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Regulation of Chloroplastic Carbonic Anhydrase[1]

EFFECT OF MAGNESIUM

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ABSTRACT

It was previously reported that magnesium ion inhibited carbonic anhydrase (Bamberger and Avron 1975 Plant Physiol 56: 481–485). Studies with partially purified carbonic anhydrase from spinach (*Spinacia oleracea* L.) chloroplasts show that the effect was the result of the chloride counterion and not the magnesium ion. Enzyme activity was reduced 50% upon addition of 3 to 10 millimolar $MgCl_2$ or KCl while all additions of $MgSO_4$ between 0.3 and 10 millimolar were mildly stimulatory.

The role of carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) in plant cells is not well defined and debate continues about the importance of the enzyme (6, 10, 12). The fact that the enzyme interconverts the major forms of inorganic carbon and the fact that many workers locate the bulk of the enzyme in the chloroplast (1, 5, 6, 9, 13) tends to focus discussion on the role of carbonic anhydrase in photosynthesis. While many reports concentrate on the role of supplying CO_2 to carboxylating enzymes (10, 12, 13) in the alkaline medium of the illuminated chloroplast stroma (4, 6, 13), others point to the excess of the enzyme relative to the apparent need for CO_2 during maximal photosynthesis (6, 10). These authors point to a possible role of carbonic anhydrase in maintaining ionic balance within and between chloroplastic compartments by rapidly interconverting CO_2 and HCO_3^- (6, 10).

Disregarding which role is predominant, high rates of carbonic anhydrase activity would favor maximum photosynthesis, thus a previous report (1) that magnesium ion inhibited carbonic anhydrase is contrary to expectations. Magnesium ion concentration is known to increase in the stroma upon transition from dark to light (2, 4) and has been shown to activate a number of reductive pentose phosphate cycle enzymes (4). The inconsistency between the reported inhibition of carbonic anhydrase by magnesium ion and the general stimulatory role of magnesium during carbon fixation (2, 4, 7) is examined in this report.

MATERIALS AND METHODS

Freshly harvested spinach (*Spinacia oleracea* L. cv Vienna) was purchased locally. Approximately 900 g of washed, destemmed leaves were homogenized in 50-g lots with 2 volumes/g of 330 mM sorbitol, 50 mM Tricine-KOH (pH 7.6), 2 mM $MgCl_2$, 2 mM Na_2EDTA in 10-s bursts with a Polytron. After filtration through Miracloth, the chloroplasts were collected by differential centrifugation (8). The resulting pellet was suspended in homogenization buffer without sorbitol. After an incubation period to insure disruption, the suspension was centrifuged for 30 min at 20,000*g* and the membrane pellet was discarded.

The stromal protein was collected by precipitation with 60% saturated ammonium sulfate, dissolved in a minimal volume of 50 mM K-phosphate (pH 7.8), 1 mM dithioerythritol, and eluted from a 3- × 86-cm Sephacryl S-300 column using the same buffer. The UV-absorbance (280 nm) and carbonic anhydrase activity (see below) of the fractions was determined (Fig. 1). The major peak of carbonic anhydrase activity was pooled and stored as a suspension in 60% saturated ammonium sulfate, 30% v/v glycerol at −20°C until use. Specific activity of this preparation was approximately 500 to 600 units (see below) /mg protein (3). Approximately 10% of the stromal protein loaded onto the column was collected in this preparation. The preparation was contaminated by a number of proteins detectable by SDS gel electrophoresis.

Immediately before use, the suspension was dissolved in 10 mM sodium barbital (pH 8.05 with H_2SO_4), 1 mM dithioerythritol and desalted over a short column (1 × 9 cm) of Sephadex G-25 equilibrated with the same buffer. While thiol is reported to be unnecessary for carbonic anhydrase activity of spinach (10, 12), it was found that substantial activity was lost within 2 h in the absence of thiol in the dilute preparation.

The enzyme was assayed by a modification of the Wilbur-Anderson electrometric method (14) using 10 mM sodium barbital as the assay buffer. The enzyme extract and test solution were equilibrated for at least 2 min in 2 ml of assay buffer. The reaction was initiated by the addition of 0.5 ml of CO_2-saturated water (approximately 76 mM at 0°C). The time for the pH to decline from 8.0 to 7.5 was recorded. Boiled enzyme and no enzyme gave identical results. Additions of test solutions were made to both

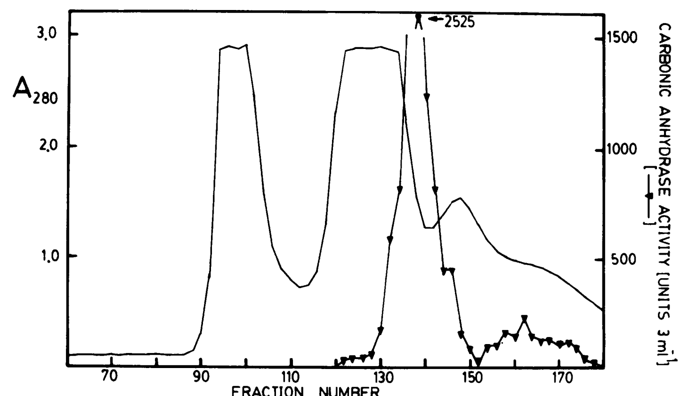

FIG. 1. Elution of chloroplast carbonic anhydrase from S-300. The plain line shows the A_{280} profile of the elution while the line with the triangles (▼) shows the activity profile for carbonic anhydrase. Fractions were 3.0 ml. Enzyme units are defined in the text.

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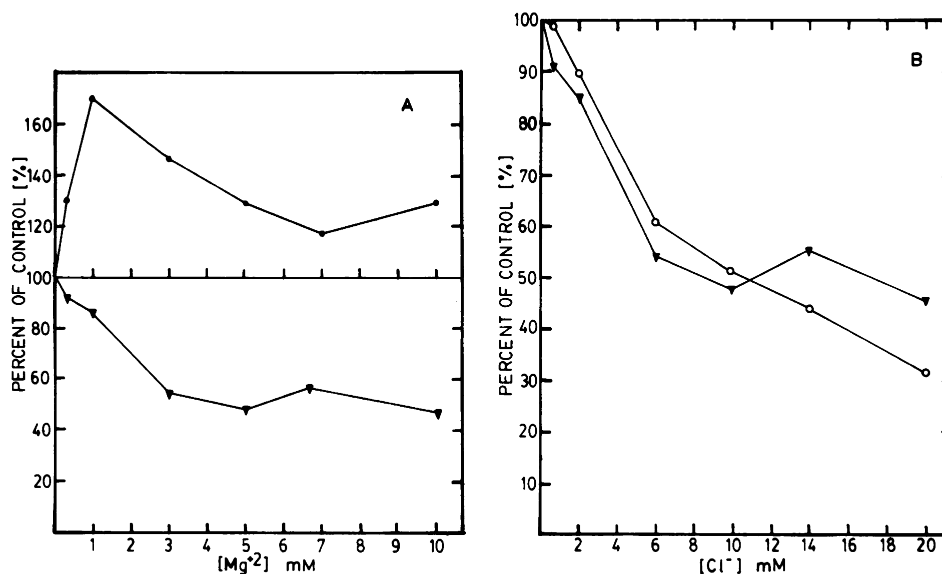

FIG. 2. The effect of magnesium and potassium salts on carbonic anhydrase. The control activity was the measured activity in the absence of any test salts and was approximately 500 units/mg protein (units are defined in the text). Assays routinely contained between 1 and 2 units. A, Effect of varying concentrations of $MgSO_4$ (●) and $MgCl_2$ (▼); B, effect of $MgCl_2$ (▼) and KCL (O).

enzyme and blank assays. Enzyme units were calculated with the formula:

$$\text{enzyme units} = (t_b/t_e - 1)$$

where t_b is the time (in seconds) for the blank pH to change 0.5 pH units and t_e is the comparable time for the enzyme assay.

RESULTS AND DISCUSSION

The alkalinization of the stroma alters the equilibrium between CO_2 and HCO_3^-, favoring the ionic form. It is generally accepted that CO_2, not HCO_3^-, is the substrate for ribulose BisP carboxylase (7), thus the transition of the stroma from dark to light acts to reduce the substrate available for carbon fixation. One major role proposed for carbonic anhydrase in the chloroplast is maintenance of the CO_2 concentration by rapidly converting HCO_3^- to CO_2.

The previous report of magnesium inhibition of lettuce carbonic anhydrase emphasizes the similar response of light-induced CO_2 fixation (by intact chloroplasts) and carbonic anhydrase activity to increasing $MgCl_2$ (1). In addition, $MgSO_4$, between 1 and 10 mM was reported to inhibit carbonic anhydrase by 50%. Both crude spinach extracts (data not shown) and a partially purified chloroplast carbonic anhydrase (Fig. 2A) showed a marked decrease in enzyme activity upon addition of $MgCl_2$ above 1 mM, a result similar to that previously reported. However, addition of $MgSO_4$ up to 10 mM did not inhibit enzyme activity but was mildly stimulatory (Fig. 2A).

Considerable literature has appeared for both plant (5, 10, 12) and animal (11) enzymes showing the inhibition of carbonic anhydrase by small monoanions. Figure 2B clearly shows that, at equivalent chloride concentrations, the response of carbonic anhydrase to KCl and $MgCl_2$ is very similar. Potassium sulfate had no effect on enzyme activity. Taken together, the data in Figure 2, A and B indicate that the inhibition of carbonic anhydrase by $MgCl_2$ was due to Cl^-, not Mg^{2+}.

Regulation of carbonic anhydrase in the chloroplast is uncertain because of ambiguities about the *in vivo* chloride concentration (2). These data suggest that Mg^{2+} fluxes in the stroma do not affect the activity of carbonic anhydrase *in vivo* and the possible compensatory role of carbonic anhydrase in the alkaline stroma in the light.

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