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Review

Inhibition of Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase by 2-Carboxyarabinitol-1-Phosphate[1]

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ABSTRACT

In some plants, 2-carboxy-D-arabinitol 1-phosphate (CA1P) is tightly bound to catalytic sites of ribulose, 1,5-bisphosphate carboxylase/oxygenase (rubisco). This inhibitor's tight binding property results from its close resemblance to the transition state intermediate of the carboxylase reaction. Amounts of CA1P present in leaves varies with light level, giving CA1P characteristics of a diurnal modulator of rubisco activity. Recently, a specific phosphatase was found that degrades CA1P, providing a mechanism to account for its disappearance in the light. The route of synthesis of CA1P is not known, but could involve the branched chain sugar, hamamelose. There appear to be two means for diurnal regulation of the number of catalytic sites on rubisco: carbamylation mediated by the enzyme, rubisco activase, and binding of CA1P. While strong evidence exists for the involvement of rubisco activase in rubisco regulation, the significance of CA1P in rubisco regulation is enigmatic, given the lack of general occurrence of CA1P in plant species. Alternatively, CA1P may have a role in preventing the binding of metabolites to rubisco during the night and the noncatalytic binding of ribulose bisphosphate in the light.

Since its discovery, rubisco[2] has remained both a fascinating as well as an enigmatic enzyme. Early workers were awed by the rather large amount of fraction I protein present in the chloroplast compared to the rather trifling amounts of the 20 or so other enzymes necessary for the operation of the Calvin cycle. In an early review, Kawashima and Wildman (7) speculated that only a minute fraction of the total rubisco may be involved in catalytic activity at any given instant. Later, based on better measurements of K_m, it was concluded that all of the rubisco had to be catalytically involved in CO_2 fixation to account for observed rates of photosynthesis in leaves (4). Conventional wisdom during the latter part of the 1970s was that the enzyme was fully active during periods of high photosynthesis and probably remained so at other times with control of carboxylation being exerted by the rate of synthesis of RuBP (4, 12). However, work during the next decade established that the fraction of catalytically competent rubisco changed with light level. In the space of a year, two mechanisms for explaining light regulation of rubisco activity *in vivo* were proposed, enzyme-mediated activation (carbamylation) of native rubisco by rubisco activase (17, 25) and the reversible binding of the CA1P to carbamylated rubisco (3, 5). Still more surprising discoveries will be made before the present enigma of multiple mechanisms of rubisco regulation are resolved.

DISCOVERY OF ENDOGENOUS INHIBITION OF RUBISCO

A number of early reports suggested that rubisco activity increased upon illumination of plant material. In retrospect, it is difficult to determine whether these increases resulted from activation (carbamylation) or total activity of the enzyme. Proper interpretation of changes in rubisco activity in response to light awaited the discovery of the mechanism of enzyme activation by the reversible binding of CO_2 and Mg^{2+} (9, 10) and a general method for measuring total activity in crude leaf extracts. Rapid extraction of leaf tissue at low temperature in buffer, followed by immediate assay in a medium containing buffer, Mg^{2+}, CO_2, and RuBP, allowed measurement of an activity resulting from only carbamylated enzyme (EC and ECM forms) (13). Perchorowicz *et al.* (13) called the activity measured immediately after extraction the initial rubisco activity. Incubation of the extract in the assay medium containing high CO_2 and Mg^{2+} for a few minutes before addition of RuBP resulted in a higher activity, total rubisco activity, the combined activity following conversion of the EC and E forms to ECM. They used the term percent activation (100% × initial activity/total activity) to describe the relative amount of enzyme existing in the leaf in the active state.

While total rubisco activity in wheat leaves was relatively independent of light level (13), initial activity increased with increasing light level. In contrast to these data from wheat, in soybean leaves both total and initial activity increased with increasing light level (11, 21, 23). The total activity present at the end of the night remained low if the dark period was extended by covering the leaf, but increased to a maximum within a few minutes after removing the cover and exposing the leaf to high light (23). Attempts to increase total activity

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[2] Abbreviations: rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; CA1P, 2-carboxy-D-arabinitol-1-phosphate; CA, carboxyarabinitol; RuBP, ribulose-1,5-bisphosphate; E, native rubisco; EC, rubisco containing bound activator CO_2; ECM, rubisco containing bound activator CO_2 and Mg^{2+}; ER, rubisco containing only bound RuBP; CABP, 2-carboxyarabinitol 1,5-bisphosphate.

by extraction of the leaf tissue in a medium containing high Mg^{2+} and CO_2 (21), followed by gel filtration (21, 23), dialysis (19), or heat activation of the extract (19) were not successful.

When the dark-inhibited enzyme was maintained in the carbamylated state during purification, the characteristic low specific activity remained unchanged (19). Further analysis revealed that a phosphate ester was bound to the inhibited enzyme in a stoichiometry of approximately 1 mol per mol of inhibited binding sites. Removal of Mg^{2+} and CO_2 from the enzyme or treatment with high concentrations of sulfate resulted in the loss of the phosphate ester and an increase in specific activity. Following the night period about half of the enzyme's active sites were catalytically inactive because of the very tight binding of a small mol wt, monophosphate ester to active sites containing bound activator CO_2 and Mg^{2+}. A light-modulated inhibitor of catalysis was also found in leaves of *Phaseolus vulgaris* (18). Treatment of the enzyme-inhibitor complex *in vitro* with alkaline phosphatase restored rubisco activity, indicating that the inhibitor was probably phosphorylated. *In vivo*, both the initial rate of disappearance and the final concentration of the inhibitor were found to vary with light level. Inhibiting electron transport with DCMU inhibited the light-dependent removal of the inhibitor. The partially purified inhibitor from bean bound tightly in a 1:1 stoichiometry to active sites on purified spinach rubisco. The dissociation constant for inhibitor binding was about 1×10^{-7} M indicating a tight binding with the active site (18).

IDENTIFICATION OF CA1P

Gutteridge *et al.* (5) purified the endogenous inhibitor from potato and established that its structure was 2-carboxy-D-arabinitol 1-P. Berry *et al.* (3) confirmed the same structure for the tight binding inhibitor from *Phaseolus*. The proton NMR spectrum suggested that the general structure of the inhibitor was $CH_2(OH)$–$CH(OH)$–$CH(OH)$–X–$CH_2OPO_3^{2-}$, X was later found to be a quarternary carbon with a carboxylic group (5). Confirmation that the natural inhibitor was a 2-carboxypentitol 1-P was achieved by GC-MS of the dephosphorylated molecule (3, 5). The mass spectra of the dephosphorylated inhibitor was identical to the spectra of authentic 2-carboxyarabinitol. To distinguish between epimers having the general carboxypentitol structure, the four possible forms were synthesized and the proton spectra of the resulting acids compared to the dephosphorylated inhibitor (5). The proton and mass spectra of authentic CA1P were similar to those for the inhibitor. CA1P closely resembles 2-carboxy-3-ketoarabinitol 1,5-bisP, the transition-state intermediate of the carboxylase reaction, and the synthetic transition-state analog, CABP. The absence of the second phosphate group on CA1P prevents it from being bound as tightly to the active site as CABP, which binds in an essentially irreversible manner ($K_d = 1 \times 10^{-11}$). Interestingly, CA5P does not bind tightly to rubisco and this difference was exploited as a method for separating CA1P from a mixture of CA1P and CA5P (3).

DEGRADATION AND SYNTHESIS OF CA1P

CA1P is progressively degraded with increasing light level. The mechanism by which light mediates the breakdown of CA1P remains to be explained. Feeding DCMU (18), nigericin, or methyl viologen (15) to leaf discs prevents the light-dependent removal of CA1P from rubisco, indicating the involvement of photosynthetic electron transport. Recently, a protein effective in hydrolyzing CA1P to CA was purified from tobacco chloroplasts (6, 16). The protein had a high affinity for hydrolyzing CA1P but was ineffective against other phosphate esters. A sulfhydryl reagent was necessary for maintaining activity and NADPH stimulated activity 2.5-fold. Control of the rate of enzymic degradation of CA1P could be by reduction of thiol groups on the protein via Fd-thioredoxin. Interestingly, this phosphatase is present, although at lower activities, in leaves of spinach (15) and wheat (AJ Keys, personal communication), species containing little or no CA1P.

While rubisco activase does not metabolize CA1P, it does to some extent relieve CA1P inhibition (14). Perhaps by facilitating removal of CA1P from rubisco, it allows CA1P to be degraded more quickly, thereby controlling both carbamylation (25) and the number of catalytic sites available for carboxylation. Interestingly, the ATP requirement to relieve CA1P inhibition may be smaller than that required to promote carbamylation (15).

Regulation involving light-dependent degradation of CA1P implies the existence of a pathway for its synthesis. CA1P is identical with D-hamamelonate 2′-P (2). Free D-hamamelose or hamamelose 2′-mono and 2′,5-bisP are present in the chloroplasts of many plant species. Likewise, hamamelose bisP was found to be labeled from $^{14}CO_2$ in isolated spinach chloroplasts in as little as 15 min (2). Conceivably, hamamelose 2′-P could be converted to CA1P simply by oxidation to hamamelonate 2′-P (2). Alternatively, CA1P could be synthesized from hamamelose, following oxidation to hamamelonate, and phosphorylation. In the latter case, CA would then be both product and substrate for CA1P degradation and synthesis. It is also possible that CA1P could be made from the transition state intermediate, 2-carboxy-3-keto-arabinitol 1,5-bisP, by removal of the phosphate at the 5 position and reduction of the 3-keto. In any scheme for generating CA1P, it might be necessary to avoid synthesis of CABP, a molecule that binds very tightly to rubisco.

A practical application of knowing the pathways of synthesis and degradation of CA1P is the potential for development of a new mode of action for herbicides. A chemical which inhibits breakdown of CA1P or stimulates its production could reduce the amount of rubisco catalytic sites available for carboxylation and reduce photosynthesis and growth. If the occurrence of CA1P is restricted to only some species, then such chemicals might have some specificity.

OCCURRENCE OF RUBISCO INHIBITION

The large variation in the amounts of CA1P present in various plant species is striking. Amounts of CA1P range from that equalling the total rubisco binding site concentration to essentially none. Vu *et al.* (24) showed that total rubisco activity in leaves collected in the dark (before dawn or predawn) and at high light (midday) was the same in maize and wheat, meaning that in the dark no inhibitor was present in leaves of these species. Servaites *et al.* (20) found that 23

of 37 species examined showed a predawn to midday activity ratio of ≤0.75, while only pepper, cucumber, soybean, tobacco, and cowpea and three *Solanum* species showed a ratio ≤0.50. *P. vulgaris* consistently showed a predawn to midday activity ratio of ≤0.10, which concurs with the prior finding of Seemann *et al.* (18). For a given species these ratios were similar whether measured in plants growing in the United States or the United Kingdom (20), indicating a genetic basis that is relatively independent of conditions in these divergent locations.

Inhibitors of rubisco may be of general occurrence in plants with only CA1P having been identified to date (1). While CA1P is the predominant inhibitor present in potato and bean, other inhibitors may predominate in other species. *Phaseolus* is an exception because of the large amount of CA1P present. Other inhibitors may have eluded discovery because they do not bind as tightly as CA1P or are labile and lost during extraction. An effective rubisco inhibitor need only bind tightly enough to avoid displacement by RuBP (1).

PHYSIOLOGICAL ROLE OF CA1P BINDING

Light-mediated regulation of carbon fixation is controlled by the rate of RuBP synthesis and its utilization. RuBP level increases from essentially zero in the dark to a substantial, steady state level in the light. The rise in RuBP level is essentially completed far below the level of light required to saturate photosynthesis and further increases in photosynthesis rate coincide with increases in rubisco activity (13). In view of the relative constancy of RuBP level with increasing light, mechanisms must exist to control rubisco activity to closely match utilization of RuBP with its synthesis. A slight imbalance between the rates of synthesis and utilization would quickly produce a dramatic change from this stable level. Because the RuBP pool represents a considerable part of the chloroplastic Pi pool size, large fluctuations in RuBP level could have significant effects on Pi:triose-P transport, photophosphorylation and on starch synthesis and breakdown rates.

Currently, we know of only two means for regulating the number of catalytically active sites on rubisco: carbamylation and binding of CA1P. The two are interrelated because if more sites contain bound CA1P, fewer sites are available for regulation by carbamylation. This is especially well illustrated in *Phaseolus* (18) and soybean (11, 21, 23) in which rubisco catalytic site concentration is principally dependent on the reversible binding of CA1P. Increasing light level reduces the amount of inhibitor, but the carbamylation state of rubisco remains nearly constant and independent of light level. Conversely, in species having little or no CA1P, light regulation of rubisco is regulated principally by changes in carbamylation state.

Activase is necessary for rubisco functioning as shown by the inability of a mutant of *Arabidopsis thaliana* which lacks rubisco activase to maintain rubisco activity at low CO_2 concentration (22). Given this evidence, the ubiquity of activase in plants, and the fact that CA1P apparently exerts no additional control on the amount of rubisco catalytic sites present at a given light level, CA1P binding may be considered a redundant mechanism for regulating rubisco (15). In support of a role in light-modulation of rubisco, CA1P has properties consistent with it being a regulatory metabolite. As with other such molecules, it reversibly inhibits at concentrations comparable to that of the enzyme's active sites. Also, both the concentration of CA1P and the initial rubisco activity in a leaf roughly follow the daily changes in light level. The rates of its synthesis and degradation are fast enough to enable it to function in dynamic regulation of rubisco (8). Although not found universally, CA1P may be but one of a class of rubisco inhibitors.

Alternatively, CA1P may have a regulatory role different from that of activase, one not related to controlling carboxylation, but to altering the binding of molecules to rubisco. When RuBP concentration is low, rubisco probably binds a number of metabolites. Some of these metabolites are effective in promoting carbamylation, as evidenced by the higher than expected carbamylation states present in leaves in the dark compared to those at low light. Similarly, species having high levels of CA1P also have high carbamylation states in the dark and these remain high in the light. A metabolite cannot be involved in metabolism if it is bound to rubisco. Because CA1P has a much higher binding affinity than other metabolites, its presence would lessen their binding and allow them to participate in metabolism. More importantly, in the light, CA1P stabilizes the ECM form and prevents the binding of RuBP and formation of the E-RuBP complex. In effect, the presence of CA1P would stoichiometrically increase the pool of unbound RuBP, allowing these plants to function at lower total concentrations of RuBP.

FUTURE RESEARCH

A primary direction for future research is resolving the enigma of multiple mechanisms for regulating rubisco activity. Specifically, understanding the relative importance of carbamylation and inhibition in regulating rubisco activity. If altering the binding of metabolites to rubisco is the primary role of CA1P, then the significance of this role needs to be addressed. Experiments are needed to determine why amounts of CA1P vary in plant species, that is, whether the pathways for CA1P synthesis and degradation are not present in some plant species or whether some species rapidly degrade any CA1P that is formed. A difficult but important question is whether other rubisco inhibitors are present and have a role in regulating rubisco activity. While evidence for a specific phosphatase for degrading CA1P exists in chloroplasts, it is not known how its activity is regulated by light and whether activase is involved. Likewise, the fate of the dephosphorylated product, the branched chain 2-carboxyarabinitol or hamamelonic acid needs to be determined. The pathway for synthesizing CA1P is not known, but could involve metabolism from hamamelose. While the level of CA1P is thought to be dynamically regulated by its rates of synthesis and degradation, it is not known to what extent either is regulated and the role of light in this regulation. Both the pathways of synthesis and degradation of CA1P may be susceptible to the action of appropriate agricultural chemicals and lead to the development of new herbicides and growth regulators based on a different mode of action.

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