FEBS Letters 444 (1999) 106–110 FEBS 21514

The localisation of 2-carboxy-D-arabinitol 1-phosphate and inhibition of Rubisco in leaves of *Phaseolus vulgaris* L.

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Received 8 December 1998

Abstract A recent controversial report suggests that the nocturnal inhibitor of Rubisco, 2-carboxy-D-arabinitol 1-phosphate (CA1P), does not bind to Rubisco in vivo and therefore that CA1P has no physiological relevance to photosynthetic regulation. It is now proved that a direct rapid assay can be used to distinguish between Rubisco-bound and free CA1P, as postulated in the controversial report. Application of this direct assay demonstrates that CA1P is bound to Rubisco in vivo in dark-adapted leaves. Furthermore, CA1P is shown to be in the chloroplasts of mesophyll cells. Thus, CA1P does play a physiological role in the regulation of Rubisco.

Key words: Ribulose 1,5-bisphosphate carboxylase/oxygenase; 2-Carboxy-D-arabinitol-1-phosphate; Dark-adapted leaves; *Phaseolus vulgaris*; Inhibitor

1. Introduction

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) activity is modulated in response to changes in light intensity, CO_2 and O_2 supply through the reversible carbamylation of lysine 201. Carbamylation is essential for catalytic activity. Full carbamylation in vivo requires an additional protein, Rubisco activase, which exerts a high degree of control over the rate of activation. The extent of carbamylation in vivo may be estimated by comparing the immediate Rubisco activity in leaf extracts (initial activity) with the greater activity attained by prior incubation with saturating concentrations of the activating cofactors, CO_2 and Mg^{2+}, to carbamylate vacant catalytic sites (total activity). Measurements of total activity may also change significantly between night and day [1]. This led to the discovery and identification of the nocturnal Rubisco inhibitor 2-carboxy-D-arabinitol 1-phosphate (CA1P) [2–4]. The structural similarity of CA1P to the carboxylation transition state intermediate, 2-carboxy 3-ketoarabinitol 1,5-bisphosphate, strongly suggests a role in Rubisco regulation. The subsequent demonstration that both CA1P and a specific phosphatase that degrades it were located in the chloroplast [5,6], and that CA1P could be released from Rubisco by Rubisco activase, gave further support to this hypothesis. In contrast, more recent claims by Anwaruzzaman et al. [7] that CA1P and Rubisco are not in the same part of the leaf and that CA1P only becomes bound during extraction is inconsistent with a role for CA1P in Rubisco regulation and needs further examination.

The crucial experiment, upon which the claim [7] depended, involved the rapid freezing of dark-adapted leaves in liquid N_2, grinding the tissue to a powder, and measuring the Rubisco activity released as the frozen powder thawed and dispersed in a reaction mixture containing appropriate substrates and cofactors. This was referred to as the 'direct assay'. Rubisco activity was not inhibited to the expected extent and fell when the tissue suspension was pre-incubated in the absence of RuBP. By contrast, tests by the same authors on conventional buffered extracts of the dark adapted leaves indicated that Rubisco activity was more strongly inhibited. This was consistent with the conclusion that the inhibitor and enzyme were initially separated in the frozen tissue but became associated with each other during the time needed to make conventional extracts. Vein-enriched tissue [7] was prepared from dark-adapted leaves by digesting with cellulase and pectinase to release mesophyll cell protoplasts. The fibrous residue remaining, referred to as vein-enriched tissue, was extracted with acid and the inhibitory effect of components of the extract on Rubisco activity compared to components in a similar extract of intact leaves. The extract of vein-enriched tissue contained more inhibitory activity per unit chlorophyll than the extract of whole leaf. This was interpreted as meaning that the inhibitor was mainly in the veins.

Reported below are experiments repeating the approach described above [7] with a commercial variety of *Phaseolus vulgaris* recently used in investigations of the biosynthesis of CA1P [8]. The results conflict with those of Anwaruzzaman et al. [7]. An explanation for the conflicting results and conclusions was sought and may be entailed in the exact conditions used to produce dark-adapted plants and the extent to which they were exposed to light during collection of samples.

2. Materials and methods

2.1. Materials

Phaseolus vulgaris c.v. Tendergreen was grown in peat-based compost in a glasshouse under supplementary lighting to give a 16 h photoperiod and a minimum photon flux density (PFD) of 200 μmol quanta/m^2/s. When the seedlings were 3 weeks old, the youngest leaves were taken either from plants kept overnight in total darkness, or from plants in the growing conditions near the middle of the photoperiod (experiments 1 and 2, Table 1; Fig. 3) or from plants after removal from darkness to a bench with lights giving a PFD of 200 μmol quanta/m^2/s (experiment 3, Table 1; Fig. 4). RuBP and CA1P were prepared as described previously [9]. RuBP from Sigma-Aldrich Company was also used in some experiments. Rubisco from *Phaseolus vulgaris* was purified by ammonium sulphate fractionation and sucrose density gradient centrifugation as in [9] but without the ion-exchange chromatography step.

2.2. Measurement of Rubisco activity

To give good temperature control at 25°C, reactions were conducted in the well of an oxygen electrode (Model DW 1, Hansatech Instruments). Reaction mixtures of 1.0 ml total volume were contin-

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PII: S0014-5793(99)00038-1

uously stirred and contained 100 mM Bicine pH 8.0, 20 mM $MgCl_2$ and 20 mM $NaH^{14}CO_3$ (0.5 μCi/μmol) with tissue or purified Rubisco from *Phaseolus vulgaris* leaves and RuBP at the concentration shown in the figures and tables. Alternatively the buffer was 0.1 M HEPES pH 8.0, with 20 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 20 mM $NaH^{14}CO_3$, with tissue and RuBP as indicated. Reaction was started by addition of the tissue or purified enzyme or, where a pre-incubation was given, by the addition of RuBP. The frozen tissue was added with a plastic or metal spatula pre-cooled with liquid N_2. All reactions were stopped by the addition of 0.2 ml of 10 M formic acid. Activity was measured by the ^{14}C in the acid-stable, non-volatile products.

2.3. Other measurements

Protein was measured by the method of Bradford [10] using a standard curve prepared for bovine serum albumin. Chlorophyll was estimated directly [11] or from measured pheophytin [12].

2.4. CA1P in veins and lamina

Dark-adapted leaves were dissected in dull light into main veins and lamina. The parts were weighed and frozen in liquid nitrogen, ground to a powder, and extracted with 0.46 M trifluoroacetic acid containing 0.15% w/v 8-hydroxyquinoline. Lipophilic substances were removed by solid phase extraction and the acid by evaporation. CA1P was estimated by inhibition of Rubisco activity compared to a pure standard as described elsewhere [9].

2.5. Non-aqueous tissue fractionation

De-veined, dark-adapted leaves of *Phaseolus vulgaris* were ground to a powder in liquid N_2 and freeze-dried. The powder was fractionated essentially as described by Gerhardt and Heldt [13]; chlorophyll, PEP-carboxylase and α-mannosidase were used as markers for chloroplast, cytosol and vacuolar components of fractions.

3. Results

Fig. 1 confirms that the direct assay can be used to assess whether CA1P, at concentrations near to the concentration of Rubisco active sites, is free or already bound to Rubisco. Purified Rubisco pre-treated with CA1P remained inhibited irrespective of the RuBP concentration present during assay of activity but when CA1P was added concurrently with RuBP to start the reaction the amount of inhibition was small and decreased as the amount of RuBP added was increased (Fig. 1). When CA1P was added concurrently with RuBP, progressive inhibition was seen during prolonged incubation of reaction mixtures, consistent with the slowed binding of the inhibitor by competition with RuBP (Fig. 2). Thus the logic of Anwaruzzaman et al. [7] concerning the use of relatively high

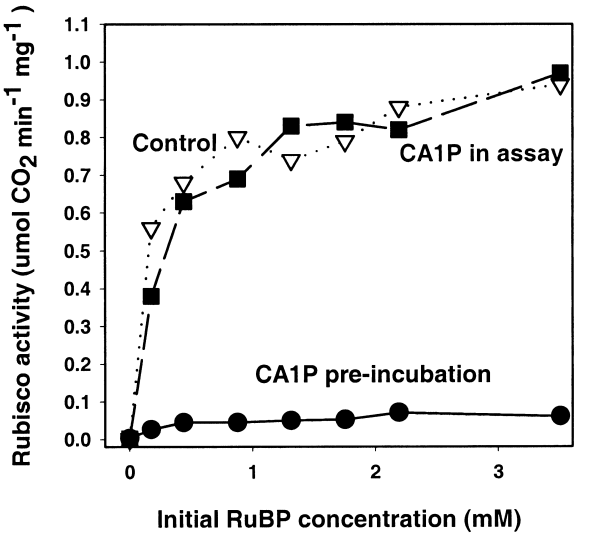

Fig. 1. Effect of RuBP concentration on the activity of purified *Phaseolus* Rubisco. Reaction mixtures contained 75 μg of Rubisco (1.09 nmol catalytic site) with or without (Control) 1.1 nmol of CA1P. CA1P was either added to the activated enzyme 10 min before the reaction was started (CA1P pre-incubation) or added at the start of the reaction (CA1P in assay).

RuBP to limit the binding of any free CA1P in the tissue added to reaction mixtures is correct.

Table 1 shows that the activity of Rubisco in leaves of *Phaseolus vulgaris* plants previously adapted to darkness was only about 10% of that in leaves of plants previously adapted to the light when measured by the direct assay described by Anwaruzzaman et al. [7]. This was irrespective of whether the buffer was Bicine or HEPES, of the concentration of RuBP, or whether the activities were expressed on the basis of soluble protein or chlorophyll in the reaction mixtures. These results are consistent with many reported measurements of Rubisco activity in extracts of leaves of *Phaseolus vulgaris* [14,15]. The activities derived from the dark-adapted leaves increased slightly when preincubated in the presence activating cofactors before the addition of RuBP whilst activities derived from the light-adapted leaves decreased. This decrease in activity was especially marked in experiment 3 (Table 2; see also Fig. 4) where light adaptation involved exposure of dark-adapted

Table 1
Rubisco activity released from frozen powder of dark- or light-adapted leaves measured immediately (Direct) or after 2.5 min pre-incubation with activating cofactors

Adaptation of the leaf	Buffer	RuBP concentration (mM)	Rubisco activity (nmol/min/mg protein)	
			Direct	Total
Experiment 1				
Dark	Bicine	0.13	34.2 ± 4.4	71.3 ± 17.2
Light			346.8 ± 31.2	324.2 ± 7.1
Experiment 2				
Dark	HEPES	0.13	27.9 ± 4.0	91.9 ± 15.2
Light			394.6 ± 24.5	370.5 ± 32.4
Experiment 3				
Dark	HEPES	1.0	48.8 ± 8.7	63.4 ± 4.9
Light			537.5 ± 81.0	244.0 ± 27.3
			(μmol/h/mg chlorophyll)	
Dark			84.9 ± 27.7	77.0 ± 14.6
light			942.7 ± 213.9	481.0 ± 186.6

Values are means of five determinations ± S.D. For experiment 3, activities are expressed both per unit soluble protein and per unit chlorophyll.

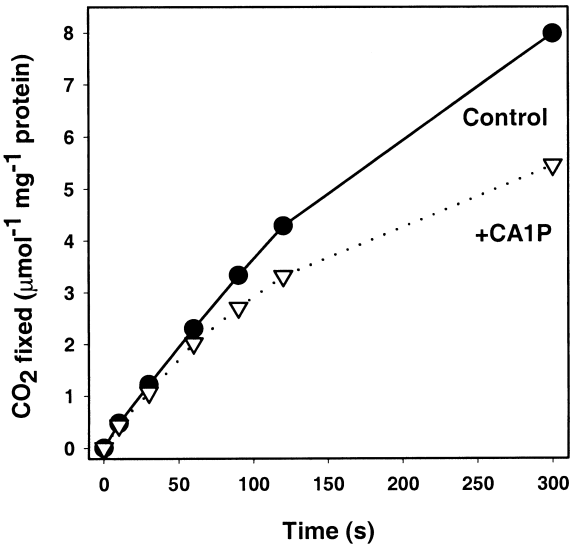

Fig. 2. Time course of the carboxylation of RuBP catalysed by purified *Phaseolus* Rubisco when CA1P was present only from the start of the reaction or absent. Inhibition develops progressively but would scarcely affect the initial rate measurements.

plants to light of 200 μmol quanta/m^2/s for 2 h rather than plants at mid-photoperiod in the growing conditions.

The experiment giving rise to Fig. 3 examined the effect of pre-incubation in the presence and absence of 0.2 M sulphate before measuring Rubisco activity in a reaction mixture in which the sulphate was diluted to 20 mM. This shows that sulphate increased the activity of Rubisco derived from dark-adapted leaves in a time-dependent manner so that it approached the activity of the enzyme from light-adapted tissue pre-incubated with sulphate. This is consistent with the established fact that sulphate releases CA1P from Rubisco but inhibits activity (by about 20% at 20 mM) [16,17].

To test how critical it was to avoid exposure of dark-

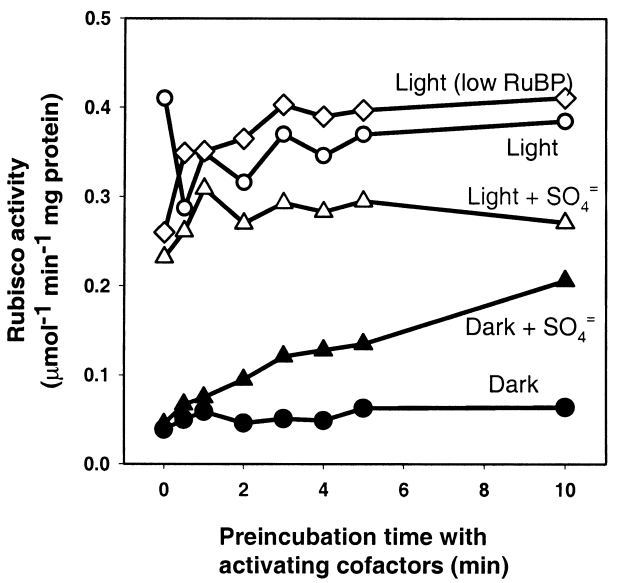

Fig. 3. Rubisco activity from frozen leaf powder from dark- and light-adapted leaves of *Phaseolus vulgaris* pre-incubated with activating cofactors (CO_2 and Mg^{2+}) with or without 0.2 M $(NH_4)_2SO_4$ and measured after 10-fold dilution into a buffer containing RuBP.

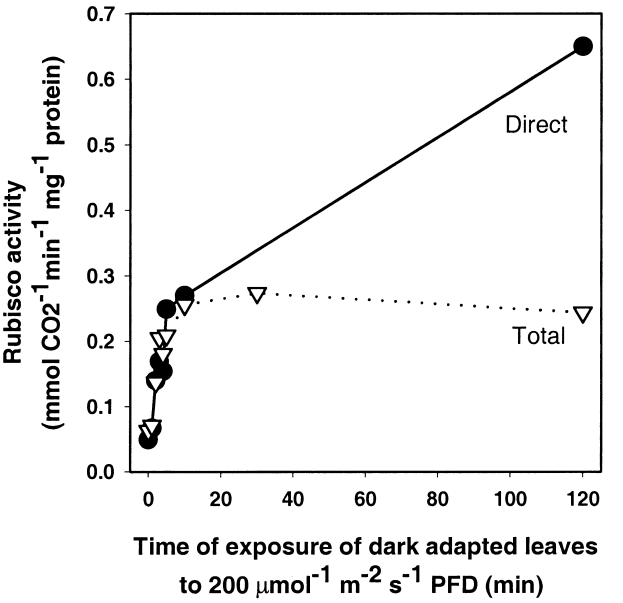

Fig. 4. Rubisco activity in frozen powder from dark-adapted leaves of *Phaseolus vulgaris* which had been exposed to light for the times shown and then was measured immediately (Direct) or after 2.5 min. pre-incubation (Total) with CO_2 and Mg^{2+} before addition of RuBP.

adapted plants to light just prior to freezing, such plants were exposed to a PFD of 200 μmol quanta/m^2/s. Powder from the leaves was used in direct assays and assays after 2.5 min pre-incubation with activating cofactors. Fig. 4 shows that Rubisco activity, measured by the direct method, rose quite quickly upon illumination; this did not seem in the short term to result in free CA1P that recombined with the Rubisco during the pre-incubation but, as already mentioned, after longer periods of illumination (>30 min) the direct assay gave greater activities than the assay after pre-incubation, suggesting that unbound inhibitor may have been present.

The claim that CA1P was in the veins and not bound to Rubisco in the chloroplasts was investigated by dissecting a dark-adapted leaf in low light into major veins and lamina, freezing the two samples in liquid N_2 and grinding the frozen samples with acid. After removal of lipids from the resulting extracts, the amount of CA1P present was estimated from the inhibitory effect on pure Rubisco. The greatest amount of CA1P per gramme fresh weight was clearly in the lamina and the distribution between the two fractions was more closely proportional to the chlorophyll content than to fresh weight (Table 2). A non-aqueous fractionation of dark adapted leaves of *Phaseolus vulgaris* was also made. Fig. 5 shows that CA1P is present in de-veined leaf tissue and fractionates with chlorophyll. The conclusion reached is that CA1P is in the chloroplast.

Table 2
CA1P in main veins and lamina of leaves of *Phaseolus vulgaris*

Leaf part	CA1P	
	nmol/g fresh weight	nmol/mg chlorophyll
Main veins	23 ± 3	81 ± 5
Lamina	110 ± 4	67 ± 7

Values are means for measurement on three leaves ± S.D.

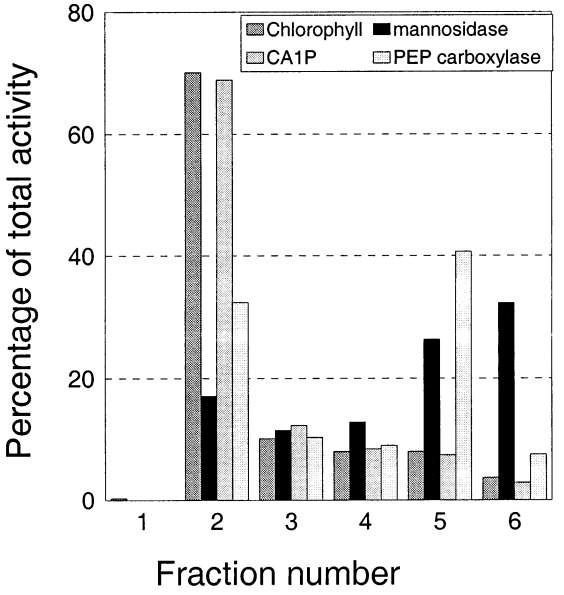

Fig. 5. Chlorophyll, PEP carboxylase, CA1P and α-mannosidase in fractions of increasing density prepared non-aqueously from freeze-dried, de-veined, dark-adapted leaves using tetrachloroethylene and heptane.

4. Discussion

The results presented here are not consistent with the conclusions of Anwaruzzaman et. al. [7] but confirm conclusions reached from earlier evidence that CA1P is bound to Rubisco in the dark in leaves of *Phaseolus vulgaris* and also in many other species. This does not preclude the presence in the chloroplast of an excess of CA1P above the Rubisco catalytic site concentration existing unbound. There already existed [5] direct evidence from non-aqueous fractionation of the leaf tissue that CA1P is in the chloroplast in the dark and this is confirmed by Fig. 5. Unless there are strong alternative binding sites for CA1P in the chloroplast, it is almost impossible to believe that it is not associated with the Rubisco in darkness when Rubisco activase is not active because of the high affinity of activated Rubisco for CA1P (K_d 3.2×10^{-8}). The evidence that it is in the veins [7] is weak since the data show an increase in CA1P with increasing chlorophyll and no corresponding fresh weight. Table 2 shows that in the vein-rich fraction CA1P content is low per gramme fresh weight compared to the lamina.

The direct assay devised by Anwaruzzaman et al. [7] to decrease to nearly zero the time between the extraction of Rubisco from tissue and the start of measurement of activity is of value in detecting initial activity. However, the amount of frozen tissue introduced is variable so it is necessary to relate activity to either chlorophyll or soluble protein. Soluble protein is more plentiful, more easily measured, and gives similar results (Table 1). Conducting the reaction in the well of a temperature-controlled oxygen electrode with adequate stirring should limit any transitional decrease in temperature – which is reported anyway to be at the most 1°C [7]. We did not check the temperature decrease; the slight increase in activity of Rubisco from the dark adapted leaf tissue during preincubation (Fig. 3) could be evidence of dissolution of the enzyme in the bulk of the reaction mixture as well as any rise in temperature. From the amounts of protein measured, the amount of frozen tissue used in the experiments of Table 1 were generally less than the 10–15 mg used by Anwaruzzaman et al. Any temperature effects should therefore be less significant.

Various circumstances were considered which may have resulted in the experimental results obtained by Anwaruzzaman et al. [7] being contrary to existing conclusions about the localisation of CA1P in leaves. The comparison of Bicine buffer and HEPES buffer was one concern that was explored since HEPES is a sulphonate and might, like sulphate, promote the release of CA1P from Rubisco. No evidence (Table 1) was found for decreased inhibition in the presence of HEPES. Furthermore, no explanation was found in the concentration of RuBP used in the assays on the activity measured either from leaf tissue (Table 1) or with pure Rubisco inhibited with pure CA1P (Fig. 2). The effect of exposure to light on the dark-adapted leaves prior to freezing was also explored since this might allow Rubisco activase to release CA1P, to produce a free pool of inhibitor if CA1P phosphatase was not sufficiently activated. Fig. 4 indeed shows the rise in activity with time of exposure to light, consistent with the release of CA1P, but only after prolonged exposure to light did the activity decrease upon pre-incubation prior to addition of the RuBP. An explanation for this may be that CA1P was present unbound in the tissue after the exposure to light and bound to Rubisco during the period of pre-incubation with activating cofactors. Whether this is the explanation needs further examination including direct measurement of CA1P in the tissue by isotope dilution or HPLC [5,8]. Alternative possible explanations must include the presence of other inhibitors [9,18] unbound in vivo but becoming bound during pre-incubation of the extract.

The bulk of evidence in the literature supports the view that CA1P is bound to Rubisco in leaves of *Phaseolus* in the dark; this is confirmed by the experiments presented which refute the conclusions drawn in [7]. Under certain circumstances, however, it seems that illuminated leaves may contain unbound inhibitors that cause the activity of Rubisco to decline following extraction. More research is needed on tight-binding inhibitors of Rubisco present in leaves in the light. It is also important that more research is directed to understanding the biosynthesis and catabolism of CA1P (hamamelonic acid 2^1-phosphate), and the relationship these processes have to hamamelose phosphates which are reported to be made in chloroplasts during photosynthesis [8,19].

Acknowledgements: IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

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