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A phosphatase from chloroplast stroma of *Nicotiana tabacum* hydrolyses 2′-carboxyarabinitol 1-phosphate, the natural inhibitor of Rubisco to 2′-carboxyarabinitol

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An enzyme has been identified in the stroma of tobacco chloroplasts that degrades the natural inhibitor of Rubisco, 2′-carboxyarabinitol 1-phosphate (2CA1P). The products of the catalysis, determined by HPLC and phosphate analysis are 2′-carboxyarabinitol and inorganic phosphate indicating that the enzyme is a phosphatase. The only other phosphate esters found to be degraded by the enzyme were the branched-chain carboxylic sugar bisphosphates, 2′-carboxyarabinitol and ribitol 1,5-bisphosphates that were converted to the corresponding 5-monophosphates. Other intermediary metabolites of the photosynthetic cycle were essentially unaffected by the phosphatase. The kinetic parameters of the enzyme are consistent with those required to relieve the inhibition of rubisco in vivo.

Carboxyarabinitol-1-phosphate phosphatase, 2′-; Carboxyarabinitol 1-phosphate, 2′-; Enzyme inhibitor; Ribulose-bisphosphate carboxylase stroma

1. INTRODUCTION

2′-Carboxyarabinitol 1-phosphate (2CA1P) is a stromal metabolite and potent inhibitor of rubisco [1,2] in some plants [3] that binds to the activated enzyme in vivo, modulating the activity in response to light [4,5]. The quantities synthesised in the chloroplasts of some species, e.g. *Phaseolus* and *Nicotiana* during the dark are about stoichiometric with respect to rubisco active site concentration. A K_d of about 10^{-8} M between 2CA1P and the activated enzyme ensures almost complete inhibition, a feature of the inhibitor that contributed to its isolation and identification [1,2]. These data combined with its rate of appearance and degradation ($t_{1/2} \sim 60$ min) [5] suggests that 2CA1P may function as an effective regulator of rubisco activity and hence CO_2 fixation as irradiance levels of leaves change.

The regulation of CO_2 fixation through rubisco has often been suspected. The requirement of the enzyme for CO_2 both as substrate and activator, along with Mg^{2+} ensures a multiple response to conditions in the stroma. Superimposed on these activation requirements is the effect of natural phosphate-containing substrate analogues of the stroma that in many cases stabilise the activated enzyme–CO_2–Mg^{2+} ternary complex [6–8]. That any of these processes acts to regulate rubisco activity in response to light has not been firmly established, although clearly the efflux of Mg^{2+} from stroma in the dark is one potential mechanism. The discovery of 2CA1P, a potent inhibitory metabolite that itself is synthesised or degraded depending on light, may provide a relatively sensitive mechanism of modulating CO_2 fixation through rubisco by stabilising the activated form of the enzyme [11].

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Abbreviations: 2CA1P, 2′-carboxyarabinitol 1-phosphate; 2CABP, 2′-carboxyarabinitol 1,5-bisphosphate

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The requirements necessary for studying the fate of 2CA1P include a ready source of stroma that contains the enzymes involved in its metabolism and a method of synthesising the inhibitor in good yield without resorting to using natural sources. It seems to be a general rule that those species of plant that produce significant quantities of the inhibitor [3] are also relatively high in starch concentrations and thus do not readily yield intact chloroplasts. However, in this instance *Nicotiana* has been grown in conditions that ensure the quantities of stored starch do not interfere in chloroplast preparations and thus allow consistently high levels of the intact organelle to be prepared.

In a previous report that described a chloroplast localised activity that relieved the inhibition of rubisco by 2CA1P [13], it was speculated that a reductive kinase might exist with a specific requirement for NADPH. We have been unable to identify such an enzyme but here describe a phosphatase activity that is localised in the stroma of *Nicotiana* chloroplasts that hydrolyses 2CA1P with high specificity and with the activity expected of an enzyme required to relieve the inhibition of rubisco by 2CA1P in vivo.

2. MATERIALS AND METHODS

2.1. *Synthesis of 2CA1P*

2′-[^{14}C]CA1P was synthesised from 2′-[^{14}C]CABP by the acid phosphatase method [9]. The monophosphate was purified as previously described [9] or by elution as the free acid from a MonoQ (5/15) column using a 0–0.5 M acetate gradient, pH 4.6. Products of the acid phosphatase reaction were analysed by ^{31}P NMR and by their retention time on an anion-exchange partisil Sax10 HPLC column [9].

2.2. *Preparation of chloroplasts from tobacco*

Nicotiana tabacum (v. *xanthi* or *samson*) seeds were sterilised by soaking in 10% chlorox, 0.1% SDS solution for 30 min and placed on Murashige media (Gibco) supplemented with 0.8% Bacto-agar and 1.0% sucrose in clear Magenta boxes. After 21–28 days growth at 25°C and 100 μM photons $\cdot m^{-2} \cdot s^{-1}$ illumination (16 h day) the upper shoot was removed, the more mature leaves trimmed away and then, in a sterile flow cabinet transferred to fresh media. The remaining plant was utilised to supply the leaves for chloroplast preparation.

The 100 g of leaves from about 24 plants were disrupted by homogenisation with a Brinkman homogeniser set at speed 6 and over 3 × 5-s pulses in 1 × Jagendorf buffer [10]. The leaf material was passed through 8 layers of cheesecloth and the chloroplasts sedimented free of cell debris by centrifugation at 7000 × *g* for 5 min at 4°C. The chloroplast pellet was resuspended in 1 ml Jagendorf buffer and layered onto a 0–50% percoll gradient. The gradient was centrifuged at 14 000 × *g* for 10 min at 4°C. The lower band in the gradient was taken as intact organelles and these finally isolated by 3-fold dilution of the percoll with a 0.1 M Hepes/KOH buffer, pH 8.0, containing 0.66 M sorbitol.

The level of intactness was determined using an O_2 electrode comparing the intact with lysed material for the ability to evolve oxygen with added ferricyanide and then decoupled with added NH_4OH. A second criterion was to sediment the chloroplasts through a 40% percoll gradient. The chloroplasts deemed intact were finally lysed in enough 20 mM triethanolamine buffer, pH 8.0, containing 5 mM DTT to give a concentration of about 2 mg/ml chlorophyll. The lysed preparation was used directly or after removal of membrane material by centrifugation at top speed on a microfuge for 5 min. Addition of 16% glycerol to these solutions allowed the lysed organelles to be stored at −80°C in the freezer.

2.3. *Analysis of 2CA1P consumption*

The enzyme solution used to degrade 2CA1P was either the freshly prepared stromal extract or after purification from a MonoQ ion-exchange column. The purification involved applying the stromal extract to the MonoQ (5/15) in the triethanolamine buffer except that the DTT concentration was 1 mM. The column was developed with a 20 mM Tris-Cl buffer, pH 8.0, containing 1 mM DTT and a KCl gradient from 0 to 1.0 M. The enzyme eluted from the column just before the rubisco peak over 0.32–0.38 M KCl and this was used without further purification.

Two methods were used to analyse the consumption of 2CA1P by stromal extracts. The first was to determine the retention time of ^{14}C-labelled products and unreacted phosphate ester on the anion-exchange HPLC column developed with an ammonium phosphate gradient, pH 4.0, as described in section 3. The treatment of the samples following incubation with the stroma was to halt the reaction by acidification with 10% acetic acid. The samples were then passed through a short column of Dowex 50H$^+$ to remove buffer cations and the resulting material, with washings, dried extensively on a heating block to drive any unreacted substrate and product to lactone. The samples were redissolved in water for application to the HPLC column.

The second method was to follow the release of P_i during the reaction using the sensitive colorimetric method with ammonium molybdate and malachite green [12].

3. RESULTS AND DISCUSSION

The ability to synthesise 2CA1P in good yield from 2CABP appropriately labelled with ^{14}C [9] has allowed the processes of initial degradation of the natural inhibitor to be investigated. The stromal isolate from lysed chloroplasts was used to determine if there was any activity that degraded synthesised 2CA1P. An ion-exchange HPLC method that could resolve degradation products of 2CA1P [9] was used to analyse the effects of exposure of the inhibitor to the freshly prepared

stroma. Because it is expected that the enzyme of interest is most active in conditions that simulate light exposed chloroplasts, in some experiments the stroma was supplemented with components that are likely to abound in these conditions, e.g. ATP, NADPH, Mg^{2+} and a substitute for reduced thioredoxin, DTT. Except for the latter, most were found to be superfluous (see below) and eventually omitted.

Fig.1 is a typical elution profile of the products of a stromal enzyme degradation of 2CA1P on the Sax anion-exchange HPLC column. After the treatment of 2CA1P with the stromal extracts for different periods of time (without supplementation apart from the DTT present in the preparation), the retention time of the radioactivity changed from 24 to 4 min. The position of elution shown in fig.1a is that obtained for the products of the reaction that have been treated as described in section 2, i.e. Dowex $50H^+$ and then extensive dehydration. Fig.1b shows the elution of a 2-h sample either added directly to the column or after readjusting the pH of a dehydrated sample to pH 10.0 overnight before the chromatography. The difference in retention time in fig.1a and b of the product of 2CA1P degradation is typical of a compound that is able to cyclise into a lactone or exist in a free acid form. Furthermore, the more rapid elution of both lactone and free acid than the 2CA1P starting material indicates that the compound has less charge than the monophosphate. The ability to recover over 90% of the radioactivity after the reaction and chromatography indicates that the loss of charge is not due to removal of the 2′-carboxy group, rather it must be due to loss of the 1-phosphate from the molecule.

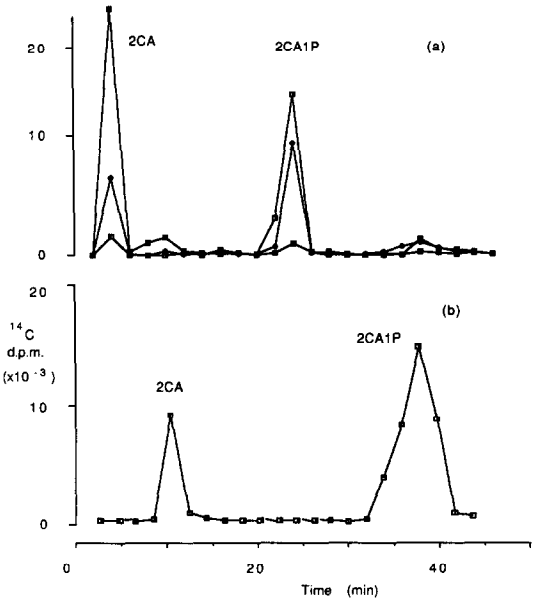

Fig.1. Analysis of the 2CA1P degradation products by a stromal preparation using ion-exchange HPLC. In (a) 40 nmol 2′-[^{14}C]-CA1P was added to 100 μl freshly prepared stroma and the reaction stopped after 1 min (■), 2 h (◆) and 16 h (■). The unreacted 2CA1P and products were lactonised by dehydration before injection. For comparison (b) shows a stromal reaction mixture (after 2 h) that has not been lactonised, to show that both the substrate 2CA1P and the dephosphorylated product can exist as free acids (see section 3). The column was run at 1 ml/min 40°C in ammonium phosphate, pH 4.0. The gradient was linear 10–30 mM phosphate over 30 min and then stepped to 0.2 M phosphate.

This was confirmed in 2 ways. The first was to synthesise 2′-carboxyarabinitol [1] and run both lactone and free acid forms on the column. The position of elution of the synthesised material coincided exactly with that of the products of the stromal reaction. The second involved following the release of P_i during stromal consumption of 2CA1P. The rate of formation of 2′-carboxyarabinitol (2CA) determined using HPLC was identical with the rate of P_i release. These data are consistent with the presence of a phosphatase in stroma that degrades the potent inhibitor 2CA1P ($K_d = 10^{-8}$ M) to the non-inhibitory compound 2CA. The results were unchanged whether the stroma was utilised directly after removal of the chloroplast membranes, or the lysed chloroplast preparation was used without further purification. To ensure that the enzyme activity was indeed localised in the chloroplast stroma, the intact organelles were washed twice in the sorbitol-containing buffer before lysis and then the membranes removed by centrifugation. No diminution of activity was detected after the washing procedure.

It was considered that there were at least 2 factors that might interfere with the analysis of the activity described above. The first was the presence of significant quantities of rubisco from the stroma that would bind the 2CA1P and might inhibit its degradation. Secondly, more than one stromal enzyme might be acting to degrade the inhibitor. The stroma was therefore fractionated further on a MonoQ FPLC column as described in section 2.

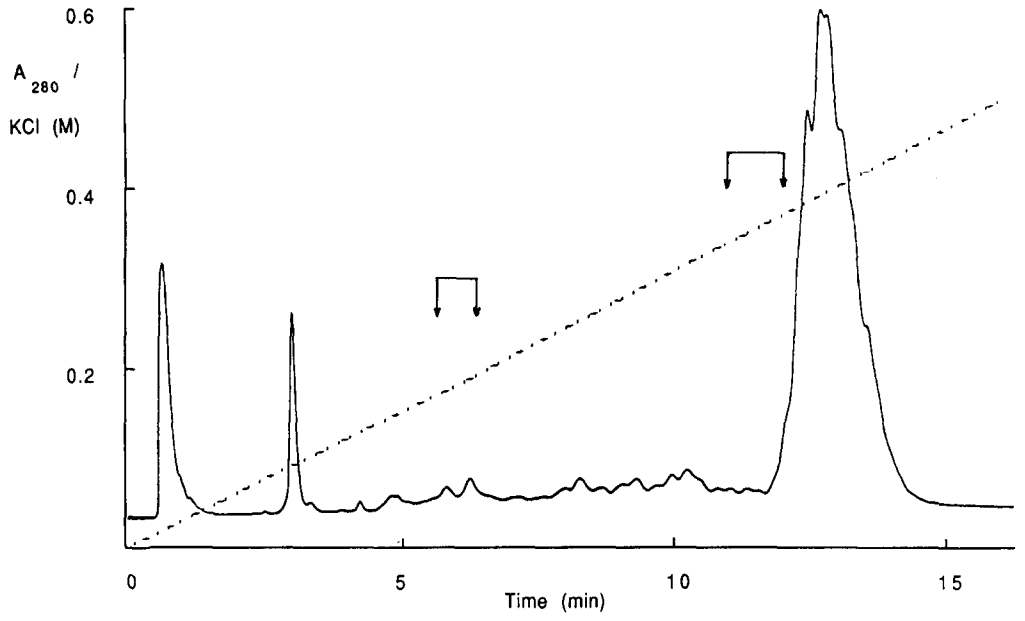

Fig.2. Partial purification of the stromal phosphatase. A MonoQ column equilibrated with a 20 mM Tris-Cl, pH 8.0, buffer containing 1.0 mM DTT was developed with a 0.0–1.0 M KCl gradient. Two peaks of phosphatase activity eluted from the column between 0.18–0.2 M and 0.32–0.38 M KCl. The latter, most active species was studied further.

The profile in fig.2 shows the position of elution of phosphatase activity that degraded 2CA1P. Two peaks of activity were identified. The first with a short elution time (between 0.18 and 0.2 M KCl) had rather low activity and accounted for about 10–20% of the total stromal 2CA1P phosphatase activity. The second peak that eluted over 0.32–0.38 M KCl just before rubisco contained the remaining 85% activity. The first peak was not due to a protein overload of the MonoQ because its elution position did not change on a second application to the column. The phosphatase with the highest activity was studied further.

The nature of the phosphatase requirement for any or all of the cofactors that might be essential for the turnover of 2CA1P in stroma was investigated. The phosphatase activity was essentially unchanged in the absence of supplemental ATP, NADPH or Mg^{2+}. However, there was clearly a need for DTT to maintain the activity of the enzyme. Over a short period of time, e.g. after ion-exchange or gel filtration without DTT-supplemented buffers, the enzyme was stable and the approx. 20% loss of activity could be reversed by addition of the reduced thiol. However, the longer the interval without DTT, less enzyme activity could be recovered. Whether this might be due to proteolytic degradation has yet to be established.

When the diurnal regulation of rubisco is followed with respect to time in plant species such as *Phaseolus* or *Nicotiana* the rate of loss of inhibition by 2CA1P has a $t_{1/2}$ of less than 60 min, with the rate of dissociation of the inhibitor from the active site of rubisco as about 10 min. Assuming 5 mg rubisco per mg chlorophyll then the catalytic rate that a putative 2CA1P degrading enzyme should attain is about 0.1 μmol/h per mg chlorophyll. The total activity determined for a typical preparation of this phosphatase at 0.3–0.5 μmol/h per mg indicates that it is quite capable of relieving the inhibition of rubisco in vivo.

3.1. *Specificity of 2CA1P phosphatase*

Having established the ability of the enzyme to hydrolyse the C-1 phosphate of 2CA1P, it was then essential to discount other phosphatases that occur in the stroma that might be implicated. Other relevant phosphate containing compounds were investigated as potential substrates. These are listed in table 1 in terms of the relative rate of phosphate release compared to 2CA1P. This table also

Table 1

Relative rates of phosphate release from various phosphate esters compared with 2CA1P

Compound	Relative rate	K_m (mM)	Product
2CA1P	1.0	0.15	2CA
2CABP	1.2	0.20	2CA5P
2CRBP	0.05	n.d.	2CR5P
2CR1P	0.05	n.d.	2CR
2CA5P	<0.05		
2P-glycolate	<0.05		
3P-glycerate	<0.05		
Fructose-P_2	<0.05		
6P-gluconate	<0.05		

n.d., not determined

establishes some range for the specificity of the enzyme. Firstly, compounds that are branched-chain carboxylic acids with adjacent phosphate ester groups are dephosphorylated. However, the simpler compounds such as 3P-glycerate and 2-P-glycolate are not. The inability of the phosphatase to hydrolyse fructose-P_2, 6-P-gluconate or 2CA5P at significant rates even when supplemented with Mg^{2+} discounts this enzyme as a general phosphatase. That both 2CA1P and 2CABP are the best substrates for the enzyme suggest that this is the activity that is expressed in the stroma. The inability of the enzyme to hydrolyse 2CR1P and 2CRBP at rates similar to those obtained with 2CA1P and 2CABP suggests that the stereochemistry around the C-2 group of the molecule is particularly important for productive catalysis.

The nature of the products of bisphosphate hydrolysis also established that the enzyme discriminates between the primary phosphate groups. On the basis of the elution positions from the HPLC column of products of 2CABP that has been hydrolysed by the phosphatase, it is suggested that it is readily converted to a monophosphate. The affinity that the enzyme has for the C-1 phosphate position as shown by the rate of consumption of 2CA1P suggests that the product in the case of 2CABP must be 2CA5P. Fig.3a shows that little conversion of the monophosphate to 2CA was detected after prolonged incubation with the phosphatase. This was confirmed by determining the amount of P_i released from 2CABP colourimetrically after prolonged incubation with the purified phosphatase (see fig.3b). Only 50% of the

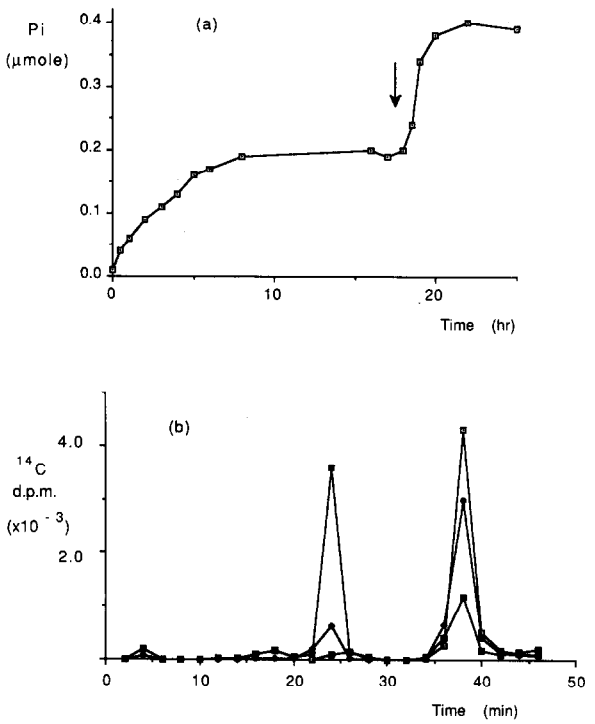

Fig.3. Hydrolysis of 2CABP to the monophosphate by the phosphatase. In (a) the release of P_i by phosphatase only accounts for 50% of the total amount of phosphate added as bisphosphate, indicating that only one of the phosphate groups is hydrolysed. The other phosphate group is rapidly released by addition (arrow) of 0.1 U acid phosphatase ([9] see also text). In (b) the products of 2CABP hydrolysis by purified phosphatase were analysed using the partisil column after 1 min (▣), 2 h (◆) and 16 h (■) to confirm that only 2CA5P and not 2CA is generated.

total organic phosphate was obtained as P_i after about 24 h. If this enzyme has converted the bisphosphate to 2CA5P, then this should be rapidly hydrolysed by acid phosphatase, an enzyme that removes the 5-phosphate of 2CABP at rates much faster than the 1-phosphate group [9]. Indeed, fig.3b shows that the remaining phosphate is released rapidly after this treatment.

The results described above establish that there is a phosphatase activity in the stroma of tobacco chloroplasts that can degrade 2CA1P with the appropriate kinetics to relieve the inhibition of rubisco and the specificity not to degrade essential intermediary metabolites of photosynthesis. In the plant 2CA1P inhibition of rubisco is maximally operative in the dark and thus an enzyme involved

in the degradation of the inhibitor should be most active in the light and presumably deactivated in the dark. The requirement of the phosphatase for reduced thiol to maintain activity is consistent with this form of regulation in the plant utilising, e.g. thioredoxin control. However, the somewhat slow inactivation of the enzyme after the removal of thiol and the diminished reversibility on readdition of the reagent is not typical of this form of regulation. Furthermore, stroma from chloroplasts of dark treated *Nicotiana* plants have about the same 2CA1P phosphatase activity as those obtained from plants that have been grown in normal lighting conditions. Clearly, further investigations are required here. Although the phosphatase exhibits rather more activity at pH 8.0 than 6.8 (data not shown) the difference is only about 30%, pH changes of the stroma accompanying Mg^{2+} movements would therefore have little effect on the activity of this enzyme. Other components that abound in the chloroplasts in the light, e.g. ATP and NADPH, are not essential for the activity of this enzyme [13]. It might be that the enzyme functions constantly, counter-balanced by an, as yet unidentified kinase that can phosphorylate the 2CA back to 2CA1P and is more responsive to the conditions of irradiation.

ADDENDUM

Salvucci and colleagues have also reported recently [(1989) Plant Physiol. 90, 673–685] the isolation and purification of a phosphatase from *Nicotiana* that degrades 2CA1P to 2CA and P_i.

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