

NADPH-dependent metabolism of the ribulose biphosphate carboxylase/oxygenase inhibitor 2-carboxyarabinitol 1-phosphate by a chloroplast protein

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Metabolism of 2-carboxyarabinitol 1-phosphate (CA 1-P), an endogenous inhibitor of ribulose biphosphate carboxylase/oxygenase, occurs in the light. A soluble protein fraction which metabolized CA 1-P in the presence of NADPH was isolated from tobacco chloroplasts. A similar fraction from spinach exhibited much lower activity. The activity in tobacco extracts was stable overnight at 4°C but its maintenance during storage required dithiothreitol. The tobacco protein responsible for CA 1-P metabolism was partially purified by ion-exchange FPLC of stromal extracts. The requirements for NADPH and dithiothreitol for activity of this protein suggest a mechanism for the light-dependent control of CA 1-P levels in plants.

Carboxyarabinitol 1-phosphate; Ribulose biphosphate carboxylase; NADPH; Light regulation; Photosynthesis; (Tobacco)

1. INTRODUCTION

Carboxyarabinitol 1-phosphate is a naturally occurring inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase [1,2] present in several agronomically important plant species including soybean, bean, potato and tobacco [3–5]. Because of its structural similarity to the carboxylation reaction intermediate 2-carboxy-3-ketoarabinitol 1,5-bisphosphate [1,2], CA 1-P binds tightly to the active site of Rubisco forming a stable enzyme-

inhibitor complex [4,5]. Under natural conditions, CA 1-P accumulates in the dark, and by early morning the inhibitor occupies from 50 to nearly 100% of the Rubisco active sites, a chloroplast concentration of about 2–4 mM [4,5]. Metabolism of CA 1-P occurs relatively rapidly in the light [5–8] and can be blocked by DCMU, an inhibitor of photosynthetic electron transport [5]. However, the specific reactions participating in the synthesis and degradation of CA 1-P are not known.

The mechanism by which photosynthetic electron transport activity controls the level of CA 1-P in plants has not been identified. Results from a previous study demonstrated that light-dependent increases in total activatable Rubisco activity in tobacco protoplasts and leaves were inhibited by methyl viologen [8], an artificial photosystem I electron acceptor. Since the level of total Rubisco activity reflects the amount of CA 1-P bound to the enzyme [5], these data suggested that reactions on the reducing side of photosystem I (i.e. Fd-thioredoxin reduction, NADP⁺ reduction) are required for the metabolism of CA 1-P. In the pre-

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Abbreviations: CA 1-P, 2-carboxyarabinitol 1-phosphate; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, ribulose 1,5-bisphosphate

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sent study, we identify the first step in CA 1-P metabolism as an NADPH-dependent reaction catalyzed by a soluble protein present in tobacco chloroplasts.

2. MATERIALS AND METHODS

Intact chloroplasts were isolated from tobacco (*Nicotiana rustica* var. *pumilia*) and spinach (*Spinacia oleracea* L.) leaves [9] following pre-illumination for 10 min at 300 $\mu\text{mol photons/m}^2 \cdot \text{s}$. The isolation procedure and all subsequent steps were performed at 4°C. Chloroplast pellets were lysed by vigorous resuspension in 20 mM Tricine-NaOH, pH 8.1, 50 mM DTT, 10 μM leupeptin, 1 mM PMSF and 0.1 mM EDTA and centrifuged at 18000 $\times g$ for 10 min. The supernatants were layered on linear 0.2 to 0.8 M sucrose gradients containing 50 mM Tricine-NaOH, pH 8.1, 5 mM MgCl_2 , 1 mM EDTA and 5 mM DTT. The <18 S fraction, which contains the bulk of the stromal proteins but is free of Rubisco, was isolated by rate zonal centrifugation [10]. The <18 S fraction was freed of low molecular mass compounds and concentrated by repeated ultrafiltration on an Amicon YM-30 membrane in 100 mM Tricine-NaOH, pH 8.0, 10 mM MgCl_2 and 10 mM NaHCO_3 (Buffer A) containing 2 mM DTT, and was then either used directly in the assays or further fractionated by anion-exchange FPLC[™] [11].

Rubisco was purified from greenhouse-grown tobacco (*N. tabacum*, KY14) using published procedures [11–13] and stored frozen as an $(\text{NH}_4)_2\text{SO}_4$ suspension in liquid N_2 . For assays, the thawed protein pellet was resuspended in buffer A containing 50 mM DTT and incubated at room temperature. After 3 h, the activated enzyme was desalted at 23°C on a column of Sephadex G-50-80 equilibrated in buffer A containing 5 mM DTT. Rubisco protein concentration was determined spectrophotometrically from the absorbance at 280 nm [13]. Soluble protein concentration in the extracts was determined by the Coomassie dye binding assay [14].

CA 1-P was isolated from darkened soybean leaves and purified by anion-exchange chromatography and BaCl_2 precipitation essentially as described in [2]. The final preparation had a total organic P_i concentration of 2.25 mM. CA 1-P metabolism was assayed at 25°C by measuring changes in the extent of Rubisco inhibition in a two-stage assay. In the first stage, 3 μl of CA 1-P (a relative concentration of 1) was incubated for 30 min in buffer A containing 20 mM DTT together with the stromal extracts (150 μg protein) or the FPLC column fractions and the additions described in the text in a total volume of 100 μl . In the second stage, 40 μg of activated Rubisco (0.57 nmol active sites) in 15–20 μl was added to allow inhibition of the enzyme by any CA 1-P remaining in the reaction mixture. After 30 min, 50 μl aliquots were assayed for Rubisco activity [8] in a total volume of 0.5 ml to determine the extent of CA 1-P inhibition.

3. RESULTS

The concentration dependence of the CA 1-P preparation, determined under the assay condi-

tions used for detecting CA 1-P metabolism, is shown in fig.1. At low concentrations of CA 1-P, inhibition of Rubisco activity was linearly dependent upon the CA 1-P concentration, while higher concentrations produced up to 90% inhibition of Rubisco activity.

Since CA 1-P is localized in the chloroplasts, intact chloroplasts were used as starting material for the isolation of a protein fraction capable of metabolizing CA 1-P. This fraction was prepared from chloroplast lysates by rate zonal centrifugation and ultrafiltration and was assayed for activity by measuring the extent of Rubisco inhibition remaining after incubation of CA 1-P with the extract (table 1). For these experiments, relatively low concentrations of the CA 1-P preparation were used to ensure that inhibition was due to CA 1-P exclusively and not to possible contaminants in the CA 1-P preparation.

Substantial inhibition of Rubisco was observed when chloroplast extracts were incubated with CA 1-P either alone, or together with ATP prior to the addition of Rubisco (table 1). In contrast, inhibition decreased considerably when CA 1-P was incubated with the stroma extract from tobacco chloroplasts in the presence of NADPH. At an equivalent protein concentration, a much smaller decrease in inhibition occurred with NADPH and stromal extracts from spinach. No decrease in the extent of inhibition was observed when CA 1-P was incubated with the tobacco extract in the presence of either NADP^+ or NADH or when tobacco stromal preparations were heated for 3 min at 100°C prior to incubation with CA 1-P and NADPH. The NADPH-dependent activity associated with the tobacco extract was stable for at least 24 h when stored at 4°C in buffer containing 2 mM DTT. However, complete loss of activity occurred in preparations that were stored overnight without DTT (not shown).

The increase in Rubisco activity which occurred following incubation of CA 1-P with the tobacco stromal extract and NADPH corresponded to an apparent decrease in CA 1-P concentration of 82%, as determined from the concentration dependence of CA 1-P inhibition shown in fig.1. Spinach, which does not exhibit a Rubisco dark inhibition effect [5], possessed only a slight capacity to metabolize CA 1-P; the increase in Rubisco activity with spinach stromal extracts and NADPH

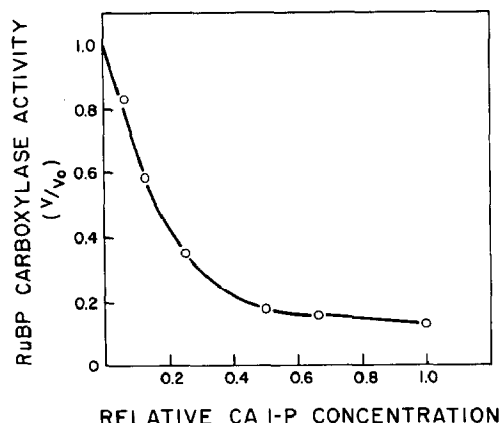


Fig.1. Effect of the relative CA 1-P concentration on the activity of purified tobacco Rubisco. Rubisco (40 μ g) was incubated in buffer A containing 20 mM DTT at 25°C with the indicated concentrations of CA 1-P in a total volume of 120 μ l. After 30 min, 50 μ l aliquots were assayed for Rubisco activity. Activity is expressed as v/v_0 , the ratio of the rate divided by the control rate without CA 1-P. A relative CA 1-P concentration of 1 is the concentration of CA 1-P required to obtain 87% inhibition of 40 μ g Rubisco under these assay conditions.

corresponded to only a 33% decrease in CA 1-P concentration.

Decreases in the extent of Rubisco inhibition which occurred following incubation of CA 1-P with stromal extract and NADPH may be due to

Table 1

Requirements for metabolism of CA 1-P by stromal extracts

Source of extract	Incubation conditions	RuBP carboxylase activity	
		U/mg	v/v_0
Tobacco chloroplasts	no additions	0.37	0.27
	+ 1 mM ATP	0.37	0.27
	+ 1 mM NADPH	1.08	0.80
	+ 1 mM NADPH, - CA 1-P	1.36	1.00
Spinach chloroplasts	no additions	0.26	0.19
	+ 1 mM ATP	0.28	0.21
	+ 1 mM NADPH	0.41	0.30
	+ 1 mM NADPH, - CA 1-P	1.37	1.00
No extract	+ 1 mM NADPH	0.19	0.14

Metabolism of CA 1-P is indicated by a loss of Rubisco inhibition. v/v_0 is the ratio of the carboxylation rate divided by the control rate without CA 1-P

either conversion of CA 1-P to a non-inhibitory metabolite during the first stage of the assay or a combined effect of NADPH and the extract on the binding of CA 1-P to Rubisco. In order to distinguish between these two possibilities, a discontinuous two-stage assay was used in which the first stage (i.e. preincubation of CA 1-P with stromal extract and NADPH) was deproteinized prior to the addition of exogenous Rubisco. In a time course experiment, the activity of Rubisco added in the second stage increased as a function of time when CA 1-P was preincubated for various lengths of time with the tobacco stromal extract and NADPH (fig.2). These results indicate that CA 1-P is actually metabolized (i.e. converted to a form which is no longer inhibitory to Rubisco) during incubation with the stromal extract and NADPH.

The changes in Rubisco activity shown in fig.2 corresponded to a hyperbolic decrease in the apparent CA 1-P concentration over time. This response is not unexpected considering the likelihood that the low concentrations of CA 1-P used in the assays were limiting for the reaction. In the absence of extract, there appeared to be no change in the level of CA 1-P after 60 min (fig.2). The addition of a large molar excess of Rubisco in

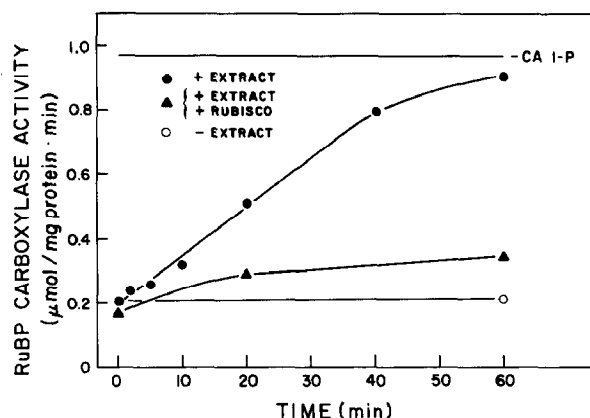


Fig.2. Time course of CA 1-P metabolism measured by the loss of Rubisco inhibition. CA 1-P at a relative concentration of 2 was incubated at 25°C with tobacco stromal extract (●), tobacco stromal extract plus 200 μ g/0.1 ml of purified tobacco Rubisco (▲) or in buffer alone (○). At the indicated times, 50 μ l aliquots were quenched by transfer to 200 μ l methanol at 4°C. Following removal of the precipitated protein, the supernatant was taken to dryness in vacuo and resuspended in 100 μ l. Rubisco (40 μ g) was added and, after 30 min at 25°C, 50 μ l aliquots were assayed for Rubisco activity.

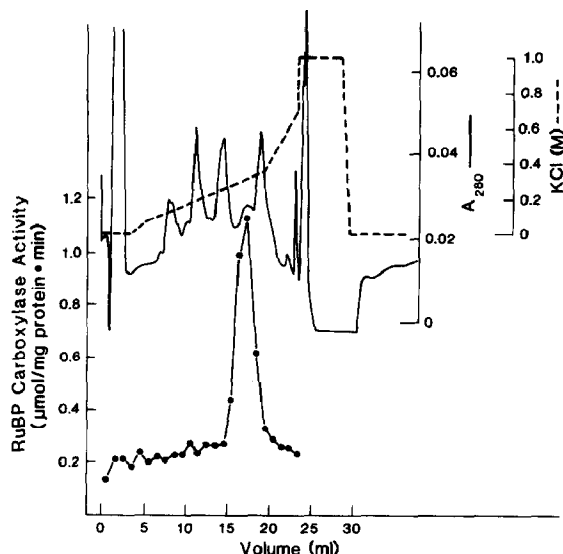


Fig.3. Partial purification of the CA 1-P metabolizing protein by anion-exchange FPLC. Elution profile of CA 1-P degradative activity (represented as a loss of Rubisco inhibition (●)) and A_{280} absorbance from a 0.5×5 cm Mono Q column. Fractions (1 ml) were collected, concentrated 10-fold by centrifugal ultrafiltration and assayed for activity in the presence of NADPH. Control rates were 0.23 without extract and 1.24 with extract and NADPH but without CA 1-P.

the first stage of the assay significantly decreased the apparent rate of CA 1-P metabolism, indicating that free CA 1-P rather than bound CA 1-P is required (fig.2).

The tobacco stromal extract was fractionated by anion-exchange FPLC to partially purify the protein active in CA 1-P metabolism (fig.3). When fractions were assayed for CA 1-P metabolizing activity, a single peak eluted from the column with about 0.3 M KCl, as shown by the peak of Rubisco activity (fig.3). The active fraction represented a minor portion of the total protein in the original stromal extract as evidenced by the A_{280} profile (fig.3) and SDS-PAGE of the individual column fractions (not shown). The FPLC-purified fraction was active only in the presence of NADPH and exhibited a rate of CA 1-P-dependent NADPH oxidation of 0.1 nmol/min at a relative CA 1-P concentration of 3.

4. DISCUSSION

In the presence of NADPH, a component of the soluble protein fraction from tobacco chloroplasts

progressively renders CA 1-P incapable of inhibiting Rubisco, presumably by facilitating its metabolism to a non-inhibitory compound. The requirement for NADPH in the degradative reaction is consistent with results from a previous study which showed that light-induced increases in total Rubisco activity in tobacco leaves and protoplasts were inhibited when electron flow was diverted away from ferredoxin [8]. Measurements of the redox state of the pyridine nucleotide pool show that the NADPH/NADP⁺ ratio is highest at very low irradiances and decreases with further increases in the irradiance level [15]. Since the concentration of CA 1-P changes in response to a fairly wide range of irradiance levels [5,7,8], metabolism of CA 1-P in the light is probably not regulated solely by the redox state of the chloroplast NADP system. Instead, we suggest that, *in vivo*, the enzyme which metabolizes CA 1-P may also require activation by Fd-thioredoxin, a requirement that would be consistent with the light-response of the process [6–8], the inhibitory effects of methyl viologen [8] and the apparent requirement for sulphhydryl reduction for maintenance of activity.

The NADPH requirement clearly distinguishes the CA 1-P metabolism reaction from the ATP-dependent Rubisco activation reaction catalyzed by Rubisco activase [16–18]. However, recent studies have shown that while Rubisco activase does not metabolize CA 1-P, it does cause a decrease in the extent of Rubisco inhibition by CA 1-P (Robinson, S.P. and Portis, A.R., personal communication), probably as a result of more rapid dissociation of the enzyme-inhibitor complex in the presence of Rubisco activase. This effect may have physiological relevance since the enzyme-inhibitor dissociation is too slow *in vitro* to account for the *in vivo* rate of CA 1-P metabolism [2,5].

The nature of the reaction responsible for CA 1-P metabolism could not be determined definitively with the indirect assay used in this study. Based on the observation that NADPH oxidation accompanies CA 1-P degradation, we suggest that metabolism of CA 1-P in chloroplasts may involve reductive conversion to either hamamelose 1-phosphate (2-C-(phosphohydroxymethyl)-ribose) or hamamelose. This reaction would require that a source of energy be available

for CA 1-P metabolism in addition to the reductant. Since exogenous ATP was not required for and did not stimulate CA 1-P metabolism (not shown), this energy is probably supplied by a source other than free ATP, for example by tightly-bound nucleotides or through rearrangement of the C-1 phosphate. Confirmation of the reaction mechanism awaits product analysis but it is of interest to note that hamamelose and hamamelose bisphosphate, two possible metabolites of the CA 1-P reduction product, occur in chloroplasts and are formed exclusively in the light [19].

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