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NADPH-dependent metabolism of the ribulose bisphosphate 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 bisphosphate 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 bisphosphate 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. *pulmilia*) and spinach (*Spinacia oleracea* L.) leaves [9] following pre-illumination for 10 min at 300 μmol photons/$m^2 \cdot s$. 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 × 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 $(NH_4)_2SO_4$ 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 conditions 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

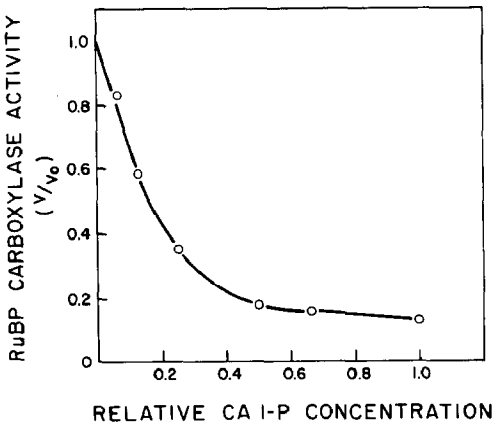

Fig.1. Effect of the relative CA 1-P concentration on the activity of purified tobacco Rubisco. Rubisco (40 μg) was incubated in buffer A containing 20 mM DTT at 25°C with the indicated concentrations of CA 1-P in a total volume of 120 μl. After 30 min, 50 μl aliquots were assayed for Rubisco activity. Activity is expressed as v/v_o, 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 μg 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 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

Table 1

Requirements for metabolism of CA 1-P by stromal extracts

Source of extract	Incubation conditions	RuBP carboxylase activity	
		U/mg	v/v_o
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_o is the ratio of the carboxylation rate divided by the control rate without CA 1-P

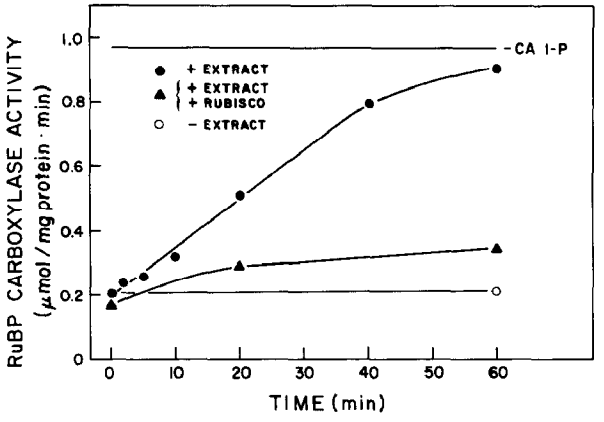

Fig.2. Time course of CA 1-P metabolism measured by the loss of Rubisco inhibition. CA 1-P at a relative concentration of 2 was incubated at 25°C with tobacco stromal extract (●), tobacco stromal extract plus 200 μg/0.1 ml of purified tobacco Rubisco (▲) or in buffer alone (○). At the indicated times,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

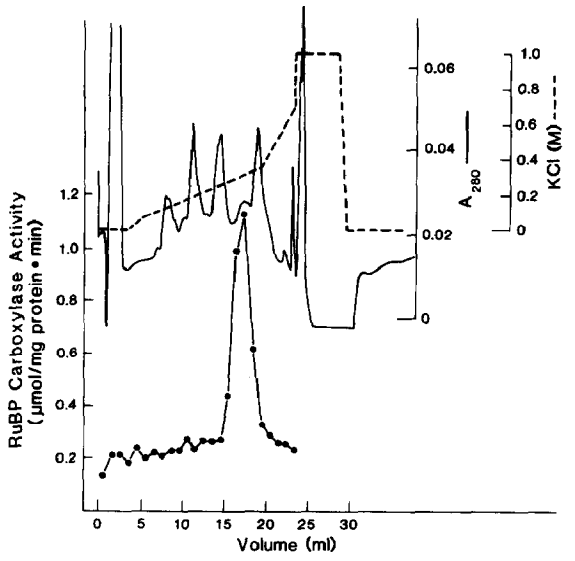

Fig.3. Partial purification of the CA 1-P metabolizing protein by anion-exchange FPLC. Elution profile of CA 1-P degradative activity (represented as a loss of Rubisco inhibition (●)) and A_{280} absorbance from a 0.5 × 5 cm Mono Q column. Fractions (1 ml) were collected, concentrated 10-fold by centrifugal 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 sulfhydryl 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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