Regulation of 2-Carboxyarabinitol 1-Phosphatase

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ABSTRACT

The regulation of 2-carboxyarabinitol 1-phosphatase (CA 1-Pase) by phosphorylated effectors was studied with enzyme purified from tobacco (Nicotiana tabacum) leaves. CA 1-Pase activity was most stimulated by fructose 1,6-bisphosphate, exhibiting an $A_{540}$ value of 1.9 millimolar and a 10-fold enhancement of catalysis. With ribulose-1,5-bisphosphate, the $A_{650}$ was 0.6 millimolar, and maximal stimulation of activity was 5.3-fold. Among the monophosphates, 3-phosphoglycerate and phosphoglycolate were more potent positive effectors than glyceraldehyde 3-phosphate, glucose 1-phosphate, glucose 6-phosphate, and dihydroxyacetone phosphate. Stimulation of CA 1-Pase by ribulose-1,5-bisphosphate and fructose 1,6-bisphosphate increased $V_{	ext{max}}$ but did not appreciably alter $K_m$ (2-carboxyarabinitol 1-phosphate) values. Inorganic phosphate appeared to inhibit CA 1-Pase noncompetitively with respect to 2-carboxyarabinitol 1-phosphate, exhibiting a $K_i$ of 0.3 millimolar. The results suggest that these positive and negative effectors bind to a regulatory site on CA 1-Pase and may have a physiological role in the light regulation of this enzyme. Related experiments with CA 1-Pase inactivated by dialysis in the absence of dithiothreitol show that partial reactivation can be achieved in the presence of a range of reducing reagents, including dithiothreitol, cysteine, and reduced glutathione. This could imply an ancillary involvement of sulphhydril reduction during light activation of CA 1-Pase in vivo. The enzyme was thermally stable up to 35°C, in contrast to ribulose-1,5-bisphosphate carboxylase/oxygenase activase which lost activity above 30°C. The activation energy for CA 1-Pase was calculated to be 56.14 kilojoules per mole.

CA 1-Pase is a chloroplastic phosphate ester that accumulates in leaves of numerous plant species in response to darkness or reduced light intensity (21, 23, 26). CA 1-P binds tightly ($K_D = 32$ nm) to the active site of Rubisco, inhibiting catalysis (3, 24). In certain species such as Phaseolus vulgaris, the kinetics of CA 1-P synthesis are consistent with a major role for this compound in the regulation of Rubisco activity (10, 21, 23). To date, relatively little is known about the metabolism of CA 1-P, despite the fact that in many species it occupies more than half the Rubisco active sites in darkness (25). A plausible sequence of events allowing the light-dependent reversal of Rubisco inhibition would involve release of CA 1-P from the enzyme facilitated by Rubisco activase (15), followed by its phosphohydrolytic degradation catalyzed by CA 1-Pase (4, 7, 21). The net reaction is dependent upon chloroplast electron transport, because treatment with DCMU inhibits reversal of Rubisco inhibition during reillumination (24). Similar consequences are observed when tobacco protoplasts are treated with methyl viologen (18) or nigericin (21). This implies an interaction between CA 1-P metabolism and reactions on the reducing side of PSI. Additionally, in preliminary studies, it has been noted that CA 1-Pase activity is stimulated in vitro by several chloroplast metabolites, including NADPH, RuBP, and FBP (7, 20). With P. vulgaris, appreciable levels of CA 1-P accumulation occur within 60 s under low-light conditions, but in other species the increase in inhibitor concentration may require more than 1 h (6, 10). The prevailing level of CA 1-P may represent a balance between its synthetic and degradative pathways (23, 26). Hence, a regulatory decrease in CA 1-Pase activity in the dark or reduced light is a likely prerequisite to a buildup of CA 1-P. If this is the case, a corresponding activation of CA 1-Pase would be expected during reillumination at high irradiance.

To elucidate possible mechanisms by which CA 1-Pase is light regulated, we examined in more detail (a) the influence of relevant effector metabolites present in the chloroplast or cytosol on CA 1-Pase activity and (b) the potential of CA 1-Pase for reductive activation akin to other chloroplast phosphatases (1). We also present information concerning the thermal stability of CA 1-Pase activity, allowing a comparison to the established heat sensitivity of Rubisco activase (17).

MATERIALS AND METHODS

Preparation and Assay of CA 1-Pase

CA 1-Pase was prepared from tobacco (Nicotiana tabacum L. var KY14) as described previously (20) and stored as a suspension in 65% (NH₄)₂SO₄ at 4°C. To desalt CA 1-Pase before assays, the enzyme suspension was diluted in medium containing 50 mM Tris-HCl (pH 7.5) and 4 mM DTT (buffer A) plus 0.2 mg/mL BSA before overnight dialysis at 4°C against several changes of buffer A. The dialysate was then concentrated by centrifugal ultrafiltration on a Centricon-30.
unit (Amicon). The inclusion of BSA reduces losses of CA 1-Pase incurred by adsorption to the ultrafiltration membrane but complicates colorimetric measurement of CA 1-Pase concentration. Absolute rates of catalysis expressed per milligram of protein are therefore approximate. CA 1-Pase activity was measured at 25°C in assays with the following components: approximately 1.2 μg of enzyme in 6 μL of buffer A, 0 to 5 μL of 0.502 mM [14C]CA 1-P (22.4 Ci/mol, ref. 7), effectors (prepared in buffer A) added to give concentrations noted in the text, and buffer A bringing the final volume to 25 μL. The concentration of [14C]CA 1-P used for the effector studies was 50 μM, a concentration close to the apparent K_m value (20). Reactions were initiated by adding the enzyme and terminated after 10 min by the addition of 0.1 mL of 0.5 N HCOOH. The [14C]CA formed was quantified after chromatography on Dowex-formate minicolumns as described previously (7). For thermal stability studies, CA 1-Pase was preincubated in buffer A containing 2 mM NADPH for 1 h at the appropriate temperature before assay at 25°C.

Preparation and Assay of Rubisco Activase

Rubisco activase was purified from tobacco leaves by a procedure modified from ref. 16. Briefly, leaf tissue was homogenized at 4°C in 50 mM Hepes-KOH (pH 7.0), 5 mM MgCl_2, 1 mM EDTA, 1 mM ATP, 5 mM DTT, 50 mM 2-mercaptoethanol, 20 mM ascorbate, 2% (w/v) polyvinylpyrrolidone, 10% glycerol, 1 mM PMSF, and 10 μM leupeptin. Following filtration through four layers of cheesecloth and one layer of Miracloth and centrifugation for 15 min at 25,000g, saturated (NH_4)_2SO_4 was added to the supernatant liquid throughout 30 min to a final concentration of 35% (v/v). Precipitated protein was collected by centrifugation and washed by resuspension in 50 mM Hepes-KOH (pH 7.0), 2 mM MgCl_2, 100 mM KCl, 5 mM DTT, and 1 mM ATP (buffer B) containing 35% (NH_4)_2SO_4. Precipitated protein was then resuspended in buffer B containing 1 mM PMSF and 10 μM leupeptin and centrifuged at 100,000g for 90 min. The supernatant liquid was chromatographed on a 330-mL Sephacryl S-300 column at 0.3 mL/min in 20 mM Hepes-KOH (pH 7.0), 2 mM MgCl_2, 50 mM KCl, 2 mM DTT, and 0.2 mM ATP. Column fractions that possessed ATPase activity were pooled and subjected to anion-exchange fast protein liquid chromatography on a Mono-Q column. Activase was isolated by elution with a linear gradient of KCl from 0 to 0.5 M in 20 mM Hepes-KOH (pH 7.0), 2 mM MgCl_2, 4 mM DTT and stored at −80°C in the presence of 0.2 mM ATP. For heat inactivation studies, 19 μg of activase were preincubated at the appropriate temperature for 1 h in 0.1 mL of solution containing 87 mM Tricine (pH 8.0), 4.36 mM MgCl_2, 17.5 mM KCl, 0.18 mM NADH, 4 mM DTT, and 0.2 mM ATP (buffer C). A coupled assay measuring the ATPase activity of activase was then performed at 25°C by adding the preincubation mixture to a spectrophotometer cuvette containing a final volume of 1 mL of solution comprising 100 mM Tricine (pH 8.0), 5 mM MgCl_2, 20 mM KCl, 0.2 mM NADH, 4.6 mM DTT, 1 mM ATP, 2 mM phosphoenolpyruvate, 12 units of pyruvate kinase (Sigma P1506), and 12 units of lactate dehydrogenase (Sigma L2518). Oxidation of NADH was measured by the decrease in A_{410}.

RESULTS AND DISCUSSION

Stimulation of CA 1-Pase by Phosphorylated Metabolites

The activation of CA 1-Pase by phosphate esters is of potential importance as a regulatory mechanism contributing to the light-dependent reversal of Rubisco inhibition. In a previous work (Table III in ref. 20), 11 different chloroplastic phosphate esters were tested for an effect on CA 1-Pase activity at fixed concentrations of either 0.1 or 1.0 mM. FBP, RuBP, 3-PGA, and NADPH showed evidence of stimulating CA 1-Pase activity, whereas ATP and ribose 5-phosphate were

Figure 1. Effect of phosphorylated metabolites on the activity of CA 1-Pase. [14C]CA 1-P concentration was constant at 50 μM. a: Response to FBP (A) and RuBP (C); b: response to glucose 1,6-bisphosphate (G16BP, □) and fructose 2,6-bisphosphate (F26BP, ▼); c: response to 3-PGA (A), phosphoglycerate (P-glycolate, ◊), glyceraldehyde 3-phosphate (G3P, ▽), and glucose 1-phosphate (G1P, ■). v_0, rate of catalysis in the presence of each effector: v_0 = 1,990 pmol/2-CA formed/mg protein/min (see "Materials and Methods").
The reported transitions and metabolites on or from measurements available from stromal I-Pase. Potent positive effectors include 1,6-diphosphogluconate and glucose-1-phosphate, and 3-phosphoglycolate, fructose-1,6-bisphosphate, and glyceraldehyde 3-phosphate, all of which are produced in the chloroplast by glycolysis. The Ca 1-Pase activity was appreciably enhanced in the presence of RuBP or FBP (Fig. 1a) with a maximal stimulation of fivefold or more than ninefold, respectively. Another bisphosphate with a 1,6 configuration, namely, glucose-1,6-bisphosphate, and the cytosolic phosphoester, fructose-2,6-bisphosphate, were also capable of a fivefold stimulation of the enzyme’s activity (Fig. 1b). However, because of their occurrence at low concentrations, the latter metabolites are unlikely to influence CA 1-Pase activity in the stroma. Among the monophosphates, 3-PGA and phosphoglycolate were more potent positive effectors than glyceraldehyde 3-phosphate (Fig. 1c). Glucose 1-phosphate (Fig. 1c) or dihydroxyacetone phosphate and glucose 6-phosphate (not shown) had minimal effects on CA 1-Pase, even at levels as high as 10 mM. The A0.5 values for metabolite concentrations causing half-maximal stimulation of CA 1-Pase activity were summarized as follows: RuBP, 0.6 mM; PGA, 3.4 mM; FBP, 1.9 mM; NADPH, 0.9 mM; and phosphoglycolate, 2.4 mM.

If chloroplastic metabolites are involved in the regulation of CA 1-Pase in vivo, it is also important to examine changes in stromal concentrations of relevant effectors associated with dark/light transitions. The present study did not include such measurements on tobacco leaves. However, similar data are available from several other sources, obtained by nonaqueous fractionation of leaf extracts, by subcellular fractionation of protoplasts, or from isolated chloroplasts. Stromal levels of metabolites calculated from each source show considerable variability. The reported values encompassing maximal dark to light concentration changes in RuBP are from 0 to 8 mM (2, 12, 13, 28); for PGA, from 1.1 to 6.0 mM (2, 9, 28); and for FBP, from 0 to 0.8 mM (2, 28). Based on the response of CA 1-Pase to phosphates esters (Fig. 1), the latter three metabolites could contribute to activation of CA 1-Pase during dark/light transitions in vivo. Stromal NADPH levels (29) may not change enough to significantly influence CA 1-Pase activity. Similarly, the minimal pool size of phosphoglycolate in normal leaves in the light (5, 27) precludes its ability to activate CA 1-Pase in vivo.

The influence of these phosphorylated effectors on the substrate saturation kinetics of CA 1-Pase is typified by FBP, which increases the VAmax value (Fig. 2). The small corresponding decrease in the Km (CA 1-P) can be dismissed as being within the limits of experimental error. Similar results were observed with RuBP and NADPH (not shown). These data, together with previous confirmation that FBP and RuBP are not substrates for purified CA 1-Pase (7), strongly suggest allosteric interactions between the enzyme and specific stromal metabolites.

Inhibition of CA 1-Pase by Pi

In contrast to the positive effectors discussed above, Pi inhibits CA 1-Pase activity with a Ki of approximately 0.3 mM (Fig. 3). Under the conditions of the assay, which included 4 mM DTT and 50 mM [14C]CA 1-P, the maximal inhibition observed was about 70%, saturating at 5 to 10 mM Pi. Further analysis showed that inhibition of CA 1-Pase by Pi decreased Vmax but did not significantly change Km (CA 1-P). Addition of 5 mM Pi decreased Vmax from 4.9 ± 0.7 (SD) to 2.3 ± 0.6 pmol 2-CA formed/min. Respective corresponding Km (CA 1-P) values were 19.6 ± 4.1 μM without Pi and 25.3 ± 9.7 μM with Pi. These observations, and the heterotropic response observed with positive effectors (Fig. 2), further support the notion that CA-1-Pase possesses a regulatory site for effector binding.

Pi is also likely to be of regulatory significance to CA 1-Pase activity in vivo. During illumination, the concentration of stromal Pi decreases due to the synthesis and autocatalytic activity of Ru5Pase II. The concentration of [14C]CA 1-P was constant at 50 μM. V0 = 1.78 pmol CA formed/min.
buildup of photosynthetic carbon reduction cycle intermediates, and its consequent incorporation into the organic phosphate pool (11, 28). Available data concerning Pi levels in the chloroplast compartment of spinach protoplasts or leaves show a dark/light fluctuation of at least 3.8 mM (8, 11, 14, 22, 28). However, in this instance, the minimum [Pi] values measured in the light were 3.0 mM (28), which would maintain CA 1-Pase activity at decreased levels (Fig. 3). On the other hand, it is difficult to assess the ratio of free and bound pools of Pi. For example, there is evidence for a nonmetabolic pool of Pi associated with thylakoid membranes, which may result in overestimation of stromal Pi levels (14). In addition, appreciable variation in leaf Pi content has been observed due to leaf age, physiological state, and species (22).

**Effect of FBP and Pi on CA 1-Pase Activity**

The results presented in Figures 1 and 3 show that the activity of purified CA 1-Pase is influenced by multiple effector metabolites present in the stroma. The catalytic rate in vivo is likely to reflect a complex interaction between positive and negative effectors at various concentrations. A simplified example of this is represented by the simultaneous effect of FBP and Pi on CA 1-Pase activity (Fig. 4). The two effectors compete to produce a net catalytic rate. At a constant concentration of CA 1-P, in the absence of Pi, 10 mM FBP stimulates

![Image of graph showing interactive effect of FBP and Pi on CA 1-Pase activity.](image)

**Figure 4.** Interactive effect of FBP and Pi on CA 1-Pase activity. Assays were initiated by adding CA 1-Pase to a reaction mixture containing the indicated concentrations of each effector and 50 µM [14C]CA 1-P. Catalytic rate with FBP alone (C), 0.5 mM Pi (C), or 10 mM Pi (Δ).

### Table I. Activation of CA 1-Pase by Reducing Agents and Inactivation by Oxidizing Agents

Purified tobacco CA 1-Pase was dialyzed overnight against four changes of 50 mM Tris (pH 7.5) at 4°C in the presence (+) or absence (−) of 4 mM DTT. After concentration of the dialysate by centrifugal ultrafiltration, aliquots were preincubated at 0°C for 1 or 28 hours with a 50 mM concentration of each reducing or oxidizing agent before initiating assays with 50 mM [14C]CA 1-P.

<table>
<thead>
<tr>
<th>Dialysis Treatment</th>
<th>Preincubation Conditions</th>
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<th>Experiment 2</th>
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* Numbers in parentheses are the ratio of treated versus control activities of CA 1-Pase.
CA 1-Pase activity 10-fold over control rates with no FBP. Addition of CA 1-Pase to a reaction medium containing 10 mM FBP and 10 mM Pi reduces the extent of this stimulation to 1.9-fold. Examination of the data indicates that CA 1-Pase is more sensitive to inhibition by Pi than to stimulation by FBP at the highest effector concentrations tested. This result is consistent with the difference in respective $K_i$ and $A_{0.5}$ values of these metabolites described above.

**Activation of CA 1-Pase by Reducing Agents**

During initial experiments on the isolation of CA 1-Pase, it was noted that maintenance of enzyme activity required storage in the presence of DTT (4, 19). For this reason, most subsequent experiments with CA 1-Pase have also routinely included 4 mM DTT in the assay medium (7, 20). The DTT requirement indicates a need to maintain the enzyme's sulfhydryl groups in a reduced state. However, it is also feasible that the extent of sulfhydryl group reduction could be of regulatory significance as exemplified by a number of chloroplastic enzymes, including fructose bisphosphatase and sedoheptulose bisphosphatase (1).

The removal of DTT by overnight dialysis of CA 1-Pase at 4°C causes inactivation to a residual activity constituting between 5 and 10% of the original. Results presented in Table I show that subsequent incubation of the enzyme with a 50 mM concentration of each of several reducing agents at 0°C causes appreciable reactivation of CA 1-Pase. DTT was the most effective reagent, restoring activity to levels up to 10-fold higher than controls lacking reductant. Expressed relative to controls maintained with DTT, this represents a 60% reactivation. With DTT and $\beta$-mercaptoethanol, reactivation of CA 1-Pase was slightly enhanced by extending the preincubation period at 0°C from 1 to 28 h. Further experiments with DTT (not shown) suggest that this effect saturated at 20 mM, and >70% of the total reactivation occurred within the first 10 min of the preincubation treatment. A key question arises as to whether CA 1-Pase is regulated by the thioredoxin system (1). The stimulatory effect of a range of monothiol and diithiol reducing agents on the enzyme (Table I) is consistent with the potential involvement of sulfhydryl reduction in the activation as well as stabilization of CA 1-Pase activity. Additionally, the inhibitory effect on CA 1-P metabolism of interrupting electron flow to the reducing side of PSI (18, 24) suggests a regulatory role for thioredoxin. However, under these conditions, the production of NADPH and the autocatalytic buildup of Calvin cycle intermediates stimulating CA 1-Pase activity would also be curtailed.

During the reverse experiment to those described above, CA 1-Pase previously kept active in the presence of DTT was incubated with 50 mM GSSG or 50 mM sodium tetrathionate. Loss of activity in the presence of these oxidizing reagents was most rapid with sodium tetrathionate, but in each case <10% of the control activity remained after 28 h (Table I). The results indicate that sulfhydryl group oxidation, and hence inactivation of CA 1-Pase, can be accelerated in the presence of these reagents compared to dialysis in air-equilibrated buffer with the simple omission of DTT or other reducing reagent. It remains to be determined whether naturally occurring oxidants such as GSSG cause measurable dark inactivation of CA 1-Pase in vivo.

**Temperature Effects on CA 1-Pase**

The response of CA 1-Pase to temperature is shown in Figure 5. The thermal stability data show a loss of catalytic activity when the enzyme is preincubated for 1 h in buffer A at temperatures $>35^\circ$C. After treatment at 42°C, <20% of the control activity remained. There was little difference in the activity loss profiles of CA 1-Pase preincubated in the presence or absence of a 2 mM concentration of the positive effector, NADPH (data not shown). Within the physiologically relevant temperature range studied (5, 10, 20, and 30°C) the Arrhenius plot (Fig. 5, inset) was linear, and the calculated activation energy for the reaction in the absence of effectors was 56.14 kJ/mol.

The relative temperature stability data of CA 1-Pase and Rubisco activase is of potential interest to experiments seeking to investigate any cooperation between these two enzymes in releasing CA 1-P inhibition of Rubisco following a period of darkness (15). In Figure 5, it can be seen that Rubisco activase from tobacco is prone to inactivation by relatively moderate temperatures and is clearly more thermally labile than CA 1-Pase. After a 1-h incubation at 40°C, 93% of purified activase activity is lost, whereas for CA 1-Pase, the equivalent inactivation is only 43% (Fig. 5). Rubisco activase from spinach exhibits a similar vulnerability to heat inactivation (17). Thus, activase is regarded as a likely component responsible for the heat sensitivity of light-dependent Rubisco activation (17, 30). In plants that accumulate CA 1-P, heat inactivation of activase would also be expected to interfere with the light-dependent release of CA 1-P bound to Rubisco during darkness or decreased irradiance.
CONCLUSIONS

The levels of certain phosphoesters in the chloroplast stroma are likely to facilitate light activation of CA 1-Pase in vivo. The concentrations of RuBP, FBP, and PGA accumulating during photosynthetic induction periods are individually capable of appreciable stimulation of CA 1-Pase activity. Pi is a negative effector of CA 1-Pase activity, and so competition between Pi and the stimulatory effect of the latter three photosynthetic carbon reduction cycle intermediates may occur to produce an overall catalytic rate. The established decrease in stromal Pi during illumination is consistent with a light-dependent increase of CA 1-Pase activity. However, more information about the light and dark levels of free intermediates is needed in plants that accumulate CA 1-P because of possible metabolite sequestration by Rubisco (2, 21). From results presented in Table I, the partial reactivation of DTT-depleted CA 1-Pase by a range of reducing reagents confirms a requirement of the enzyme for stabilization of sulfhydryl groups. Involvement of thioredoxin in the activation of this enzyme cannot be excluded at this time, particularly in view of the apparent inhibitory effect on CA 1-P metabolism associated with DCMU or methyl viologen treatments (18, 24).

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LITERATURE CITED