointed out, with the aim of suggesting where future research efforts will be particularly beneficial.

II. The *Arabidopsis rca* mutant and other rubisco activation enigmas

The impetus for the eventual discovery of rubisco activase was the isolation of a nuclear gene mutant of *Arabidopsis thaliana*, named *rca*, that was not able to activate rubisco in the light although the rubisco enzyme in the mutant seemed to be identical to the wildtype [93]. The isolation of the *rca* mutant was fortuitous, since it was recovered using a screen, a high CO_2 requirement for growth, designed to isolate photorespiratory mutants. Interestingly, despite several attempts, no other mutants with the rubisco activase phenotype have been found to date (Somerville, C.R., personal communication). The phenotype of this mutant was somewhat unexpected because the activation of rubisco in vitro had been well characterized and shown to involve a pH-dependent and ordered addition of CO_2 and Mg to the enzyme which proceeded spontaneously to equilibrium [46]. This in vitro mechanism seemed to form the basis for an in vivo mechanism of light activation [34] in which the light-induced increases of stroma pH [102] and Mg [60] observed in chloroplasts would reversibly modulate the extent of activation of the enzyme. Nonetheless, further studies with the *rca* mutant indicated that the light-induced stromal pH and Mg changes were probably normal [76].

In addition to the question raised by the *rca* mutant, other observations indicated that this mechanism could not fully account for the activation of rubisco as it occurs in the leaf. In the first studies of rubisco activation in leaves [47,57], rubisco was found to be nearly fully activated under high light with atmospheric levels of CO_2 and activation varied with light intensity over a similar range as photosynthesis. In contrast, an examination of the pH, Mg and CO_2 dependence of the in vitro activation process indicated that the enzyme should be less than half-activated under in vivo conditions [3,50]. Additionally, the light dependent change in pH (and presumably Mg) was found to saturate at very low light intensities [25]. Therefore considerable interest arose from the observation that various phosphorylated compounds could markedly lower the pH and CO_2 dependence of activation in vitro (reviewed in Refs. 34 and 51) and thereby possibly regulate rubisco activation. It later became clear that they could not actually vary the amount of catalytically active enzyme because these compounds bind to the same site as RuBP [3,50]. Furthermore, the RuBP levels measured in leaf extracts were normally much greater than those measured for the most effective compounds and were in excess of that required for catalysis except at the lowest light intensities [56,57]. It also was quite surprising when a reexamination [36] of rubisco activation in vitro confirmed earlier indications [20,42,59] that RuBP and not a possible impurity [54], was itself a potent inhibitor of the in vitro activation process. The early studies of the activation of rubisco in leaves further showed that activation was relatively insensitive to CO_2 in the light [47,56] but not in the dark [47] and that it was sensitive to O_2 [56,80]. The in vitro activation mechanism also could not account for these observations. Finally, the isolated rubisco enzyme lost activity after the addition of RuBP [20,34,42], but this obviously did not occur in vivo. These enigmas have proven to be important bench marks on the way to uncovering the actual nature of the activation process.

III. Discovery and isolation of rubisco activase

An analysis of the soluble polypeptides in chloroplasts isolated from the *rca* mutant and wild-type *Arabidopsis thaliana* by two-dimensional polyacrylamide gel electrophoresis revealed that two polypeptides were missing in the mutant [75]. A genetic analysis confirmed that the *rca* mutant phenotype was associated with the missing polypeptides and prompted the search for a new rubisco activation assay. After a preincubation step with RuBP in the dark, a light-dependent activation of rubisco was found with either lysed chloroplasts or a reconstituted system consisting of thylakoids, rubisco, and a partially purified stromal extract consisting of proteins greater than 30 kDa, but freed of rubisco by sucrose density gradient centrifugation. A light-dependent activation of rubisco was not observed with either lysed chloroplasts or a stroma extract isolated from the *rca* mutant. These results demonstrated that the activation observed in the assay was dependent on the presence of the two rubisco activase polypeptides absent in the mutant [75].

The activation assay allowed the development of a purification scheme based on the fractionation of spinach chloroplast stromal extracts by anion exchange followed by gel filtration [77]. Polyacrylamide gel electrophoresis of the preparation indicated the presence of two major polypeptides at 45 and 41 kDa, consistent with the absence of 43 and 47 kDa polypeptides in the *Arabidopsis rca* mutant. Since the gel filtration data suggested a molecular mass of about 200 kDa, rubisco activase seemed to be a rather large protein comprised of two subunits. Rubisco activase prepared using this original procedure was found to be unstable and sometimes extensive proteolysis was observed. During this work it was found that rubisco activase could be precipitated with only a few contaminating proteins in a 0–35% ammonium sulfate fraction, but because the resulting protein had little activity, it was not reported.

This observation later proved useful in the purification of rubisco activase for antibody production [105]. Polyclonal antibodies to a spinach rubisco activase preparation were obtained from tumor-induced mouse ascites fluid and used to confirm that the 47 and 43 kDa polypeptides absent in the *Arabidopsis rca* mutant were associated with rubisco activase activity [77]. Using these antibodies, the presence of rubisco activase polypeptides was observed in soluble protein extracts of numerous plant species, including representatives with C3 and C4 photosynthesis and plants containing CA1P (see below). These results indicated that the rubisco activase dependent regulation of rubisco activation is ubiquitous in higher plant species [77].

The discovery of an ATP requirement for the activation of rubisco by rubisco activase [94] led to a simplified assay system and the development of an improved purification procedure that did not require the isolation of chloroplasts [70]. The key factor was the maintenance of activity before and during the 0–35% ammonium sulfate step by addition of ATP and the use of a lower pH. With this procedure, it was found that up to 18 mg of rubisco activase could be obtained from 100 g of spinach leaves. The use of protease inhibitors and conditions that maintained activity during storage facilitated work on the protein. Gel filtration of a rubisco activase preparation isolated in this manner resulted in an asymmetric peak with a maximum suggesting a molecular mass of about 500 kDa (Robinson, S.P. and Portis, A.R., unpublished results). Changing the column conditions (+ATP, +ADP, etc) did not greatly alter the elution profile. Similarly, broad bands were obtained when the protein was electrophoresed on nondenaturing gels. However, a Ferguson plot analysis performed at pH 9.0 with high reductant indicated a molecular mass of 255 kDa (Chatfield, J.M. and Ogren, W.L., personal communication). The simplest conclusion that can be drawn from these confusing results is that rubisco activase self-associates which makes its functional size unclear until factors affecting the process can be defined.

IV. Activities of rubisco activase

Rubisco activase was named to signify its ability to promote the activation of rubisco [75]. In the initial studies to characterize the protein [61], emphasis was placed on establishing that rubisco activase was physiologically relevant and indeed was sufficient to account for several aspects of rubisco activation in vivo that were otherwise inexplicable at the time. The two most important observations in this regard were the ability of rubisco activase to promote the near maximal activation of rubisco at atmospheric CO_2 concentrations (10 μM) and the activation of rubisco in the presence of RuBP. By illuminating a reconstituted system containing thylakoid membranes, rubisco, partially purified rubisco activase, and 3 mM RuBP, the CO_2 concentration required for half-maximal activation of the enzyme was found to be about 4 μM as compared to 23 μM in the absence of RuBP where the spontaneous process occurred [61]. In the dark, there was little rubisco activity in the presence of RuBP even with 70 μM CO_2. In order to achieve these results, a relatively large amount of rubisco activase relative to rubisco and long incubation periods were required to reach an apparent steady-state condition. At the time there was less concern for the kinetics of the process, which would be necessary to determine the rubisco activase mechanism. The studies required to establish the exact mechanism are still far from complete, but the studies conducted to date are outlined below and provide a general outline to formulate possible mechanisms.

IVA. Spontaneous activation of rubisco versus activation with rubisco activase

The spontaneous activation of rubisco (Fig. 1) in the absence of rubisco activase is itself a rather complex process [46] involving the pH dependent and rate limiting addition of CO_2 to form a carbamate on the enzyme, followed by the rapid addition of Mg to produce the only active form (the ECM complex) of the enzyme *. More complexity is introduced if RuBP (or other sugar phosphates) are present because in principle they may bind to all forms of the enzyme [3,36,42,50]. The kinetic parameters needed for a complete description of the process are not obtainable with current methods of analysis. Nevertheless, several activation rate and equilibrium [6,42,46,52,83] and sugar phosphate binding [3,36,37,50] studies have provided relevant information on the approximate values for some of these rate constants.

Although unlikely, it was conceivable that the activation of rubisco by rubisco activase proceeds by a mechanism that does not involve carbamate formation. To examine this, the kinetics of carbamate formation and the increase of rubisco activity dependent on rubisco activase were assayed simultaneously and found to be identical [104]. This observation indicates that carba-

* Some uncertainty still exists as to whether the ECM form of the enzyme is always accurately assayed when rubisco samples are diluted into an assay solution containing RuBP and generally different CO_2 and Mg concentrations [6,42,46,50,83]. With reference to Fig. 1 there are two concerns. First, how effectively does RuBP convert E, EC and ECM species into ER, ECR and ECMR without change? Second, when other phosphorylated compounds are present, do they always dissociate rapidly from the various enzyme forms to be replaced by RuBP such that the relative amounts of ES, ECS and ECMS do not change?

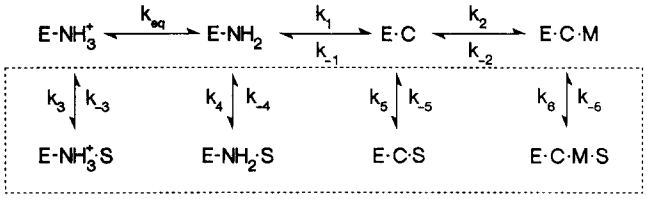

Fig. 1. Scheme for the spontaneous activation of rubisco which is dependent on CO_2, Mg and pH. The box outlines additional intermediates possible in the presence of a sugar phosphate (S) that binds to the active site. Interconversion between ES, ECS, and ECMS is not indicated, since the 3-dimensional structure of rubisco [18,79] indicates that access to the carbamate site is probably blocked by sugar phosphate binding. Some evidence exists [37,96] that an even more complex kinetic scheme is necessary to explain the effect of certain compounds like 6-phosphogluconate on rubisco activation.

mate formation is an integral part of the activation process catalyzed by rubisco activase.

Given that rubisco activates rubisco by ultimately producing the same form (ECM) of the enzyme, it must be introduced into an already complex picture. One might postulate the existence of either rubisco-rubisco activase complexes during one or more stages of activation or a modified form of rubisco produced by some kind of interaction with rubisco activase. The task at hand is to define the rate constants for the activation process which, in principle, include those for the forms previously defined, the interaction rate constants for rubisco activase and the various rubisco species, and new rate constants for the CO_2 and Mg addition processes. It should be noted that the latter might not have the same values as those of the spontaneous process. A model incorporating these features is presented in Fig. 2.

Examination of Fig. 2 makes it obvious that some simplifying conditions and assumptions must be made in order to begin to make any progress. In the work to date, the system has been simplified by starting with

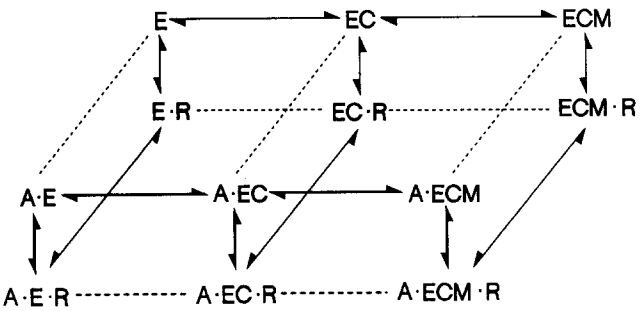

Fig. 2. Scheme for possible intermediates in the activation of rubisco by rubisco activase in the presence of RuBP (R). A·E represents a complex between rubisco and rubisco activase, but with our current knowledge A·E also could represent a modified form of rubisco, resulting from an effect of rubisco activase on the enzyme. By analogy with Fig. 1, addition of CO_2 and Mg to RuBP-bound forms is unlikely and thus is indicated by the dashed lines. A direct binding of rubisco activase to rubisco enzyme forms without bound RuBP is also somewhat less likely because rubisco activase has no effect on rubisco activation in the absence of RuBP. ATP hydrolysis is not included in the scheme because the step where this occurs has not been determined.

only one form of rubisco (ER), limiting the studies to rate-limiting amounts of rubisco activase with saturating amounts of CO_2 and Mg and focusing on initial rates [70]. The inclusion of an ATP-regenerating system (to avoid the complications of ADP; see below) and the use of a large excess of RuBP such that the formation of active enzyme would not lead to RuBP depletion and the complications of spontaneous activation, also were essential.

With such initial conditions, the rate of appearance of rubisco activity (and the final extent although, as discussed below, this now appears to be a more complicated situation) increased when either the amount of rubisco or rubisco activase in the assay was increased [70]. The increase in rubisco activity in the absence of rubisco activase or ATP was comparatively minor. Analysis of the data led to the definition of an operational unit of rubisco activase activity as that sufficient to result in the increase in the activity of rubisco by one unit (i.e., the observed activity normalized to the amount of rubisco in the assay) in 1 min. The defined unit was linearly related to the rubisco activase concentration in the assay. Unfortunately, the defined unit was not completely independent of the amount of rubisco present in the assay, as it decreased by about 30% when rubisco was increased by 8-fold from 0.2 mg/ml. The small, but significant, effect of rubisco concentration needs further study and may indicate the formation of rubisco-rubisco activase complexes before the formation of the active enzyme.

Rubisco activase has no effect on the activation state of rubisco in the absence of RuBP [61]. This result has been confirmed in more recent studies (Lilley, R.M. and Portis, A.R., unpublished results). With reference to Fig. 2 several possibilities exist which might account for the RuBP requirement. Rubisco activase may bind to rubisco only when the active site is occupied. Binding could be followed by substrate release such that rubisco activase-rubisco complexes do exist as shown in Fig. 2. Alternatively, RuBP may be functioning to stabilize the ECM complex [37,42], while rubisco activase specifically promotes RuBP release from the other forms. Finally, rubisco activase itself may be dependent on RuBP. These possibilities are discussed more below, but

further studies are needed to distinguish better between them.

Studies of the spontaneous activation process at limiting CO_2 concentrations allowed an estimate of a pseudo-first-order rate constant for the addition of CO_2 to be made [6,42,46]. In principle, similar studies of the rubisco activase catalyzed activation of ER should be possible and the measured rate constant would reflect the actual rate constant for CO_2 addition to the extent that rubisco activase was not limiting the process. Such studies are currently in progress (Lilley, R.M. and Portis, A.R., unpublished results). The value of this rate constant as compared to that observed for the spontaneous process is important for understanding the rubisco activase mechanism because it would indicate if the lower CO_2 requirement for activation (4 μM vs. 23 μM) observed in the equilibrium measurements [61] is the result of a rubisco activase-induced change in the intrinsic affinity of rubisco for CO_2.

Regardless of the results of such studies, another possibility exists in which the binding of RuBP to the various forms of the enzyme may contribute to the lower CO_2 requirement observed with rubisco activase. At the high RuBP concentrations normally used with rubisco activase, only rubisco forms with bound RuBP need be considered. By altering the affinity of the various rubisco species for RuBP, rubisco activase could alter the relative amounts of the various forms to favor more ECM-R at a given CO_2 concentration. In other words, by analogy to the effects of sugar phosphates on the spontaneous activation process [3,36,50], rubisco activase might alter rubisco binding kinetics such that RuBP changes from being a transient negative effector (binding to E and EC > ECM) to being a positive effector (binding to E and EC < ECM). In contrast to the other sugar phosphates, the ECM-R complex is catalytically competent in situ. Although it may be difficult to test experimentally, this mechanism implies that the CO_2 requirement for activation of ER with rubisco activase should depend on RuBP concentration over some range.

At this point it should be apparent that future progress in studying rubisco activase catalyzed activation will be extremely limited if one were to continue to rely only on the assay of rubisco activity to follow the course of the reaction. Although it is not yet possible, ideally one needs to be able to 'see' the various forms of rubisco and rubisco activase that exist in solution such that the rates of interconversion could be determined. Fortunately rubisco activase has been found to have several activities in addition to its ability to catalyze the activation of rubisco. For the present, it is reasonable to assume that these activities represent partial reactions in the more complex overall process. As such they could be important in breaking down the activation process into a specific sequence of steps.

IVB. ATP requirement, ATP hydrolysis and ATP/ADP regulation of rubisco activase

The requirement for thylakoids and light in the early studies with rubisco activase was later shown to be unnecessary if ATP and a ATP regeneration system was utilized [94]. Subsequently, it was not surprising to find that purified rubisco activase hydrolyzed ATP with specific activities of up to 1.5 μmol per min mg protein [68]. The hydrolysis reaction is specific for Mg, as replacement with other cations results in activities of less than 16%. The hydrolysis reaction also is specific for ATP as other nucleotides give activities of less than 9%. Hydrolysis of sugar phosphates like RuBP and FBP was not detected.

Substantial evidence exists to indicate that ATP hydrolysis is an intrinsic property of rubisco activase and is part of the overall activation process. The ATPase activity does not seem to be due to contamination of the purified preparations with other phosphatases or ATPases because various typical inhibitors of such activities had minimal effect [68]. Both the activation activity and the ATPase activity have an identical sigmoidal response to ATP concentration (Hill coefficient of 1.7 and half-maximal activity at 50 μM). Both activities are inhibited by ADP to a similar extent. The activities copurify and show the same heat lability. Finally, the derived amino acid sequence of the spinach rubisco activase has two areas of high similarity [105] with nucleotide binding domains identified in a variety of proteins [22,27], most of which have an ATPase activity (Table I). Studies of the nucleotide binding properties of rubisco activase will be useful in attempts to define the role of ATP in the activation process.

The inhibition by ADP allows the activation of rubisco by rubisco activase to respond to both ATP and ADP concentrations [68,94]. This form of regulation would allow the activity of rubisco and therefore the rate of CO_2 fixation to the coordinately regulated with the rate of ATP formation by the thylakoids. A close correlation in vivo between the steady-state ATP concentration and rubisco activity in intact isolated chloroplasts under a variety of conditions has been observed [67]. Changes in the ATP/ADP ratio in leaves also have been found to be associated with changes in rubisco activation [12,81,92]. Nevertheless, at the present time, activation seems to be more sensitive to ADP in vitro than observed in vivo [68] and as discussed below the light intensity dependence of rubisco activation does not seem to be the result of an ATP/ADP regulation of rubisco activase activity.

If ATP hydrolysis is a partial step in the overall activation mechanism, one generally would expect the reaction to be tightly coupled to the activation of rubisco. Unfortunately, the rate of ATP hydrolysis was not significantly affected by the presence of rubisco in

TABLE I

Consensus nucleotide binding regions of spinach rubisco activase and other representative enzymes

Sequences were obtained from Refs. 22 and 27. The particularly close homology with adenylate kinase in region 1 is interesting, since the 3-dimensional structure of the area is known in detail [27].

Enzyme	Region 1		Region 2	
Rubisco activase	103–119	V W G G K G Q G K S F Q C E – L V F	158–173	A K G K M C A L F I N D L E P G
Adenylate kinase	13– 30	V V G G P G S G K G T Q C E K I V Q	109–123	K I G Q P T L L L Y V D A G P E
E. coli F_1-beta	147–164	L F G G A G V G K T V N M M E L I R	232–246	D E G R D V L L F V – D N I Y R
EF-Tu	17– 34	T I G H V D H G K T T L T A A I T T	98–113	M D G A I L V V A A T D G P M P
Transcarboxylase	43– 60	A A G G A G A G K A G E G E I P A P	77– 92	K A G E T V L V L E A M X M E T
ras protein	8– 25	V V G A G G V G K S A L T I Q L I Q	46– 61	I D G E T C L L D I L D T A G L

the form of either ER or ECM [68]. Phosphorylated forms of rubisco and rubisco activase also have not been observed thus far (Streusand, V.J., Chatfield, J.M., Ogren, W.L. and Portis, A.R., unpublished results). Therefore an ATP-hydrolysis step cannot be placed with any confidence in the scheme shown in Fig. 2. The significance of the lack of tight coupling is not yet clear, but as discussed [68] other instances of a similar nature have been reported. It is also possible that conditions appropriate for a tight coupling of the activities just have not been found as yet. For example the effect of ADP, which is normally present in vivo, on coupling has not been determined and the two proteins are present in the chloroplast stroma at concentrations that are not easily accessible in in vitro studies. The lack of strict coupling between the activities and the apparent absence of phosphorylated intermediates will make it more difficult to investigate the role of ATP hydrolysis in relation to the steps in the activation process.

IVC. Release of CA1P, RuBP and other sugar phosphates from rubisco

In addition to changes in activation, the activity of rubisco can be modulated by another mechanism involving carboxyarabinitol 1-phosphate (CA1P). CA1P, which is sugar phosphate somewhat similar to the 6-carbon intermediate, 2-carboxy-3-keto-arabinitol 1,5-bisphosphate, formed during the carboxylation reaction [58,78], binds very tightly to the activated form of the enzyme (ECM) and inhibits catalysis in essentially a non-competitive manner. CA1P was discovered as a result of the changes in maximal extractable activity observed in some species [8,82,85,86,98]. These and other studies [84] led to the eventual isolation and identification of the compound [8,30] causing this effect. Based on activity measurements, the inhibitor is present at variable levels in the various species after the plants are kept in the dark for long periods. The inhibitor decreases after only a short time in proportion to increasing irradiance and therefore can limit rubisco activity as irradiance increases. In contrast, decreases in irradiance generally result in a slower decline in maximal activity (presumed to correspond to reformation of the compound) as compared to the decline in rubisco activation [41,72,86,97]. A rapid response has been reported in *Phaseolus vulgaris* [40,41]. It has been suggested [40] that the regulation of rubisco activity by light intensity potentially varies from an almost complete regulation by the amount of CA1P (as in *Phaseolus*) to regulation only by changes in activation (as in spinach) with most species probably being somewhere in between. A specific phosphatase that degrades the inhibitor has been discovered [33,73], but many aspects of its synthesis and degradation are not yet known. The physiological implications of the regulation of rubisco activity by this mechanism in addition to that already provided by the rubisco activase system are not clear.

CA1P was identified at about the same time as rubisco activase and there was a possibility that they were connected. The finding that rubisco activase was present in every higher plant species examined [77] whereas CA1P could be found only in certain plants [82,85,98] argued against this possibility. Furthermore, it was shown that the two could be mechanistically separated in tobacco protoplasts [72]. Nevertheless, these observations only indicated that control of rubisco activity by rubisco activase and by CA1P were not directly related. A more crucial test was provided by examining the effect of rubisco activase on rubisco which had been inhibited by preincubation with CA1P. It was found that rubisco activase enhanced the reversal of CA1P inhibition, but did not appear to metabolize the compound [66]. The half-time for reversal of inhibition by rubisco activase was about 3-fold greater than that achieved with alkaline phosphatase [8]. The results indicated that rubisco activase increases the rate of release of CA1P from the active site of the rubisco. It was not determined if the binding constant, which is the ratio of the on- and off-rate constants, was changed. It has been suggested [66] that this activity is physiologically important because the rate of reversal obtained with rubisco activase was similar to the rate of reversal observed in vivo [82]. Thus rubisco activase does appear to function in the CA1P regulation of rubisco activity by controlling the release of CA1P from rubisco for its

subsequent metabolism in a light-dependent manner. A rubisco activase-deficient plant that contains CA1P would be useful to test this idea more thoroughly.

Since rubisco activase catalyzes the activation of the ER form of the enzyme, one obvious approach to defining the mechanism would be to determine what happens to the bound RuBP. It was found that ^{3}H-RuBP bound to inactive enzyme was rapidly exchanged with RuBP in the medium during the activation process (Portis, A.R., unpublished results) with 66% of the label released by the time (1 min) that only 20% of the rubisco has been activated. Both exchange and activation were nearly complete by 3 min and an analysis of the label at that point indicated that only 19% had been converted to 3-phosphoglycerate. The same amount of 3-phosphoglycerate was formed in a control experiment in which the label was added after ER formation. These results indicate that the bound RuBP leaves the active site before CO_2 and Mg addition occurs to form the carbamate and converts the enzyme into a catalytically active form. Because the exchange kinetics in the experiment were too fast to be properly analyzed by the centrifugal ultra-filtration method used to determine the amount of free and bound label, an alternative method is being developed to study the reaction further (Larson, E.M. and Portis, A.R., unpublished results). Additional studies of the properties (e.g., whether exchange is dependent on CO_2 or on ATP) and the kinetics of the exchange reaction, will be important in developing a better understanding of the activation process.

The above studies with RuBP and CA1P raise several questions. Does rubisco activase promote the release and alter the binding of a wide range of sugar phosphates? What, if any, specificity exists for the form of rubisco (e.g., E with RuBP vs. ECM with CA1P)? Attempts to determine the answer to such questions are only beginning. Already clear evidence that rubisco activase also promotes the release of fructose bisphosphate and ribose 5-phosphate from inactive rubisco was obtained by observing that an increased rate of activation occurs in the presence rubisco activase, ATP, and either of these these compounds as compared to the rate in the absence of rubisco activase (Lilley, R.M. and Portis, A.R., unpublished results). These results probably account for the positive effect of FBP on rubisco activation reported in a lysed chloroplast system [55].

IVD. Maintenance of the catalytic activity of rubisco

As mentioned earlier, a long-standing problem in the study of the rubisco kinetics has been the decline in catalytic activity, termed fallover *, that occurs after the addition of RuBP to the activated form of the enzyme (for various reports, see Refs. 5, 20, 42, 50, 52 and 88). For many years the problem was simply avoided by restricting assays to less than 1 min, or by producing RuBP in situ [44] with ribose 5-phosphate and ATP in the presence of ribose 5-phosphate isomerase and ribulose 5-phosphate kinase. The latter system usually provides a more stable reaction rate, but rubisco activity still declines (Pierce, J., personal communication). Rubisco activity in situ in isolated chloroplasts is stable in the presence of large amounts of RuBP [88] and is obviously stable in leaves. It was shown that inhibitory degradation products can be present in RuBP preparations and might account for fallover [54]. Alternatively, since RuBP binds more tightly to the inactive form of the enzyme [36,106], it was thought that formation of inactive rubisco-RuBP complex might be responsible for the decline [50]. Evidence against this possibility was obtained by showing that the activator CO_2 was not lost from the enzyme during the loss of activity (Ref. 23; and also Edmonson, D. and Andrews, J., and Pierce, J., personal communications; unfortunately, these observations have not been published and the general lack of awareness of these data has certainly hampered the resolution of the problem).

Because of the fallover phenomenon, it now appears that a complete understanding of the activation of rubisco by rubisco activase will require that the decline in rubisco activity due to fallover (carbamylated enzyme without activity) be differentiated from the decline in activity due to true deactivation (reformation of decarbamylated enzyme), especially in in vitro experiments. Some recent experiments on the ability of rubisco activase to maintain rubisco activity in vitro have begun to address this question. It was found that the addition of rubisco activase (and ATP) could largely prevent the typical loss of rubisco activity in the presence of RuBP [69]. More revealing perhaps was the observation that rubisco activase could reverse the process after it had already occurred. The relationship between ER formation and the status of the enzyme after fallover was further examined by gel-filtration experiments. These indicated that whereas the ER form of the enzyme could be reactivated by gel filtration in the presence of CO_2 and Mg, enzyme which been subjected to fall-over only partially recovered activity. Otherwise, treatment of the inhibited enzyme with alkaline phosphatase completely restored activity [69]. These results indicated that fall-over results from the formation of enzyme with a sugar phosphate that is relatively tightly bound. Other extensive studies of the fallover phenomenon have led to a similar conclusion [23]. Therefore the reversal of fallover by rubisco activase might simply represent an increase in the dissociation rate of the bound compound as suggested by the effect of rubisco activase on the binding of RuBP and CA1P. The possibility that the

* This very descriptive term was introduced by D. Edmonson and J. Andrews at the Rubisco 1987 Conference in Tucson, AZ, U.S.A.

inhibitor associated with fallover is actually a contaminant present in the RuBP cannot yet be disproved, but seems unlikely [23]. Instead, it has been suggested that fallover results from a low-frequency catalytic event during the carboxylation reaction [23,69]. The ability to gel filter the inhibited enzyme without recovery of activity has allowed sufficient quantities of the inhibitor to be isolated and characterized. Phosphate analysis and inhibitor titration experiments indicate that the compound is a bisphosphate with a K_d of about 0.1 μM (Portis, A.R. and Robinson, S.P., unpublished results). Further analysis by mass spectrometry and NMR should reveal the actual structure of the compound and thus might provide clues to its metabolism.

IVE. Deactivation of rubisco

The spontaneous activation process (Fig. 1) is readily reversible in the absence of sugar phosphates and a priori one would expect the same to be true for the rubisco activase catalyzed process outlined in Fig. 2. Therefore in the initial studies of rubisco activase [61], the decline of rubisco activity observed when the sample was returned to darkness was believed to indicate the reformation of ER (i.e., a true decarbamylation of the enzyme). The fallover process described above has complicated this simple interpretation and the role of rubisco activase in deactivation is presently unclear. Attempts to obtain a true deactivation (as measured by directly assaying carbamylation vs activity) of rubisco after activation with rubisco activase, by inhibiting the activation process with addition of ADP, were unsuccessful (Chatfield, J.M., Portis, A.R. and Ogren, W.L., unpublished results) and the results were more indicative of fallover. In contrast, a true deactivation seems to occur in vivo as measured by activator CO_2 exchange [7], the binding of 2-carboxyarabinitol 1,5-bisphosphate [13], the formation of enzyme with tightly bound RuBP [11,17], and the fairly rapid in vitro reactivation of the enzyme in extracts with high CO_2. Further studies of the deactivation process which occurs in the *Arabidopsis rca* mutant, as compared to the wild-type plant, might be a good approach to resolve this issue. For example, it could be determined if the light-dependent decline of rubisco activity in the mutant is associated with decarbamylation and with the formation of enzyme with tightly bound RuBP as shown previously for the wild-type plant. If these processes do not occur in the *rca* mutant, a role for rubisco activase in a true deactivation of rubisco would be evident. The failure to observe these processes in vitro would then suggest that additional factors or requirements were not satisfied in the experiments attempted to date. Otherwise, if these processes do occur in the *rca* mutant, it would indicate that deactivation occurs by an unknown mechanism independent of rubisco activase.

V. Primary structure and synthesis of rubisco activase

Using λgt11 cDNA libraries constructed from spinach, *Arabidopsis* [105] and barley mRNA (Rundle, S. and Zielinski, R.E., unpublished data), rubisco activase cDNA clones were recovered immunologically and sequenced. Interestingly, two types of rubisco activase cDNA were isolated [103]. Part of the deduced amino acid sequences of the polypeptides encoded by the two forms of rubisco activase cDNA are shown in Fig. 3. The sequences are identical except at the C-terminal end, where the larger polypeptide in spinach consists of 37 more amino acids. A search for sequence homology with other proteins did not prove to be informative (Werneke, J.M. and Ogren, W.L., unpublished data), except for the two short regions associated with nucleotide binding that were discussed previously (Table I).

As expected for a nuclear-encoded protein localized in the chloroplast, rubisco activase is initially synthesized as a precursor polypeptide containing a transit peptide (not shown in Fig. 3) of about 58 amino acids [103,105]. The in vitro synthesis of rubisco activase mRNA from a cDNA clone to produce the precursor polypeptide with a wheat germ translation system permitted chloroplast import and processing of the precursor polypeptide to be demonstrated in vitro [105]. Thus rubisco activase is another example of a chloroplast protein which is encoded by a nuclear gene, synthesized on cytoplasmic ribosomes, and imported into the chloroplast [24].

The two cDNAs were nearly identical and a genomic Southern blot analysis of both spinach and *Arabidopsis* indicated that there was only a single rubisco activase gene [105]. A priori several processes might be responsible and have been examined [103], but an analysis of the structure of the genomic clones from spinach and *Arabidopsis* proved to be most informative. An intron was found at the 3′-end of the gene which could produce by an alternative splicing process (Fig. 4), two mRNAs of nearly identical size that direct the synthesis of each corresponding polypeptide.

Alternative mRNA splicing has been associated with both developmental and tissue specific expression in animal systems [43]. When the cellular differentiation gradient of the first leaf of 7-day-old barley was examined, it was found that the two polypeptides and the mRNA encoding rubisco activase accumulate progressively from the leaf base in a qualitatively similar pattern [108]. The pattern was similar to that observed for the two rubisco subunit polypeptides and mRNAs. Thus differential expression associated with development does not appear to be related to the rubisco activase alternative mRNA splicing process, at least in barley.

The other possibility, tissue specific expression, was

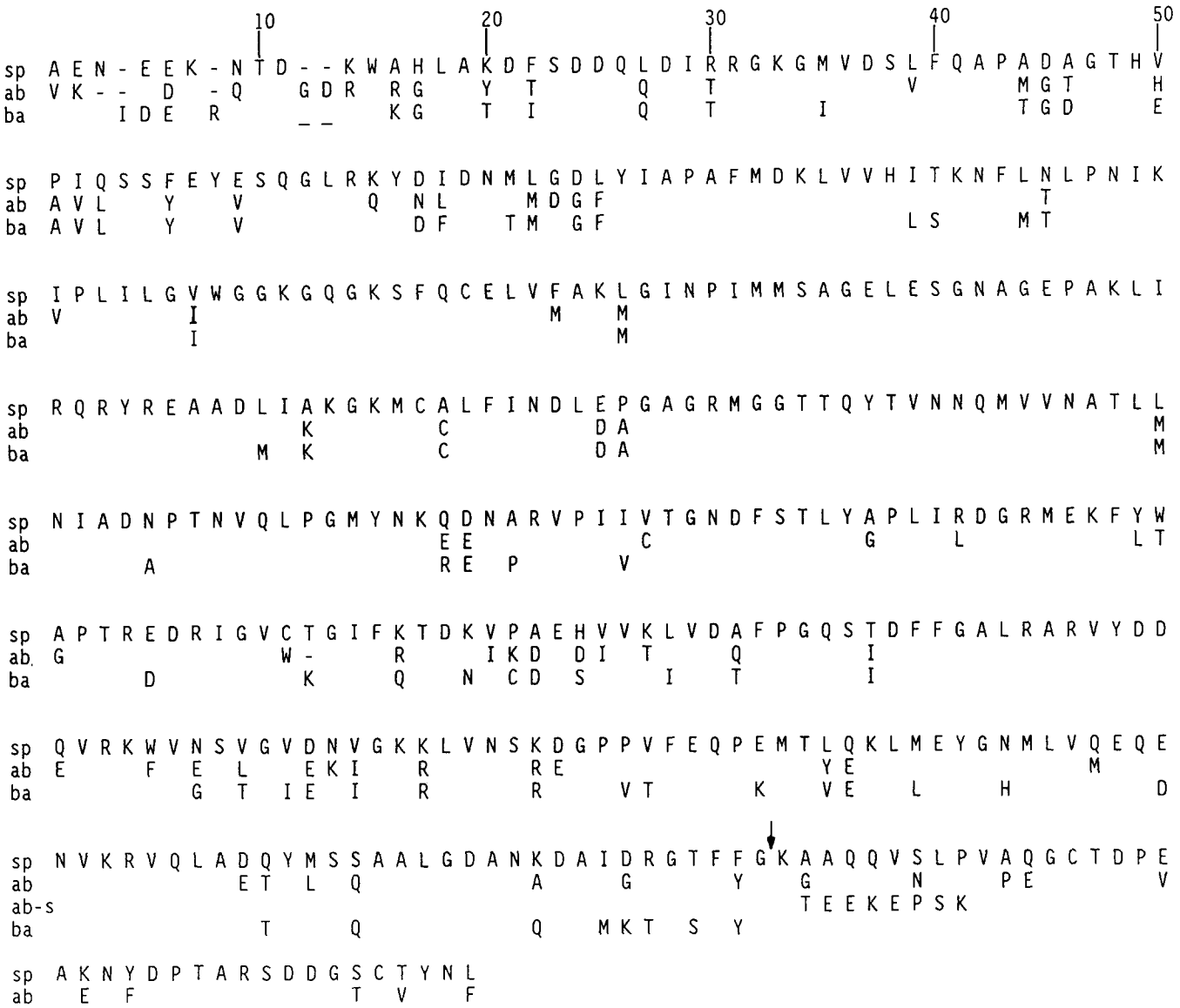

Fig. 3. A comparison of the amino acid sequences of rubisco activase polypeptides as derived from cDNA sequence data from (sp) spinach, (ab) *Arabidopsis*, and (ba) barley (Ref. 103; see also Rundle, S. and Zielinski, R.E., unpublished data). Only differences from the spinach sequence are shown. Gaps necessary for maximal homology are indicated by a hyphen(-). The arrow near the C-terminal region of the spinach sequence defines the end of the 41 kDa spinach polypeptide. The N-terminal and C-terminal amino acids of the spinach polypeptides have been confirmed by analysis of the isolated polypeptides. N-terminal start for the *Arabidopsis* and barley sequences has not been defined. C-terminal information on the 42 kDa *Arabidopsis* polypeptide is indicated by ab-s, and ends at a nonsense codon. Sequence information on the large barley polypeptide has not been obtained as yet.

investigated by obtaining cryosections of leaf tissue from the upper (palisade cells) and lower (mesophyll cells) surfaces of a spinach leaf [103]. Proteins in extracts of the samples were separated by gel electrophoresis and blots probed with rubisco activase antibodies. The results indicated that both polypeptides were present in each sample in the same relative amounts as the whole leaf extract. Therefore contrary to the majority of examples of alternative splicing in animal systems, alternative splicing of rubisco activase mRNA appears to be a constitutive process and its significance remains unknown.

The cDNA sequencing work [103,105] has definitively confirmed the initial indications [75,77] that rubisco activase consists of two polypeptides in the range of 40–47 kDa. An analysis of the maize genome will be interesting because a clear evidence of two polypeptides in this species was not obtained. The two polypeptides do not necessarily combine to form a functional holoenzyme as initially thought [75], since they can be partially resolved under native conditions using ion-exchange chromatography [104]. Furthermore, either polypeptide is capable of independently activating rubisco. This was demonstrated by the cloning, expression, and purification of each polypeptide from *E. coli* (Shen, J., Ogren, W.L. and Orozco, E.M., unpub-

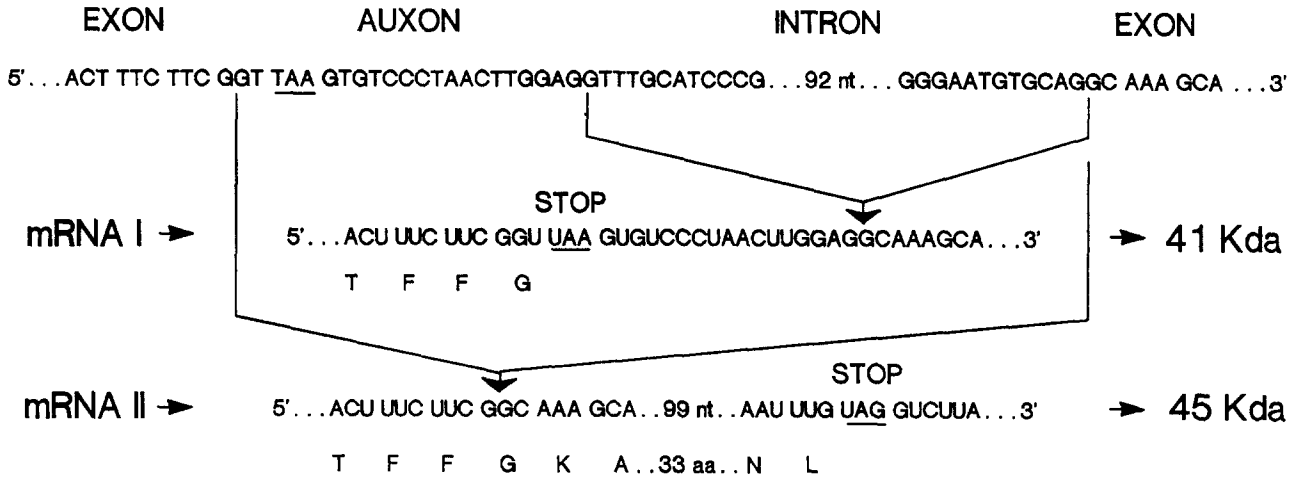

Fig. 4. Scheme for the alternative splicing process in intron III of spinach rubisco activase mRNA (from Werneke [103]). The top line shows the relevant portions of the genomic sequence of intron III and some flanking sequence. Shown below are the two types of mRNA produced by alternative splicing. When the second 5′-splice site is used, a 22-nucleotide auxiliary exon or 'auxon' is retained. Since an ochre termination signal occurs at the first codon in the auxon, an mRNA is produced which encodes a 41-kDa polypeptide (center). When the first 5′-slice site is used, the auxon is absent in the mRNA and translation continues for an additional 37 amino acids, producing a 45-kDa polypeptide (bottom). A similar, but not identical, process occurs in *Arabidopsis* [103].

lished results). Using these clones some significant differences in the kinetic properties of the two polypeptides have been detected recently (Shen, J., Ogren, W.L. and Orozco, E.M., unpublished results), but the physiological significance of this observation awaits further work. In addition to the present uncertainty about the polypeptide composition of native rubisco activase(s), the number of polypeptides forming the holoenzyme is unclear as discussed previously.

Rubisco activase polypeptides were not found [77] and did not appear to be synthesized in the *rca* mutant of *Arabidopsis* [103]. Nevertheless, the mutant was found to contain two rubisco activase mRNA species of different size than the wild type, one that was 0.2 kilobases larger and the other 0.2 kilobases smaller than the 1.9 kilobase mRNA found in spinach, barley, pea and the wild-type *Arabidopsis* [105]. These results indicate that an error in mRNA processing probably occurs in the *rca* mutant and leads to the absence of the protein. The exact nature of the lesion in the *rca* mutant will become more clear when the cDNA and genomic clones from the mutant have been completely sequenced.

VI. Physiological aspects of rubisco activation by rubisco activase

As already outlined, the characteristics of rubisco activase account for several aspects of rubisco activation as it occurs in vivo: activation in the presence of RuBP, lowered CO_2 requirement for activation so that near maximal activation occurs at atmospheric CO_2 levels, maintenance of constant rubisco activity in the presence of RuBP in vitro, and regulation of rubisco activation by ATP/ADP.

Rubisco activation state in leaves can be influenced by numerous environmental factors. These include light intensity [47,57], P_i [10,63] and nitrogen [49] deficiencies, O_2 [56,81,91], super-optimal CO_2 [71] and temperature [80,101]. Many of these effects remain rather difficult to explain at a biochemical level, but the discovery of rubisco activase now provides us with a new way to evaluate the influence of these factors on rubisco activation.

The most important regulatory aspect of rubisco activation on a daily basis is the dependence on light intensity. The effect of light intensity was observed first in *Triticum aestivum* [47,57] and subsequently has been found in *Raphanus sativus* [14], *Beta vulgaris* [40,95], *Spinacia oleracea* [10,12,40,62], *Glycine max* [86,97], *Trifolium repens* [80], *Arabidopsis* [11,76], *Nicotiana rustica* (protoplasts) [72], and *Helianthus annuus* (Sharp, R. and Portis, A.R., unpublished data). A regulation of activity also occurs in *Phaseolus vulgaris*, but CA1P binding accounts for all the variation [40,41] as discussed previously. With a rapid decrease in light intensity, the decrease in activation occurs slowly such that following a rapid decline in RuBP, a reciprocal return of the RuBP level to saturating levels is observed [11,53,57,62]. On the basis of the initial studies of rubisco activase [61], it was proposed that light intensity was sensed and communicated by the thylakoids, through rubisco activase to modulate rubisco activation. It was subsequently shown that the presence of thylakoids was not an essential component and could be replaced by use of an alternative ATP regenerating system [94]. This finding raised the possibility that light intensity was sensed by rubisco activase through corresponding changes in ATP/ADP. Unfortunately, direct

comparisons of these parameters in spinach has indicated that ATP/ADP ratios do not vary over a range of irradiance where changes in rubisco activation can be observed [12]. Unfortunately, a knowledge of the actual relationship between the measured and thermodynamically active amounts of these compounds continues to be a problem [64] due to the large fraction of these nucleotides that can be bound to proteins. Nevertheless, a light-dependent increase in the activation of rubisco was observed at low ATP levels [67] in chloroplasts (with estimated ATP/ADP ratios of less than one) whereas in the in vitro system, rubisco activase activity was severely inhibited under similar conditions [68,94]. Interestingly, the light activation of rubisco is inhibited by glyoxylate [19] and the inhibition could not be attributed to an effect on rubisco, rubisco activase, pH, or ATP/ADP [16]. Otherwise the effect of light intensity on rubisco activation has been shown to be related to thylakoid energization, as monitored by light scattering [74]. Although the present evidence would indicate that rubisco activase is certainly involved, the biochemical basis for the regulation of rubisco activity by light intensity remains to be determined.

Rubisco activation is reduced in phosphate-deficient plants [10,63] and in isolated chloroplasts in low phosphate media [31,48]. The chloroplast studies led to a suggestion [48] that rubisco activation was more closely related to chloroplast ATP level than it was related to stromal phosphate, although an effect due to a reduced stromal pH could not be excluded. Further studies of rubisco activation and stromal ATP in isolated chloroplasts under various conditions confirmed that these parameters were related [67]. Direct effects of stromal phosphate now appear to be less likely and instead the effects of phosphate seem to be the result of associated changes in ATP/ADP that can influence the activity of rubisco activase [67].

A recent report on the effect of nitrogen nutrition on the regulation of photosynthesis in wheat seedlings indicated that increased rubisco activation partly compensated for the decreased rubisco content as nitrogen was reduced [49]. Changes in ATP/ADP levels in the leaves also were observed in this case which may account for the change in rubisco activation.

The effect of O_2 on rubisco activation is complex in that it is determined by other conditions. Under high irradiance, high CO_2 and/or subambient temperature, photosynthesis often becomes O_2 insensitive [90]. Under such conditions, a decrease in O_2 below 21% results in a deactivation of rubisco [81,91,92]. The deactivation of rubisco again can be attributed to a decrease of the ATP/ADP ratio [81,92]. Under low irradiance, the activation of rubisco in wheat was further decreased by low O_2 when the CO_2 was either equal to or even much greater than atmospheric levels [56]. This effect of O_2 on rubisco activation under low irradiance also has been observed in *Arabidopsis* (Salvucci, M.E., Portis, A.R. and Ogren, W.L., unpublished results) and sunflower (Sharp, R. and Portis, A.R., unpublished results). Simultaneous measurements of ATP/ADP have not been reported. If the effects of O_2 under low irradiance are not explicable by changes in ATP/ADP, further studies of the effect may provide new insights into the regulation of rubisco activase and rubisco activation.

Increasing the CO_2 above ambient was reported recently [71] to induce a substantial reduction of rubisco activation at both saturating and subsaturating light intensities in *Phaseolus vulgaris*. Some evidence of a similar effect was observed earlier with *Triticum aestivum* [47] and *Raphanus sativus* [14], but it has not always been observed [15,56,76] which may indicate that other factors may be involved. Measurements of ATP/ADP during the experiments were not reported.

Temperature also interacts with other parameters to influence rubisco activation. Exposure of plants to subambient temperatures at high light intensities can promote a stromal and cytoplasmic P_i deficiency that reduces the ATP/ADP ratio and rubisco activation as outlined above. In contrast, the light intensity response of rubisco activation was reported to be altered by temperature [80], such that at low light intensities activation was actually higher at the lower temperatures. Additional studies of the effects of low temperature on rubisco activation, with measurements of the various metabolites, are warranted. At the other extreme, exposure of spinach [100, 101] and wheat [39] to high temperatures led to reduced photosynthesis and reduced activation of rubisco. In the studies with spinach, the effect on photosynthesis seemed to be related specifically to the reduction in rubisco activation as all the other processes examined were not significantly affected. Because isolated rubisco activase is very heat sensitive, it has been suggested that this sensitivity might be important in determining the effect of high temperatures on photosynthesis [68].

The changes in rubisco activation that can be induced by this wide variety of environmental factors must represent some kind of advantageous adaptive response. For changes associated with a decrease in irradiance, or an increase in CO_2, and presumably most other cases as well, the alteration in activation does not seem to optimize the photosynthetic rate. Following a short period of readjustment immediately following the reduction of light [62], or increased CO_2 [71], the photosynthetic rate then remains unchanged while rather dramatic changes in rubisco activation and metabolite levels occur over periods of up to 1 h. Therefore some other aspect of photosynthesis must be modified by the change in rubisco activation. The possible importance of the regulation of rubisco activation for the stabilization of the level of inorganic phosphate and other metabolites, which among other things could minimize

feedback effects from limitations in either energy production, energy utilization, or sucrose export, has been discussed extensively in a recent review [107]. Because the potential importance of changes in rubisco activation has become clear only recently, critical experimental verifications of most of these ideas have not been reported.

Examples of how two specific ideas might be investigated are presented. The first idea is that if a change in rubisco activation serves to increase metabolic stability then it should be reflected in a reduction of the oscillatory potential of the reductive pentose phosphate cycle. Oscillations in photosynthesis can be induced by various treatments and it has been suggested that explaining these oscillations can extend the limits of our understanding of the regulatory systems that must be involved [99]. Nevertheless, homeostasis, not oscillation, is generally the norm with an oscillatory behavior sometimes occurring under conditions where normal regulation has broken down [65]. Thus the system is actually uncontrolled in a physiological sense. As the conditions and changes that induce oscillations in photosynthesis are fairly well characterized [99], a determination of rubisco activation and the oscillatory potential of photosynthesis at various times following a decrease in light intensity would provide a good test of this hypothesis.

The second idea is that the alterations in rubisco activation reestablish conditions suitable for the proper partitioning of the newly formed photosynthate into starch and sucrose [4,89]. The regulation of starch and sucrose metabolism is complex [21] and it is difficult to evaluate the conditions over which the regulatory processes can properly function to maintain the proper rates for each. Nevertheless, one can envision that a relatively homeostatic environment for many of the metabolites might be required. This environment could be provided in part by maintaining a large and otherwise inert RuBP pool, thereby keeping the rubisco active sites occupied with RuBP, rather than occupied with other metabolites [2]. As a specific example, the PGA level was observed to increase dramatically following the reduction of light intensity in spinach [62]. If no further change occurs, the high PGA level should favor proportionately more starch synthesis even though total photosynthate production is reduced. Indirect evidence of a continued high rate of starch synthesis after the decrease in irradiance was found [62]. In contrast, translocation studies indicate that export of photosynthate from mature leaves is maintained at the expense of starch reserves when current photosynthate production is reduced [26,32]. Accordingly, the PGA level does not remain high, but slowly decreases as rubisco deactivates [62], sequestering the phosphate in the form of RuBP. A similar response of PGA was seen with the effect of increased CO_2 on rubisco activation in *Phaseolus vulgaris* [71]. The adjustments of the metabolite pools then allow the relative rates of starch and sucrose to be regulated properly by other factors. A critical test of this hypothesis can be made only by direct measurements of whether or not short term changes in the rates of sucrose and starch synthesis are associated with the changes in rubisco activation that are induced by the various factors.

VII. Concluding remarks

Rubisco catalyzes two unique reactions in biochemistry; the carboxylation of RuBP with CO_2 rather than bicarbonate and the oxygenation of RuBP by molecular O_2 without the participation of a metal cofactor [51]. Activation of the enzyme by carbamate formation also is unique although other instances of carbamate formation for biochemical purposes are known. On the basis of these precedents, perhaps we should not be surprised that the mechanism used by rubisco activase to promote the activation of this unique enzyme is not obvious at present. There could be useful analogies between rubisco activase and the G-protein system [29], but except for the nucleotide binding area, significant sequence homologies do not exist.

Regardless of the remaining questions about the mechanism, the phenotype of the *Arabidopsis rca* mutant has made it clear that rubisco activase is a very important component for photosynthesis in eukaryotic organisms. Whether or not rubisco activase is present in the photosynthetic bacteria is not known. A customary growth in high CO_2 environments or the presence of CO_2 concentrating mechanisms coupled with the absence of a strong inhibitory effect of RuBP on the spontaneous activation of rubisco in the few representatives examined [1,28,36,38] might imply that there would be less of a need for rubisco activase in these prokaryotes. Nevertheless, only additional experiments can provide a definitive answer.

The regulation of rubisco activity once seemed to be superfluous because of the extensive regulation of enzyme activity at many other steps in the reductive pentose phosphate cycle [51]. The existence of a regulator of rubisco activation state, rubisco activase, together with the repeated observation that rubisco activity is coordinately regulated with the rate of photosynthesis under steady-state conditions when light intensity changes, and the variations in rubisco activity that have been found to occur in other situations, have made this viewpoint no longer tenable. Yet our understanding of the physiological implications of the changes in rubisco activation is largely inferential. Further studies of rubisco activase should make an important contribution to the resolution of this problem.

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A report on the alternative mRNA splicing process in spinach and Arabidopsis has recently appeared [109].