Plant Physiol. (1989) 90, 679-685
0032-0889/89/90/0679/07/$01.00/0

Received for publication December 14, 1988
and in revised form February 10, 1989

Purification and Properties of 2-Carboxy-D-Arabinitol 1-Phosphatase[1]

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ABSTRACT

Carboxyarabinitol 1-phosphatase (2-carboxy-D-arabinitol 1-phosphate phosphohydrolase), a chloroplast enzyme that metabolizes the naturally occurring inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase, was isolated from tobacco (*Nicotiana tabacum*) leaves. The enzyme was purified more than 3500-fold using a protocol that included ammonium sulfate fractionation and gel filtration, ion-exchange, and hydrophobic interaction chromatography. Analysis of the final preparation by sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the presence of a single polypeptide with a molecular mass of 53 kilodaltons. The enzyme exhibited an apparent K_m (carboxyarabinitol 1-phosphate) of 33 micromolar and a pH optimum of 7.5. Enzyme activity did not require divalent cations and was unaffected by the metal chelators EDTA and cysteine. Carboxyarabinitol 1-phosphatase activity was inhibited by zinc, copper and molybdate and stimulated by sulfate. Chloroplast metabolites that affected activity included inorganic phosphate and ATP, which were inhibitory, and ribulose-1,5-bisphosphate, fructose-1,6-bisphosphate and NADPH which stimulated activity 2.5-fold. Activation of carboxyarabinitol 1-phosphatase activity by these positive effectors, together with the previously reported requirement for dithiothreitol, explain the light/dark modulation of carboxyarabinitol 1-phosphatase activity *in vivo*.

Three phosphohydrolytic reactions occur as steps in the pathways for photosynthetic and photorespiratory carbon metabolism. In the reductive pentose phosphate cycle, sedoheptulose-1,7-bisphosphate and fructose-1,6-bisphosphate are metabolized to the corresponding monophosphate esters by hydrolysis of their C-1 phosphate (22). These reactions are catalyzed by separate chloroplast-specific enzymes, sedoheptulose- and fructose-bisphosphatase. Both enzymes exhibit a number of regulatory properties that modulate their activation state in response to irradiance (1, 7, 14, 19). The underlying mechanism for this regulation is the reduction of specific sulfhydryl groups on the enzyme, a reaction which is mediated *in vivo* by the ferredoxin-thioredoxin system (6, 21). In the chloroplast, changes in stromal pH, the concentration of Mg^{2+}, and the levels of the bisphosphate substrates all affect the activation state of sedoheptulose- and fructose-bisphosphatase (11, 16, 17, 20) presumably by modifying the accessibility of critical sulfhydryl groups (2, 21).

In the photorespiratory carbon oxidation cycle, a chloroplast phosphatase has been described that catalyzes the phosphohydrolysis of phosphoglycolate (8, 23), one of the products of the ribulose-1,5-bisphosphate oxygenase reaction (4). The enzymic properties of phosphoglycolate phosphatase include inhibition of activity by ribose-5-phosphate and activation by both anions and divalent cations (13); however, aside from these factors, phosphoglycolate phosphatase does not appear to be under extensive regulatory control. This apparent lack of regulatory control may be indicative of a housekeeping function for this enzyme of rapidly catalyzing the metabolism of phosphoglycolate. The conversion of phosphoglycolate to glycolate is not of trivial importance for it prevents the accumulation of phosphoglycolate, a potent inhibitor of triose-phosphate isomerase (31), and initiates the recycling of the carbon lost in photorespiration back to the Calvin Cycle (4, 8, 28).

In a companion study (12), a newly discovered plant phosphatase was shown to be involved in the metabolism of 2-carboxyarabinitol 1-phosphate (CA 1-P[2]), an endogenous inhibitor of Rubisco. In the present study, we report a detailed protocol for the purification of CA 1-Pase (2-carboxy-D-arabinitol 1-phosphate phosphohydrolase) from tobacco leaves and describe some of the properties of the isolated enzyme.

MATERIALS AND METHODS

Plant Material

Leaf material for the purification of CA 1-Pase was obtained from 2- to 3-month old tobacco plants, *Nicotiana tabacum* L. var. KY14. Plants were grown in soil in a glasshouse under natural lighting.

Purification of CA 1-Pase

All procedures were conducted at 4°C. Buffers were adjusted to the indicated pH at 23°C before cooling to 4°C. One hundred g of deveined tobacco leaves were homogenized in a

[1] Kentucky Agriculture Experiment Station Journal Series, No. 89-3-6.

[2] Abbreviations: CA 1-P, 2-carboxyarabinitol 1-phosphate; CA 1-Pase, 2-carboxyarabinitol 1-phosphatase; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; FPLC, fast protein liquid chromatography.

Waring[3] blender for 1 min in 250 mL of 100 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, pH 8.0, containing 20 mM ascorbate, 50 mM 2-mercaptoethanol, 5 mM DTT, 2% (w/v) polyvinylpolypyrrolidone and 10% (v/v) glycerol. The homogenate was filtered through 1 layer of Miracloth and 8 layers of cheesecloth, and the filtrate was centrifuged at 20,000*g* for 15 min. Saturated $(NH_4)_2SO_4$, adjusted to pH 7.0 with NH_4OH, was added slowly to the supernatant to bring the final concentration to 40%. Following the removal of precipitated material by centrifugation at 20,000*g* for 20 min, solid $(NH_4)_2SO_4$ was added slowly to the supernatant to bring the concentration of $(NH_4)_2SO_4$ to 65%. The amount of $(NH_4)_2SO_4$ required to precipitate CA 1-Pase was established in preliminary experiments using crude tobacco homogenates that were centrifuged and then freed of Rubisco by gel-filtration chromatography (see below).

Following precipitation with $(NH_4)_2SO_4$, the protein pellet was collected by centrifugation at 20,000*g* for 20 min and resuspended in 20 mL of 100 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 10 mM $NaHCO_3$, pH 8.0, containing 2 mM DTT and 20 mM 2-mercaptoethanol (buffer A). The resulting solution, which was quite turbid, was centrifuged at 150,000*g* for 1 h to remove subchloroplast particles and other membranous material. Fifteen mL of the supernatant were immediately loaded on a 2.6 × 62 cm Sephacryl-S 300 column (Pharmacia Inc.) and chromatographed overnight at 0.3 mL/min in buffer A. Five ml fractions were collected and those eluting after the Rubisco peak were assayed for CA 1-Pase activity as described below. Peak fractions, corresponding to an elution volume of 149 to 216 mL, were stored overnight at 4°C while the entire procedure (*i.e.* extraction, $(NH_4)_2SO_4$ fractionation, ultracentrifugation and gel-filtration) was repeated for a second 100 g patch of leaves.

The peak Sephacryl fractions stored from the first extraction were pooled and combined with those from the second extraction and then concentrated by ultrafiltration on a YM-30 membrane (Amicon). The sample was clarified by centrifugation, and loaded on a 1.6 × 33 cm Q-Sepharose column equilibrated with 20 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, pH 8.0, containing 2 mM DTT (buffer B). This column was scaled up 65-fold from the FPLC ion-exchange column described previously (25). After elution with 1 column volume of buffer B at 1 ml/min, the sample was fractionated using a scaled-up version of the multistep gradient described previously (25). The total volume of the 0 to 1 M KCl gradient was 1300 mL. Thirteen mL fractions were collected and assayed for CA 1-Pase activity. Peak fractions from the Q-Sepharose column were pooled and protein precipitated by the addition of solid $(NH_4)_2SO_4$ to 65%.

Following storage overnight, the precipitated protein from the Q-Sepharose peak was collected by centrifugation (17,000*g* for 10 min) and resuspended in 27 mL of 50 mM Tris-HCl, 1.2 M $(NH_4)_2SO_4$, pH 7.5, containing 4 mM DTT. The solution was concentrated to 5 mL by ultrafiltration. Precipitated protein in the retentate was removed by centrifugation at 17,000*g* for 10 min. The supernatant, which contained all of the CA 1-Pase activity, was loaded on a 1.0 × 10 cm column of phenyl-Sepharose equilibrated with 50 mM Tris-HCl, 1.7 M $(NH_4)_2SO_4$, pH 7.5, containing 4 mM DTT, and was fractionated at 1 mL/min using a linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 7.5, and 4 mM DTT (buffer C). The elution profile was identical to that shown in Figure 1 for a 0.5 × 5.0 cm FPLC phenyl-Superose column except that the chromatographic conditions were increased eightfold. Four mL fractions were collected and assayed for CA 1-Pase activity. Protein in the pooled peak fractions was precipitated by the addition of solid $(NH_4)_2SO_4$ to 65% and stored overnight.

Precipitated protein from the phenyl-Sepharose column was collected and resuspended, and the solution was ultrafiltered and centrifuged using the procedure described in the preceding paragraph for the Q-Sepharose material. The sample was then subjected to a second hydrophobic interaction separation, this time at 0.5 mL/min using a 0.5 × 5.0 cm FPLC phenyl-Superose column. The gradient consisted of a two-segment linear $(NH_4)_2SO_4$ gradient in buffer C from 1.7 to 0.85 M over the first 5 mL and from 0.85 to 0 M over the next 16 mL. One mL fractions were collected and assayed for CA 1-Pase activity.

Peak fractions from the phenyl-Superose separation were resuspended by dilution in 20 mM Tris-HCl, pH 7.4, containing 4 mM DTT, and were desalted by ultrafiltration. The material was chromatographed on a 0.5 × 5.0 cm ion-exchange FPLC Mono Q column at pH 7.4 as described previ-

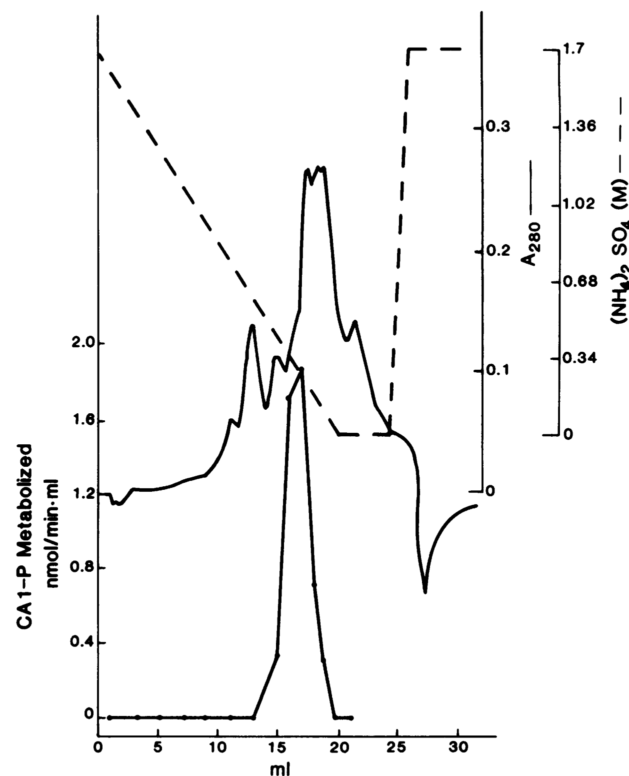

Figure 1. Elution profile of CA 1-Pase activity and A_{280} absorbance from a 0.5 × 5 cm FPLC phenyl-Superose column.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

ously (25). Saturated ammonium sulfate was added to a final concentration of 65% to the two fractions that contained most of the CA 1-Pase activity. Aliquots of these fractions were diluted with one volume of 100 mM Hepes-KOH, pH 7.5, containing 4 mM DTT and subjected to gel filtration FPLC on a 1.0 × 30 cm Superose-12 column.

For some preparations, dye-ligand chromatography was used as the final purification step. Precipitated protein from the FPLC phenyl-Superose column was collected by centrifugation at 17,000*g* for 10 min and resuspended in buffer C containing 1 mM fructose-1,6-bisphosphate (buffer D). Fructose bisphosphate increased the recovery of CA 1-Pase from the Reactive-red column (see below). The solution was repeatedly concentrated and resuspended by ultrafiltration on a YM-30 membrane to reduce the concentration of residual $(NH_4)_2SO_4$ by at least 10-fold. The solution was concentrated to 2 mL and loaded on a 2 ml column of Reactive-red 120-agarose, type 1000-CL (Sigma), which was equilibrated in buffer D. Following application of the sample, the column was eluted at 0.5 mL/min with 10 mL of buffer D followed by a linear gradient from 0 to 0.75 M of KCl in buffer D. One ml fractions were collected and assayed for CA 1-Pase activity. Fractions containing CA 1-Pase activity were pooled, made to 65% $(NH_4)_2SO_4$ and stored at 4°C.

Assay of CA 1-Pase

CA 1-Pase, stored as an $(NH_4)_2SO_4$ slurry, was diluted in 50 mM Tris-HCl, pH 7.5, containing 4 mM DTT, and the $(NH_4)_2SO_4$ concentration was reduced to a concentration less than 1 mM by repeated centrifugal ultrafiltration on a Centricon 30 unit (Amicon). CA 1-Pase activity was assayed at 25°C in 25 μL reactions containing 50 mM Tris-HCl, pH 7.5, 4 mM DTT, 2 mM NADPH, and 19.2 μM [2′-^{14}C]CA 1-P. Reactions were terminated after 10 min by the addition of 0.1 mL of 0.5 N HCOOH. The reaction product was quantified after chromatography on Dowex 1-X8 (HCOOH) as described in a companion study (12). Changes in the basic assay are noted in the text.

General Methods

Protein concentration was determined by the dye-binding assay (5) using BSA as the standard. SDS-PAGE was performed in 9 to 15 or 10 to 17% polyacrylamide gradient gels as described previously (9). Gels were stained with either Coomassie blue (9) or silver-reagent according to the directions of the manufacturer (Bio-Rad).

RESULTS

Purification of CA 1-Pase

A summary of the protocol for the purification of CA 1-Pase from tobacco leaf tissue is presented in Table I. The initial steps in the protocol were modified from the procedure described for the isolation of CA 1-Pase from tobacco chloroplasts (25). For example, gel filtration through Sephacryl S-300 and low pressure ion-exchange chromatography on Q-Sepharose replaced the rate zonal centrifugation and FPLC ion-exchange steps used previously (25). The current purification scheme for leaves also includes an $(NH_4)_2SO_4$ fractionation step and a brief ultracentrifugation; these steps were used primarily to concentrate and clarify the sample prior to column chromatography. The extent of enzyme purification after these two steps could not be determined directly due to interference of the CA 1-Pase assay by endogenous Rubisco (25). However, a 1.5-fold purification can be estimated based on the reduction in protein level after the first two purification steps (Table I). Gel filtration on Sephacryl S-300, which was used early in the purification scheme to remove Rubisco, reduced the total protein level by more than fivefold (Table I).

Isolation of CA 1-Pase from leaf tissue made it necessary to increase the capacity of the ion-exchange step used previously for the isolation of CA 1-Pase from chloroplasts (25) to accommodate greater amounts of protein. Therefore, the original procedure was increased 65-fold by direct scale-up from a 1 mL FPLC Mono-Q column to a 65 mL Q-Sepharose column. CA 1-Pase activity eluted in two peaks from the Q-Sepharose column, one which emerged in a position corresponding to that described for the enzyme from chloroplasts (25) and a second peak which eluted later in the gradient. The elution behavior of the two peaks was identical on the hydrophobic interaction and dye-ligand columns. Since the first peak had higher specific activity and contained the majority of the CA 1-Pase activity, it alone was carried through the subsequent purification steps.

The specific activity of CA 1-Pase was increased 7-fold by ion-exchange chromatography on Q-Sepharose (Table I). Further purification was achieved by hydrophobic interaction chromatography on phenyl-Sepharose and phenyl-Superose (Table I). The chromatographic elution behavior of CA 1-Pase from leaves was identical to that of the enzyme isolated from intact tobacco chloroplasts (data not presented). Hydrophobic interaction chromatography was followed by ion-exchange chromatography on a 1 mL FPLC Mono Q column. CA 1-Pase activity eluted from the Mono Q column in a single peak, and the specific activity was increased 2.8-fold.

Nonspecific binding of CA 1-Pase to the ultrafiltration membranes was a problem at the later stages of purification, making it difficult to concentrate the protein for subsequent purification. For assays, bovine serum albumin could be added to the solution at 0.5 mg/mL to enhance recovery (data not shown). However, for further enzyme purification, it was necessary to chromatograph small aliquots of the FPLC ion-exchange fractions directly on the Superose-12 gel filtration column. CA 1-Pase exhibited anomolous behavior on this column, eluting in a region corresponding to an apparent mol wt of <10,000. Recovery of CA 1-Pase activity after gel filtration was nearly 100%, and the specific activity of the enzyme was increased 2.3-fold to 0.26 units/mg.

Partial purification of CA 1-Pase following hydrophobic interaction chromatography was also achieved by subjecting material purified through the phenyl-Superose step to dye-ligand chromatography on reactive red 120-agarose. CA 1-Pase activity eluted late in the KCl gradient with 0.5 M KCl (data not shown). The specific activity of CA 1-Pase increased

Table I. *Purification of CA 1-Pase from Tobacco Leaves*

Purification Step	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mg	*nmol/min*	*nmol/min·mg*	*%*	*-fold*[a]
Homogenate	2557.0				(1.0)
Ammonium sulfate (40–65%)	2191.4				(1.2)
Ultracentrifugation	1700.1				(1.5)
Gel-filtration (Sephacryl S-300)	319.2	197.8	0.6	100	1 (8)
Ion-exchange (Q-Sepharose)	20.5	88.6	4.3	49	7 (58)
Hydrophobic interaction					
(phenyl-Sepharose)	10.7	70.7	6.6	36	11 (88)
(phenyl-Superose)	1.7	68.0	39.9	34	66 (532)
FPLC ion-exchange (Mono-Q)	0.3	30.6	112.7	16	187 (1502)
FPLC gel-filtration[b] (Superose-12)	0.2	49.6	261.2	25	435 (3482)

[a] The numbers in parentheses indicate the approximate purification based on the reduction in total protein that occurred in the first three steps of the protocol. [b] Chromatography was conducted on portions of the post-FPLC ion-exchange material and the results were extrapolated to the entire sample.

2.8-fold with this step, but recovery of enzyme activity from this column was less than 15%.

CA 1-Pase prepared by the procedure outlined in Table I was purified more than 400-fold compared to the enzyme isolated after Sephacryl S-300 gel filtration. Taking into account the reduction in total protein concentration that occurred in the first three steps of the protocol (Table I), the enzyme was purified more than 3500-fold. The enzyme preparation was stable when stored at 4°C as an $(NH_4)_2SO_4$ suspension, but slowly lost activity when stored at 4°C without $(NH_4)_2SO_4$ or frozen at −20°C. Recovery of activity through the entire procedure was 25% with major losses occurring at the ion-exchange steps. Approximately 50% of the total activity was lost at the Q-Sepharose step, most of which appeared as a second peak of CA 1-Pase activity. This second CA 1-Pase peak may have represented a pool of CA 1-Pase that was associated with Rubisco since the second CA 1-Pase peak eluted from the ion-exchange column in the same position as the small amount of residual Rubisco that carried through from the Sephacryl separation.

An analysis of the polypeptides after the various stages of purification is shown in Figure 2. Several polypeptides were present in the active fractions from the FPLC ion-exchange column. However, when SDS-PAGE analysis was conducted on the individual column fractions from this column, only a single polypeptide at 53 kD was strictly associated with CA 1-Pase activity (Fig. 2B, indicated by the arrow). This polypeptide was present in the active fractions from the dye-ligand column along with a contaminated polypeptide at 63 kD (data not presented). Further purification of CA 1-Pase by FPLC gel filtration confirmed the identification of the 53 kD polypeptide as CA 1-Pase (Fig. 2).

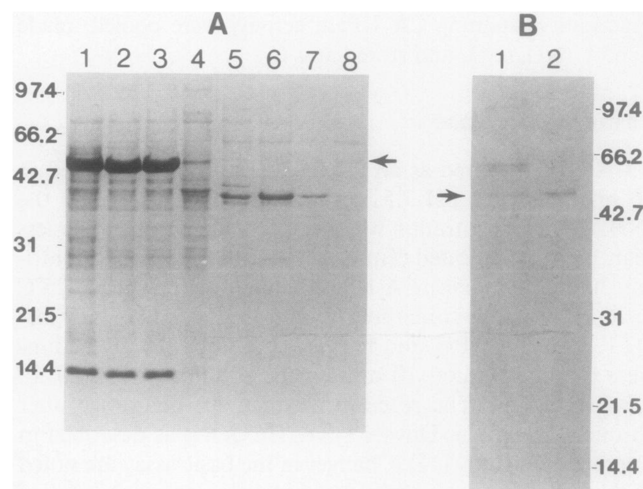

Figure 2. SDS-PAGE separation of the polypeptides in the CA 1-Pase preparations following each step in the purification. A, Coomassie blue-stained gel of the material from: 1, crude leaf homogenate; 2, 40 to 65% ammonium sulfate; 3, ultracentrifugation; 4, Sephacryl S-300; 5, Q-Sepharose; 6, phenyl-Sepharose (12.5 μg); 7, phenyl-Sepharose (2.5 μg); 8, phenyl-Superose (2.5 μg). B, Silver-stained gel of CA 1-Pase after FPLC ion-exchange chromatography (lane 1) and gel filtration FPLC (lane 2). The numbers in the margins indicate the positions and molecular mass of standards expressed in kilodaltons. The arrows indicate the position of CA 1-Pase on the gels.

Properties of CA 1-Pase

The effects of various metal ions on the activity of purified CA 1-Pase were examined (Table II). At a concentration of 5 mM, magnesium, manganese, and potassium had little effect on CA 1-Pase activity, whereas copper, zinc, and especially molybdenum were inhibitory. Enzyme activity was not affected by 1 mM EDTA or 0.2 mM cysteine (data not shown), but was enhanced by the inclusion of the sulfate anion as either the potassium, sodium, or ammonium salt (Table II). Concentrations of $(NH_4)_2SO_4$ from 10 to 50 mM were optimal for stimulation of CA 1-Pase activity, but concentrations greater than 0.7 M were inhibitory. Substantial inhibition of

Table II. *Effect of Various Metals and Other Ions on the Activity of CA 1-Pase*

All assays were conducted in the presence of 2 mM NADPH with enzyme purified through the phenyl-Superose step.

Compound	Concentration	CA 1-Pase Activity
	mM	%
None[a]		100
KCl	5	106.1
$MgCl_2$	5	96.1
$MgSO_4$	5	89.0
$MnCl_2$	5	75.0
$ZnCl_2$	5	32.3
$ZnSO_4$	5	37.7
$(NH_4)_6Mo_7O_{24}$	5	1.4
$CuSO_4$	5	44.3
K_2SO_4	50	109.5
Na_2SO_4	50	131.1
$(NH_4)_2SO_4$	50	130.3
NH_4Cl	50	111.1
NH_4HCO_3	50	72.6
KH_2PO_4	2	60.0
KH_2PO_4	50	16.7

[a] Assays were conducted in the presence of 1 mM EDTA.

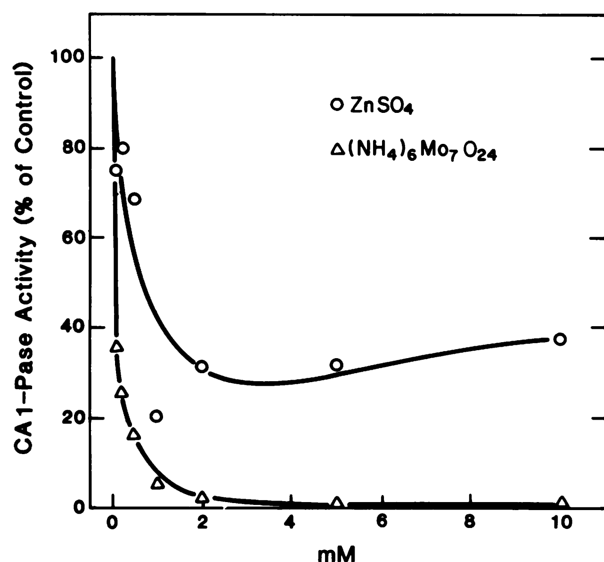

Figure 3. Effect of zinc sulfate (O) and ammonium molybdate (Δ) concentration on CA 1-Pase activity. Carboxyarabinitol 1-phosphatase was purified through the phenyl-Superose step and assayed for activity at pH 8.0 in the presence of 2 mM NADPH.

CA 1-Pase activity also occurred when the enzyme was assayed in the presence of inorganic phosphate (Table II).

The concentration dependence for inhibition of CA 1-Pase activity by ammonium molybdate and zinc sulfate is presented in Figure 3. These compounds were inhibitory in the micromolar range; the K_i values for molybdate and zinc were less than 100 μM and 500 μM, respectively (Fig. 3). Molybdate was an especially potent inhibitor causing almost complete inhibition of CA 1-Pase activity when included as either the ammonium or sodium (not shown) salt at 1 to 2 mM.

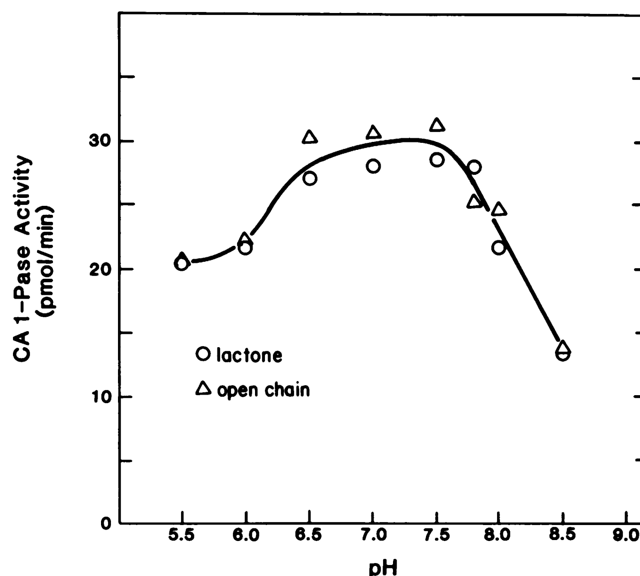

Figure 4. Effect of pH on the activity of CA 1-Pase. [2-^{14}C]CA 1-P adjusted to either pH 4.0 (lactone, O) or pH 9.0 (open chain, Δ) was used to initiate 5 min assays buffered to the indicated pH with 200 mM Mes (6.0–6.5), Hepes (7.0–7.8), or Tricine (8.0–8.5). Assays were conducted in the presence of 2 mM NADPH with enzyme that was purified through the dye-ligand chromatographic step.

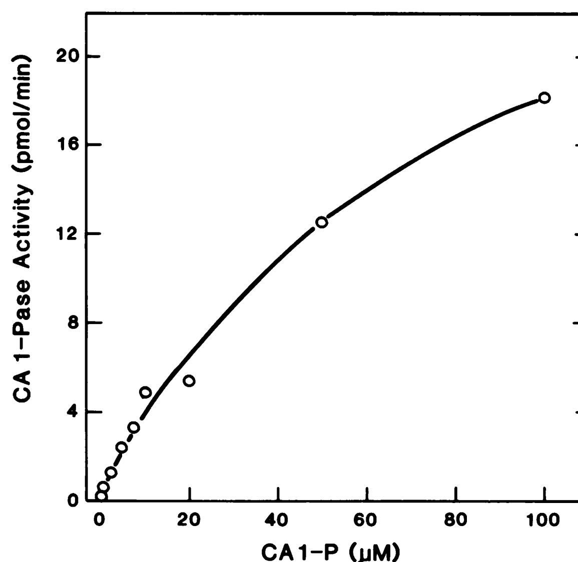

Figure 5. Effect of CA 1-P concentration on the activity of CA 1-Pase. CA 1-Pase was purified through the phenyl-Superose step and assayed for activity at pH 8.0 in the presence of 2 mM NADPH.

The response of CA 1-Pase activity to pH is presented in Figure 4. Since CA 1-Pase undergoes lactonization (3, 10), the effect of pH on CA 1-Pase activity was examined in experiments in which CA 1-Pase assays were initiated with either the open chain form of the substrate or the lactone. With either form of the substrate, the pH profile of CA 1-Pase activity was identical, exhibiting a broad maximum between pH 7.0 and 7.5. Activity was optimal at pH 7.5, but declined by more than 50% with increasing pH from 7.5 to 8.5.

Table III. *Effect of Metabolites on the Activity of CA 1-Pase*

CA 1-Pase purified through the FPLC ion-exchange step was thoroughly desalted by ultrafiltration and assayed at 25°C in the presence of the indicated concentrations of metabolites. Except where indicated, assays were conducted in the absence of NADPH.

Metabolite	CA 1-Pase Activity with Metabolite Concentration:		
	0.1 mM	1.0 mM	2.0 mM
		V_e/V_o[a]	
NADPH			2.5
Ribulose bisphosphate	1.5	2.4	
Fructose bisphosphate	1.2	2.3	
3-Phosphoglycerate	1.0	1.5	
ADP	1.0	1.2	
6-Phosphogluconate	0.9	1.1	
Fructose-6-P	0.9	1.0	
Glucose-1-P	0.9	1.0	
Ribulose-5-P	0.9	0.9	
Ribose-5-P	0.8	0.6	
ATP	0.7	0.6	

[a] Activity is expressed as the ratio of the activity in the presence (V_e) and absence (V_o) of effector.

The response of CA 1-Pase activity to the concentration of substrate is presented in Figure 5. CA 1-Pase activity exhibited typical Michealis-type kinetics in response to CA 1-P concentration, approaching saturation at 100 μM CA 1-P (Fig. 5). An apparent Km value for CA 1-P of 33 μM was determined from double reciprocal plots of the data in Figure 5.

Several chloroplast phosphoesters were examined for an effect on CA 1-Pase activity (Table III). Of those tested, NADPH, ribulose bisphosphate, fructose bisphosphate, and to a lesser extent 3-phosphoglycerate, were positive effectors of CA 1-Pase activity. The stimulatory effect of NADPH on CA 1-Pase activity has been reported previously (12). Of the other chloroplast metabolites that were tested, most had little or no effect on CA 1-Pase activity, with the exception of ATP and ribose-5-P, which were inhibitory.

DISCUSSION

CA 1-Pase catalyzes the reaction which converts CA 1-P to carboxyarabinitol (12). In the accompanying paper, we demonstrated that the phosphohydrolytic activity of the enzyme was specific for CA 1-P, an endogenous inhibitor of Rubisco which is present in the chloroplasts of some plant species (3, 10, 26). In most of these species, CA 1-P accumulates only after prolonged periods of dark or low irradiance requiring several hours to reach a final concentration sufficient to block one-half of the Rubisco active sites (10, 26, 27, 29). An exception to this occurs with *Phaeseolus vulgaris*, a species in which CA 1-P has been reported to accumulate relatively rapidly, within minutes following a transition from high to low irradiance (15). Degradation of CA 1-P following a step increase to high irradiance is generally much more rapid than its synthesis (*i.e.* $t_{1/2}$ = 6.7 min [24]) in all species that have been examined (15, 24, 26, 27, 29). The rapid rate at which CA 1-P is metabolized compared with the slow rate at which it accumulates suggests that even if synthesis of CA 1-P continued unabated in the light it would be superceded at high irradiance by a much more rapid rate of degradation. Under natural conditions, however, CA 1-P degradation occurs at a much more gradual rate presumably due to modulation of CA 1-Pase activity by the low incident radiation encountered during sunrise (27, 29).

CA 1-Pase was identified as a soluble enzyme present in the chloroplasts of tobacco (12, 25). Purification of the enzyme from tobacco leaves was relatively straightforward, but its quantification was complicated by the large amount of Rubisco protein present in the leaf extract. Rubisco binds CA 1-P with high affinity, thus its abundance in leaves precluded the direct determination of CA 1-Pase activity in the initial plant extract (25). Based on the specific activity of the purified enzyme and the total activity measured after chromatography on Sephacryl S-300, CA 1-Pase appeared to comprise 0.03% of the total protein in the tobacco leaf. However, if CA 1-Pase can associate with Rubisco, a large amount of the CA 1-Pase may have been lost when the bulk of the Rubisco was removed on the Sephacryl column. More precise information regarding the abundance of CA 1-Pase in leaves and possible associations between CA 1-Pase and Rubisco can be determined with immunological techniques once anti-CA 1-Pase antibodies are produced.

The enzymic characteristics of CA 1-Pase differ considerably from those of the other chloroplast phosphatases. For example, CA 1-Pase exhibited a lower pH optimum than has been reported for the bisphosphatases (17) or for P-glycolate phosphatase (13). Most phosphatases including phosphoglycolate phosphatase, sedoheptulose- and fructose-bisphosphatase require a divalent cation, usually Zn^{2+} or Mg^{2+}, for activity (8, 13, 17). In contrast CA 1-Pase activity was relatively unaffected by the presence of Mg^{2+} and Mn^{2+}, or the metal chelators EDTA and cysteine, and was inhibited by low concentrations of Zn^{2+} as well as by copper and molybdate. Inhibition of phosphohydrolytic activity by these metals has also been reported for glucose-6-phosphatase, a multifunctional microsomal enzyme most extensively studied from rat liver (18). The apparent requirement for sulfhydryl reduction for activity (18) constitutes another similarity between CA 1-Pase and glucose-6-phosphatase. However, the enzymes differ in many ways, including their response to metal chelators, their subcellular distribution, and their substrate specificity (18).

Several properties of CA 1-Pase could provide the regulatory control necessary to explain the increased rate of CA 1-P degradation occurring in response to rising irradiance. For example, we have previously noted (25) that the requirement for DTT for maintenance of activity during storage indicated a probable involvement of sulfhydryl reduction in the light activation of CA 1-Pase. A regulatory effect of pH on CA 1-Pase activity was not readily apparent from the pH profile shown in Figure 4, since the pH optimum is somewhat lower than the pH values that have been reported for the stroma in illuminated chloroplasts (30). However, the highest rates of CA 1-P degradation occur during sunrise, a time when low irradiance may limit stromal alkalization (11). Thus, the pH response of CA 1-Pase may actually be optimized to ensure

that maximal enzyme activity occurs during the time when substrate levels are at their highest.

A most important regulatory property of CA 1-Pase was the observed modulation of enzyme activity by chloroplast metabolites, particularly activation by ribulose bisphosphate, fructose bisphosphate, and NADPH, and inhibition of activity by inorganic phosphate. In a companion study (12), we showed that ribulose bisphosphate and fructose bisphosphate were not substrates for the purified enzyme; thus, their stimulatory effect on activity must involve allosteric interactions. Since the concentrations of these metabolites increase substantially upon illumination, their effect on enzyme activity, together with the effects of pH and sulfhydryl reduction, would provide a mechanism for light/dark regulation of CA 1-Pase activity.

ACKNOWLEDGMENTS

We wish to thank J. C. Anderson for her many essential contributions to this study.

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