Plant Physiol. (1994) 105: 731–737

Evidence That 2-Carboxyarabinitol 1-Phosphate Binds to Ribulose-1,5-Bisphosphate Carboxylase in Vivo[1]

Brandon d. Moore* and Jeffrey R. Seemann

Department of Biochemistry, University of Nevada, Reno, Nevada 89557–0014

An important question concerning the role of carboxyarabinitol 1-phosphate (CA1P) metabolism in the light-dependent regulation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity is the extent to which CA1P is bound to Rubisco in vivo. We report here the development of an extraction procedure using ammonium sulfate that stabilizes CA1P bound to Rubisco. This procedure exploits the ability of sulfate to bind at the catalytic site of Rubisco and to competitively balance the binding and release of CA1P from Rubisco. In darkened bean leaves about 75% of the Rubisco catalytic sites were found to be bound with CA1P. This confirms previous indirect estimates from gas exchange measurements. We have used this extraction procedure to examine CA1P-Rubisco interactions in bean during a natural transition from darkness to light. With increasing light intensity following sunrise, CA1P degradation proceeded in two distinct phases: first, a majority of the unbound CA1P pool was degraded at very low light levels (≤30 μmol quanta m^{-2} s^{-1}); second, CA1P initially bound to Rubisco was then degraded at increasing light levels (>30 μmol quanta m^{-2} s^{-1}). These results indicate that there is a low-fluence activation of CA1P phosphatase that can occur prior to CA1P release by Rubisco activase. This activation may be mediated by NADPH. During sunrise in bean, the level of the catalytically competent form of Rubisco was regulated by CA1P metabolism.

Researchers have recognized for about 10 years that the total extractable activity of Rubisco may be considerably lower from darkened leaves of certain species than from high-light-treated leaves (Ku et al., 1982; Vu et al., 1983). This inhibition was attributed to the presence of a tight-binding, phosphorylated metabolite (Seemann et al., 1985; Servaites, 1985) that was identified as CA1P (Gutteridge et al., 1986; Berry et al., 1987). CA1P is present in darkened leaves of most species but at levels varying from <7% of the Rubisco catalytic site concentration in *Spinacia oleracea* and *Triticum aestivum* to 150% in *Phaseolus vulgaris* (Moore et al., 1991). During illumination of dark-treated leaves, CA1P is likely removed from Rubisco by Rubisco activase (Robinson and Portis, 1988) and then degraded by a specific chloroplast enzyme, CA1P phosphatase (Holbrook et al., 1989).

The light-dependent regulation of Rubisco activity occurs by adjustment of the level of catalytically competent enzyme within the chloroplast (ECM; for review, see Portis, 1992). In some species the level of the ECM complex is adjusted by reversible carbamylation/decarbamylation. There is also substantial evidence that other species may regulate Rubisco activity by a combination of reversible carbamylation and tight binding of CA1P to carbamylated enzyme (e.g. *Nicotiana rustica* [Salvucci and Anderson, 1987], *Beta vulgaris* [Kobza and Seemann, 1988], *P. vulgaris* [Kobza and Seemann, 1988; Sage et al., 1993]). Much of the evidence for such regulatory binding of CA1P to Rubisco in leaves has been obtained from examination of the time course and levels of CA1P accumulation in relation to the light-dependent level of Rubisco activity and whole leaf photosynthesis (for review, see Seemann et al., 1990). However, it has been questioned whether the observed decreases in extractable Rubisco activity from darkened leaves are solely due to CA1P that is bound to Rubisco in vivo or are in part due to free CA1P that becomes bound to Rubisco during extraction (Portis, 1992). If CA1P were not extensively bound to Rubisco in vivo, then new explanations would be required to account for the reduced photosynthetic rate of some C_3 plants under moderately low-light conditions, when Rubisco is highly carbamylated and the carboxylation reaction is not substrate limited (Kobza and Seemann, 1988; Sage et al., 1990, 1993).

Until now there has been no way to assess the actual level of CA1P binding to Rubisco in vivo. To determine such binding, an ideal method would prevent both the dissociation of any ECM-CA1P present in vivo and the binding of any free CA1P during extraction and activity assay. Since the calculated half-time for dissociation of ECM-CA1P is about 20 min (Berry et al., 1987), the magnitude of such dissociation during rapid leaf extraction and assay is probably minimal. Therefore, the question of the in vivo binding status of CA1P could be most simply addressed by including an appropriate exogenous substance during extraction that would prevent binding of CA1P but would not inhibit binding of RuBP during assay.

Both inorganic and organic oxyanions such as sulfate, phosphate, Fru-1,6-bisP, and 6-P-gluconate can have complex interactions with Rubisco. Such compounds will often increase the enzyme activation state at low CO_2 but will also competitively inhibit the carboxylation reaction with respect to RuBP (Keys and Parry, 1990). Vu et al. (1984) noted that, following ammonium sulfate fractionation of extracts from darkened soybean leaves, Rubisco was then capable of being activated in vitro (i.e. CA1P was removed during fractiona-

[1] This work was supported by U.S. Department of Agriculture-National Research Initiative grant 93–37306–9240 to J.R.S. and B.d.M.

* Corresponding author; fax 1–702–784–1419.

Abbreviations: CA1P, 2-carboxyarabinitol 1-phosphate; E, inactive form of Rubisco; ECM, active (carbamylated with active site Mg^{2+}) form of Rubisco; RuBP, ribulose-1,5-bisphosphate.

tion). Servaites (1985) exploited this observation to obtain fully activatable enzyme following a 20-min treatment of tobacco leaf extracts with 0.2 M ammonium sulfate. Thus, since high levels of ammonium sulfate can remove CA1P from Rubisco, such levels must also prevent CA1P from binding to Rubisco. We reasoned that it should be possible to extract leaf tissue with more modest levels of ammonium sulfate and thereby limit the artifactual binding of any free CA1P, while maintaining the short-term stability of any ECM-CA1P present in vivo. Such a procedure should stabilize ECM-CA1P or at least poise the binding equilibria such that there would be no net change during extraction in the amount of CA1P that was bound to Rubisco. The experiments reported herein demonstrate the validity of this procedure and provide evidence that CA1P is bound to Rubisco in vivo to an extent largely predicted by previous measurements. Furthermore, we utilize this technique to show that CA1P degradation in *Phaseolus* during a natural dawn proceeds in two distinct phases: first, unbound CA1P is degraded and then CA1P initially bound to Rubisco is degraded, presumably after its release by Rubisco activase.

MATERIALS AND METHODS

Plant Material

Plants of *Phaseolus vulgaris* L. cv Linden were grown as previously described (Moore et al., 1991). Leaves were collected into liquid N_2 at midday or as otherwise indicated. Principal veins were removed, and the material was stored at −80°C until used. To minimize variation in plant material, most experiments were done using a single, large collection of high-light-treated leaves.

Assay of Leaf CA1P

[2-^{14}C]CA1P (specific radioactivity about 1.5 Ci mol^{-1}) was synthesized as described previously (Gutteridge et al., 1989; Moore et al., 1991). Leaf CA1P levels were measured using an isotope dilution assay (Moore et al., 1991; Moore and Seemann, 1992).

Leaf Extractions

About 0.6 g fresh weight were ground in a frozen mortar with 20% (w/w) insoluble PVP and 10 volumes of CO_2-free grinding buffer. The buffer generally contained 100 mM Bicine (pH 7.8), 5 mM $MgCl_2$, 1 mM EDTA, and 5 mM DTT. Varying amounts of other components were sometimes added to the mortar ([^{14}C]CA1P) or grinding buffer (ammonium sulfate) as indicated in the figure legends and table heading. The leaf homogenate was filtered through Miracloth (4°C) and centrifuged at 10,000*g* for 10 s (20°C), and the supernatant was then used for assays.

Assay of Rubisco Activity

Rubisco activity was assayed in a 250-μL volume containing 50 μL of leaf extract in 100 mM Bicine (pH 8.2), 20 mM $MgCl_2$, 10 mM NaH$^{14}CO_3$ (specific radioactivity 0.2 Ci mol^{-1}), 5 mM DTT, 1 mM EDTA, and 1.0 mM RuBP. Assays were done in triplicate for 30 s at 25°C and were terminated by addition of 250 μL of 2 N HCl. The reaction mixture was dried with moderate heat and then resuspended in H_2O, and the radioactivity was measured by liquid scintillation spectroscopy. "Initial" Rubisco activity was measured directly from the leaf extract. This assay was initiated within 4 min from the time that the leaf material in the mortar began to thaw. "Total" Rubisco activity was measured after incubating 450 μL of extract with 50 μL of activation buffer, which gave final solution concentrations of 100 mM Bicine (pH 7.8), 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM DTT, and 1 mM EDTA. Other additions to the activation buffer and the incubation temperatures are indicated below. Where ammonium sulfate is present in the extraction buffer and CA1P is also present, either through addition or because it is present in the leaf, activation was carried out ultimately at 4°C. The increased activity was regarded as being due to carbamylation of vacant sites.

Assay of Rubisco Content

Rubisco catalytic site contents were measured by saturation binding of [2-^{14}C]2-carboxyarabinitol-1,5-bisphosphate (specific radioactivity 53 Ci mol^{-1}) to activated enzyme, followed by immunoprecipitation using anti-Rubisco polyclonal antibody (Collatz et al., 1979; Evans and Seemann, 1984).

Chl Measurement

Aliquots of leaf extracts were taken prior to centrifugation for measuring Chl content using 96% (v/v) ethanol (Wintermans and De Mots, 1965).

RESULTS

Effect of Ammonium Sulfate on Rubisco Activity

We first examined the influence of varying levels of ammonium sulfate on Rubisco activity following extraction of high-light-treated bean leaves. When ammonium sulfate was present only in the assay medium, there was little effect on enzyme activity at concentrations up to 20 mM (<7% inhibition, Fig. 1). At higher ammonium sulfate levels more substantial inhibition was observed. Thus, since the extract was always diluted 5-fold in the activity assay, ammonium sulfate could be present in the grinding or activation buffers at up to 100 mM concentration without there being significant direct inhibition of the activity assay. Most of the experiments described below utilized 25 or 50 mM ammonium sulfate in the grinding or activation buffers (thus, 5 or 10 mM in the activity assay), unless otherwise indicated.

We tested whether addition of 25 or 50 mM ammonium sulfate during in vitro activation of Rubisco had any effect on the measured total enzyme activity in leaf extracts that had no CA1P. Such additions did not change the total activity whether the activation procedure was done at 25 or at 4°C (data not shown). Also, the total Rubisco activity in such solutions was generally stable for 45 min.

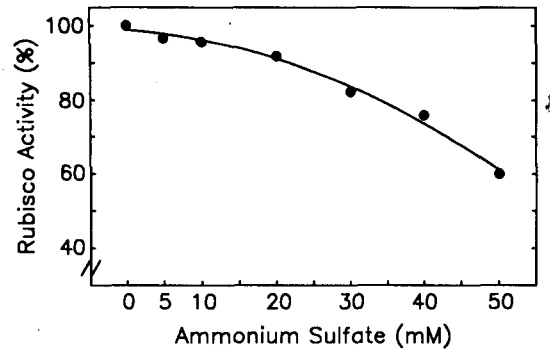

Figure 1. Ammonium sulfate inhibition of Rubisco activity. High-light-treated bean leaves were extracted in buffer without ammonium sulfate. The extract was then incubated with 10 mM $NaHCO_3$ and 20 mM $MgCl_2$ for 10 min at 25°C. Rubisco activity was measured in assay medium that contained various levels of ammonium sulfate. In this and subsequent experiments, activity measures were means of triplicate assays, and in vitro catalytic site concentrations were about 8.5 μM.

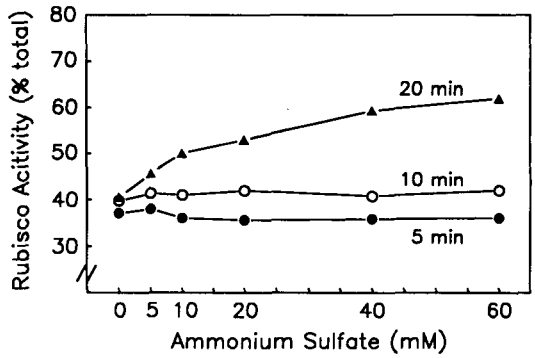

Figure 2. Influence of ammonium sulfate on Rubisco activity after its partial inhibition from binding CA1P. Activated Rubisco was incubated for 10 min at 25°C with CA1P at 1.27 times the Rubisco catalytic site concentration. The Rubisco/CA1P mixture was put on ice for 5 min, after which various levels of ammonium sulfate (or H_2O) were added at time zero. Rubisco activity was then assayed after further incubation on ice for an additional 5 min (●), 10 min (○), or 20 min (▲).

Effect of Ammonium Sulfate on Stability of ECM-CA1P

We examined the stability of the ECM-CA1P complex formed in vitro in solutions plus or minus ammonium sulfate. This stability was assessed by measuring any increase in Rubisco activity due to dissociation of CA1P. We reasoned that RuBP in the assay medium likely would out-compete the low amount of CA1P present in solution for binding at the substrate site, since RuBP would be present at levels about 2000-fold in excess of CA1P. Thus, any dissociation of ECM-CA1P due to the presence of ammonium sulfate would result in increased Rubisco activity and could be directly measured. By using ^{14}C-labeled CA1P, we could also monitor the stability of the CA1P in the leaf extracts by using HPLC to look for other labeled products such as carboxyarabinitol (Moore et al., 1992).

We tested the influence of varying levels of ammonium sulfate on a constant level of ECM-CA1P that was formed after incubation of enzyme with a relative excess of CA1P (Fig. 2). The inhibition level of Rubisco activity was essentially constant for 10 min at 4°C in the presence of ammonium sulfate at up to 60 mM. There was a substantial increase in enzyme activity after a longer, 20-min incubation at 4°C. Such increased enzyme activity was much more rapid if the incubation with ammonium sulfate was done at 25°C rather than 4°C (data not shown). Metabolite analysis by HPLC of the incubation mixtures showed that the [^{14}C]CA1P was stable after incubation at 4°C (data not shown). Thus, the observed increase in Rubisco activity was a direct consequence of ammonium sulfate-induced removal of CA1P and not due to CA1P degradation. Of primary importance, however, is that the ECM-CA1P complex was stable for at least 10 min at 4°C with up to 60 mM ammonium sulfate.

We also examined whether ECM-CA1P complexes with different ratios of inhibitor to enzyme were also stable in 25 mM ammonium sulfate at 4°C (Fig. 3). We did this experiment because, within a bean leaf under different conditions, there can be present different ratios of inhibitor to enzyme. Such complexes were found to be stable, particularly at inhibitor to enzyme ratios <1.0 mol CA1P/mol Rubisco sites.

Effect of Ammonium Sulfate on Formation of ECM-CA1P

We tested the effect of ammonium sulfate on the binding of CA1P to Rubisco by adding CA1P directly to the mortar at the time of extraction of high-light leaf material with buffer plus or minus ammonium sulfate. First, we extracted leaves in the presence of a constant ratio of CA1P to Rubisco sites (1.14) and with varying levels of ammonium sulfate (Fig. 4). Extraction in the absence of ammonium sulfate resulted in a 45% reduction of initial enzyme activity due to CA1P binding. Extraction with CA1P plus ammonium sulfate resulted in <5% reduction of initial activity when ammonium sulfate was present at 25 to 50 mM. Decreased initial activities at

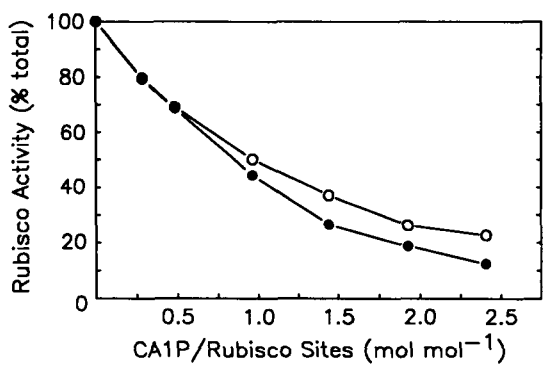

Figure 3. Influence of 25 mM ammonium sulfate on Rubisco activity after its partial inhibition from binding various levels of CA1P. Activated Rubisco was incubated for 10 min at 25°C with different levels of CA1P as indicated. The Rubisco/CA1P mixture was put on ice for 5 min, after which was added either H_2O (●) or ammonium sulfate (25 mM final concentration, ○). Rubisco activity was then assayed after an additional 5 min.

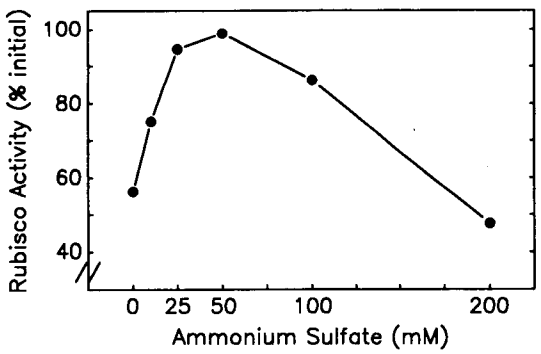

Figure 4. Effect of ammonium sulfate on protection of Rubisco from binding CA1P. High-light-treated leaves were extracted in the presence of CA1P (1.14 times the catalytic site content) and various levels of ammonium sulfate. Rubisco activity was then measured in the initial extract. For the control, initial activity was measured in the absence of CA1P and ammonium sulfate.

higher ammonium sulfate levels were attributed to direct inhibition of the activity assay.

Next, we extracted high-light leaf material in the presence of various ratios of CA1P to Rubisco catalytic sites and with either 25 or 50 mM ammonium sulfate (Fig. 5). In the absence of added ammonium sulfate, the extractable initial Rubisco activity declined in proportion to the increasing relative levels of CA1P. At the highest relative level of CA1P, the initial activity was reduced by about 75%. However, inclusion of either 25 or 50 mM ammonium sulfate effectively maintained the initial enzyme activity in the presence of added CA1P at a level up to 1.2 times that of Rubisco catalytic sites. At lower relative CA1P levels, both 25 and 50 mM ammonium sulfate gave somewhat higher initial rates than in control extracts without CA1P. At higher relative CA1P amounts, greater initial enzyme activity was measured from extracts with 50

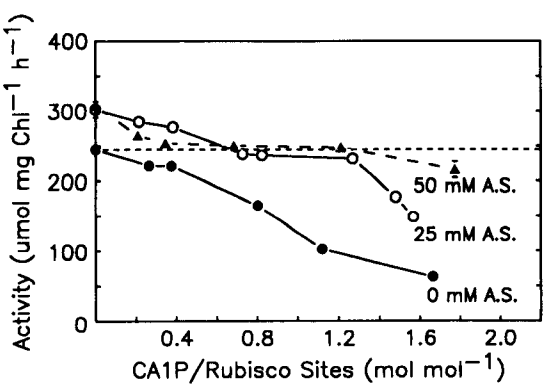

Figure 5. Effect of varying the CA1P to ECM ratio on ammonium sulfate protection of Rubisco from binding CA1P. High-light-treated leaves were extracted with different levels of CA1P as indicated and with either 0 mM (●), 25 mM (○), or 50 mM (▲) ammonium sulfate. Rubisco activity was then measured in the initial extracts. The horizontal dashed line represents the initial enzyme activity in leaves extracted without CA1P or ammonium sulfate. The average SD values for assays from the single extractions were 3.1% of the indicated average mean values. A.S., Ammonium sulfate.

mM ammonium sulfate than from extracts with 25 mM ammonium sulfate.

We also attempted to activate Rubisco from high-light-treated leaves that were extracted with 25 or 50 mM ammonium sulfate and a relative excess of CA1P to Rubisco catalytic sites (approximately 1.2-fold). No increase in enzyme activity occurred when the activation was done at 25°C. However, when incubated at 4°C, Rubisco activity increased substantially; after 10 min it reached a rate of about 90% of the total activity of control extracts that had no CA1P (i.e. a 30% increase from initial rates; data not shown).

Rubisco Activity from Dark-Treated Bean Leaves

Our first objective was to demonstrate that CA1P is bound to Rubisco in vivo in dark-treated bean leaves. We did initial activity assays from extracts of light- and dark-treated leaves, with or without ammonium sulfate in the grinding buffer (Table I). After extraction of light- or dark-treated leaves with ammonium sulfate, there was a small increase in initial activities. The initial rate from dark-treated leaves was still quite low relative to light controls. We also attempted to activate Rubisco from the leaf extracts (Table I). With light-treated leaves, the same total activity was obtained from extracts made with or without ammonium sulfate. When dark-treated leaves were extracted without ammonium sulfate, there was a small decrease in the total activity relative to the initial activity. This decrease was presumably due to additional binding of CA1P. However, when dark-treated leaves were extracted with ammonium sulfate, there was a modest increase in total Rubisco activity relative to the initial activity. We assume that such increased activity was due to carbamylation of sites that initially had no CA1P and were decarbamylated. The total dark activity, however, was still much less than the total activity from light-treated leaves due to CA1P bound to Rubisco. Thus, we can extract bean leaves with ammonium sulfate to partially characterize Rubisco catalytic sites under a physiological condition of interest.

Table I. *Rubisco activity in light- or dark-treated bean leaves*

Leaves were collected either at midday or after a 16-h dark treatment. Leaves were extracted in grinding buffer with or without 25 mM ammonium sulfate. Total Rubisco activity was measured in leaf extracts that were incubated under activating conditions for 10 min at 4°C. CA1P content of the dark leaves was measured by HPLC, using an isotope dilution assay (Moore et al., 1991). The amount of CA1P was 167 nmol g^{-1} fresh weight, or 1.46 times the amount of Rubisco catalytic sites (measured in light-treated leaves). Values are means ± 1 SD of three extractions.

Light Treatment	Extracts Plus/Minus Ammonium Sulfate	Rubisco Activity	
		Initial	Total
		μmol mg^{-1} Chl h^{-1}	
Light	Minus	245 ± 12	426 ± 31
Light	Plus	302 ± 6	433 ± 23
Dark	Minus	102 ± 5	93 ± 2
Dark	Plus	128 ± 5	156 ± 8

CA1P Levels and Rubisco Catalytic Sites Characteristics before and after Sunrise

We examined Rubisco activity and CA1P levels in bean leaves collected at various levels of natural irradiance before and after sunrise (Fig. 6). Rubisco activity was assayed after leaf extraction using 0, 25, or 50 mM ammonium sulfate added to the grinding buffer. When leaves were extracted with ammonium sulfate, the initial activities were the same in leaves collected either from the dark or at 30 μmol m^{-2} s^{-1} irradiance (30 min after sunrise). Thereafter, the initial activity increased with further increases in irradiance. However, CA1P levels were highest in darkened leaves and then declined continually with increasing light after sunrise. That is, although the level of active catalytic sites was constant during the first 30 min after sunrise, the total leaf CA1P pool decreased by almost one-third.

As determined from calculations described in Figure 7, about 25% of the Rubisco catalytic sites were inactive in high light, and about 70% of the sites were inactive in the dark. The number of inactive sites can be further partitioned into those that were apparently decarbamylated and those that were bound to CA1P. The number of CA1P-inhibited sites decreased in a linear fashion as the CA1P-to-Rubisco site ratio decreased from 1.05 times the catalytic site concentration. However, the number of CA1P-inhibited sites present before dawn was the same as the number at 30 min after dawn. These data indicate that about one-half of the dark CA1P pool is not bound to Rubisco in vivo, and the majority of this pool is metabolized at a lower light level than is the CA1P that is bound to Rubisco. The number of decarbamylated sites was relatively constant during the different irradiance conditions, such that the change in the number of total active sites during this time was determined by CA1P degradation.

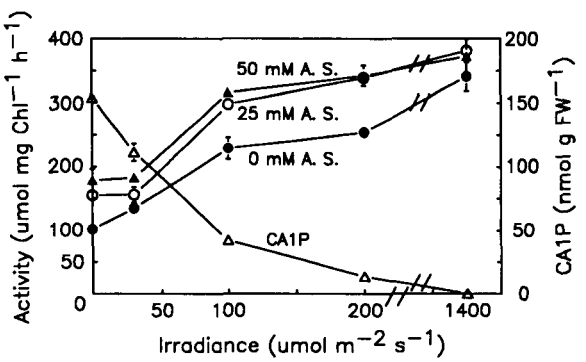

Figure 6. Initial extractable Rubisco activity and CA1P levels in bean leaves collected at different irradiances, before and after sunrise. Leaves were collected before dawn (dark), 30, 60, and 90 min after dawn (30, 100, and 200 μmol quanta m^{-2} s^{-1}, respectively), and at midday (1400 μmol quanta m^{-2} s^{-1}). Initial Rubisco activity was measured from leaves extracted with 0 mM (●), 25 mM (○), or 50 mM ammonium sulfate (▲). CA1P content (△) was measured from the same collection of leaf material as used for activity assays. Values are means ± 1 SD of three or four extractions. For data points with no indicated SD values, the SD range was less than the symbol size. A.S., Ammonium sulfate; FW, fresh weight.

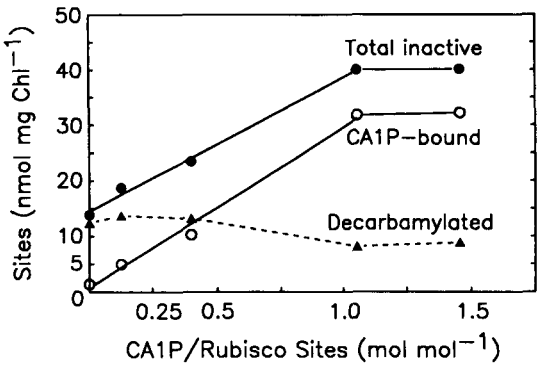

Figure 7. The number of inactive Rubisco catalytic sites as a function of leaf CA1P levels present before and after sunrise. The total number of inactive catalytic sites (decarbamylated plus CA1P bound, ●), the number of CA1P-inhibited Rubisco sites (○), and the number of decarbamylated sites (▲) were calculated using Rubisco activities measured from leaves extracted with 25 mM ammonium sulfate. Rubisco activities in leaves from low irradiances were measured after extraction with 25 mM ammonium sulfate and either assayed immediately (initial activity, Fig. 6) or assayed after activation for 10 min at 4°C (total activity). Assays of high-light total Rubisco activity were done with leaves collected at midday and extracted without ammonium sulfate. For all of the irradiance conditions, the apparent activation state was 73 ± 4% (SD). Total Rubisco catalytic site content was 58.1 nmol mg^{-1} Chl, measured in high-light-treated leaves. Site content in other leaves was calculated from Chl content. The number of inactive catalytic sites was calculated from the difference in total sites and active sites. The number of active sites = (total sites) × (initial activity ÷ light total activity). The number of CA1P-inhibited sites = (total sites) × [1 − (dark total activity ÷ light total activity)]. The number of decarbamylated sites = (total inactive sites) − (CA1P-inhibited sites).

DISCUSSION

Ammonium sulfate has long been recognized as an inhibitor of Rubisco activity (Paulsen and Lane, 1966), but the manifestations of its interaction are complex and not clearly understood. The sulfate anion is known to interact with the RuBP-binding site (McCurry et al., 1981), and in so doing may stabilize the ECM form of the enzyme by inhibiting the dissociation of the activator CO_2 and Mg^{2+} (Badger and Lorimer, 1980). Depending on experimental conditions, sulfate can either inhibit or mildly stimulate Rubisco activity (Parry and Gutteridge, 1984). In this study, we also observed both effects of ammonium sulfate on catalysis by bean Rubisco (Fig. 1; Table I).

We were able to exploit the ability of ammonium sulfate to compete with CA1P at the RuBP-binding site to demonstrate that CA1P is bound to Rubisco in bean leaves. The validity of this method is based on the following observations: (a) Rubisco in the ECM-CA1P form was stable for at least 10 min at 4°C in the presence of ammonium sulfate at up to 60 mM (Figs. 2 and 3). This time period is sufficiently long to allow leaf extraction and activity assays without dissociation of any ECM-CA1P that may be present. (b) Extraction of high-light-treated bean leaves (with Rubisco at ≥75% carbamylation) with 25 or 50 mM ammonium sulfate

prevented binding of CA1P at levels up to 1.2 mol CA1P mol^{-1} Rubisco catalytic sites (Figs. 4 and 5). This experiment is somewhat different from the situation in vivo. In the leaf, CA1P levels may never be in large excess of the Rubisco catalytic site content at the same time that there is a large number of available, carbamylated sites with which to bind. Generally, several hours are required for CA1P to accumulate to such levels in the leaf (Sage et al., 1993), during which it will have already bound to Rubisco. (c) Extraction of dark-treated bean leaves with 25 mM ammonium sulfate resulted in a slightly higher initial Rubisco activity than measured in control extracts (Table I). This may indicate that a small amount of artifactual binding of CA1P occurred during leaf extraction under control conditions. However, this small increase was also evident in extracts of light-treated leaves and could be due to ammonium sulfate limiting any decarbamylation that may have occurred in control extracts (Parry and Gutteridge, 1984). (d) Extraction of dark-treated bean leaves with 25 mM ammonium sulfate yielded an extract from which Rubisco could be activated, with a 25 to 40% increase in activity (Table I; Fig. 6). The difference in total enzyme activity in light versus dark extracts is still quite large due to CA1P binding to catalytic sites.

Ammonium sulfate at 25 to 50 mM was optimal for inclusion in the grinding medium to inhibit artifactual binding of CA1P to ECM during extraction of bean leaves (Fig. 4). Whether CA1P binds to decarbamylated Rubisco in vivo is unknown but is likely. Methods used in this study would not have explicitly detected any E-CA1P complex since our measure of bound CA1P relied on the inhibition of catalytic activity. However, if any E-CA1P were stable during the leaf extraction and activity assay, then ammonium sulfate in the grinding buffer also may have stabilized this enzyme form. This aspect remains to be investigated.

We used grinding buffer plus ammonium sulfate to partially characterize the Rubisco catalytic sites during sunrise (Fig. 7). Almost one-third of the leaf CA1P was degraded by the time the PPFD had increased to 30 μmol quanta m^{-2} s^{-1}; yet there was no change in the level of CA1P bound to Rubisco. This initial decrease in CA1P level probably resulted from an increased rate of CA1P degradation. CA1P that is bound to Rubisco is likely released by Rubisco activase (Robinson and Portis, 1988) and then degraded by CA1P phosphatase (Holbrook et al., 1989). The phosphatase is active on free inhibitor but will not degrade CA1P bound to Rubisco (Salvucci et al., 1988). Since there is no evidence for a change in the level of CA1P bound to Rubisco during the initial sunrise period, then the observed decreased CA1P level suggests that the initial response to light involved CA1P phosphatase but not Rubisco activase. Salvucci and Holbrook (1989) showed that CA1P phosphatase is activated 2- to 3-fold by certain chloroplast metabolites, including NADPH, Fru-1,6-bisP, and RuBP. Levels of Fru-1,6-bisP and RuBP are probably quite low in bean leaves close to their light compensation point (Dietz and Heber, 1984). However, at an irradiance of only 30 μmol quanta m^{-2} s^{-1} the turnover rate of photochemical products would be rather slow and NADPH levels may be relatively high. Heber et al. (1982) showed that total NADPH levels increased at least 2-fold in dark-adapted spinach chloroplasts treated with a light intensity of 60 μmol quanta m^{-2} s^{-1}. Thus, in bean leaves, the initial CA1P-related response during sunrise may be an activation of CA1P phosphatase by NADPH.

These experiments provide evidence that not only is CA1P bound to Rubisco in vivo to a degree suggested by previous experiments but also that, during the natural transition from darkness to daylight in bean leaves, Rubisco activity is regulated by CA1P. At all levels of irradiance before and after dawn, the Rubisco activation state was constant at about 75%. During this time, the available pool of carbamylated Rubisco was regulated predominantly by release and degradation of CA1P.

ACKNOWLEDGMENTS

We thank Kitty Spreeman for valuable technical assistance and Dr. Rowan Sage for providing anti-Rubisco antibody. We also appreciate useful comments by two anonymous reviewers.

Received January 24, 1994; accepted February 24, 1994.
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LITERATURE CITED

Berry JA, Lorimer GH, Pierce J, Seemann JR, Meeks J, Freas S (1987) Isolation, identification, and synthesis of 2-carboxyarabinitol 1-phosphate, a diurnal regulator of ribulose-bisphosphate carboxylase activity. Proc Natl Acad Sci USA **84:** 734–738

Badger MR, Lorimer G (1980) Interaction of sugar phosphates with the catalytic site of ribulose-1,5-bisphosphate carboxylase. Biochemistry **20:** 2219–2225

Collatz GJ, Badger MR, Smith C, Berry JA (1979) A radioimmune assay for RuP_2 carboxylase protein. Carnegie Inst Washington Year Book **78:** 171–175

Dietz K-J, Heber U (1984) Rate-limiting factors in leaf photosynthesis. I. Carbon fluxes in the Calvin cycle. Biochim Biophys Acta **767:** 432–443

Evans JR, Seemann JR (1984) Differences between wheat genotypes in specific activity of RuBP carboxylase and the relationship to photosynthesis. Plant Physiol **74:** 759–765

Gutteridge S, Parry MAJ, Burton S, Keys AJ, Mudd A, Feeney J, Servaites JC, Pierce J (1986) A nocturnal inhibitor of carboxylation in leaves. Nature **324:** 274–276

Gutteridge S, Reddy GS, Lorimer G (1989) The synthesis and purification of 2′-carboxy-D-arabinitol-1-phosphate, a natural inhibitor of ribulose bisphosphate carboxylase investigated by ^{31}P NMR. Biochem J **260:** 711–716

Heber U, Takahama U, Neimanis S, Shimizu-Takahama M (1982) Transport as the basis for the Kok effect. Levels of some photosynthetic intermediates and activation of light-regulated enzymes during photosynthesis of chloroplasts and green leaf protoplasts. Biochim Biophys Acta **679:** 287–299

Holbrook GP, Bowes G, Salvucci ME (1989) Degradation of 2-carboxyarabinitol 1-phosphate by a specific chloroplast phosphatase. Plant Physiol **90:** 673–678

Keys AJ, Parry MAJ (1990) Ribulose bisphosphate carboxylase/oxygenase and carbonic anhydrase. *In* PM Dey, JB Harborne, eds, Methods in Plant Biochemistry, Vol 3. Academic Press, London, pp 1–14

Kobza J, Seemann JR (1988) Mechanisms for the light regulation of ribulose-1,5-bisphosphate carboxylase activity and photosynthesis in intact leaves. Proc Natl Acad Sci USA **85:** 3815–3819

Ku SB, Prickril BC, Reger BJ, Pallas JE (1982) Diurnal fluctuation of RuBP carboxylase activity in peanut leaves (abstract No. 284). Plant Physiol **69:** S-51

McCurry SD, Pierce J, Tolbert NE, Orme-Johnson WH (1981) On the mechanism of effector-mediated activation of ribulose bisphosphate carboxylase/oxygenase. J Biol Chem **256:** 6623–6628

Moore Bd, Kobza J, Seemann JR (1991) Measurement of 2-carbox-

yarabinitol 1-phosphate in plant leaves by isotope dilution. Plant Physiol **96:** 208–213

Moore Bd, Seemann JR (1992) Metabolism of 2′-carboxyarabinitol in leaves. Plant Physiol **99:** 1551–1555

Moore Bd, Sharkey TD, Kobza J, Seemann JR (1992) Identification and levels of 2′-carboxyarabinitol in leaves. Plant Physiol **99:** 1546–1550

Parry MAJ, Gutteridge S (1984) The effect of SO_3^{2-} and SO_4^{2-} ions on the reactions of ribulose bisphosphate carboxylase. J Exp Bot **35:** 157–168

Paulsen JM, Lane MD (1966) Spinach ribulose diphosphate carboxylase. I. Purification and properties of the enzyme. Biochemistry **5:** 2350–2357

Portis AR Jr (1992) Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. Annu Rev Plant Physiol Mol Biol **43:** 415–437

Robinson SP, Portis AR Jr (1988) Release of the nocturnal inhibitor, carboxyarabinitol-1-phosphate, from ribulose bisphosphate carboxylase/oxygenase by Rubisco activase. FEBS Lett **233:** 413–416

Sage RF, Reid CD, Moore Bd, Seemann JR (1993) Long-term kinetics of light-dependent modulation of ribulose-1,5-bisphosphate carboxylase/oxygenase activity in C_3 plants. Planta **191:** 222–230

Sage RF, Sharkey TD, Seemann JR (1990) Regulation of ribulose-1,5-bisphosphate carboxylase activity in response to light intensity and CO_2 in the C_3 annuals *Chenopodium album* L. and *Phaseolus vulgaris* L. Plant Physiol **94:** 1735–1742

Salvucci ME, Anderson JC (1987) Factors affecting the activation state and the level of total activity of ribulose bisphosphate carboxylase in tobacco protoplasts. Plant Physiol **85:** 66–71

Salvucci ME, Holbrook GP (1989) Purification and properties of 2-carboxy-D-arabinitol 1-phosphatase. Plant Physiol **90:** 679–685

Salvucci ME, Holbrook GP, Andersen JC, Bowes G (1988) NADPH-dependent metabolism of the ribulose bisphosphate carboxylase/oxygenase inhibitor 2-carboxyarabinitol 1-phosphate by a chloroplast protein. FEBS Lett **231:** 197–201

Seemann JR, Berry JA, Freas SM, Krump MA (1985) Regulation of ribulose bisphosphate carboxylase activity in vivo by a light-modulated inhibitor of catalysis. Proc Natl Acad Sci USA **82:** 8024–8028

Seemann JR, Kobza J, Moore Bd (1990) Metabolism of 2-carboxyarabinitol 1-phosphate and regulation of ribulose-1,5-bisphosphate carboxylase activity. Photosynth Res **23:** 119–130

Servaites JC (1985) Binding of a phosphorylated inhibitor to ribulose bisphosphate carboxylase/oxygenase during the night. Plant Physiol **78:** 839–843

Vu CV, Allen LH Jr, Bowes G (1983) Effects of light and elevated CO_2 on the ribulose bisphosphate carboxylase activity and ribulose bisphosphate level of soybean leaves. Plant Physiol **73:** 729–734

Vu CV, Bowes G, Allen LH Jr (1984) RuBPCase dark/light modulation in various photosynthetic groups (abstract No. 1063). Plant Physiol **75:** S-189

Wintermans JFGM, De Mots A (1965) Spectrophotometric characteristics of chlorophylls *a* and *b* and their pheophytins in ethanol. Biochim Biophys Acta **109:** 448–453