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INTRACELLULAR DISTRIBUTION OF CARBONIC ANHYDRASE IN SPINACH LEAVES

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SUMMARY

Intact spinach chloroplasts, capable of high rates of photosynthetic CO_2 fixation contain 44.3% of the total leaf carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) activity. The intact chloroplasts upon increasingly severe osmotic shock treatment lose progressively larger amounts of carbonic anhydrase activity. This loss parallels the removal of the envelope membranes and soluble material from the stroma. Correction for this leakage from the fraction of damaged chloroplasts present in the intact chloroplast preparations yielded a maximal value of 63.0% of the total leaf carbonic anhydrase activity being found in chloroplasts. This carbonic anhydrase activity was associated with the stroma portion of fractionated chloroplasts, and it exhibited a distribution pattern similar to that of ribulose diphosphate carboxylase, a known stroma enzyme. It is suggested that an association between carbonic anhydrase and ribulose diphosphate carboxylase activities occurs *in vivo*.

INTRODUCTION

The carbonic anhydrase activity in spinach leaves exhibits a distribution pattern similar to that of chloroplast enzymes, since it accompanies the ribulose diphosphate carboxylase activity among various fractions isolated by a non-aqueous technique[1]. This technique, which yields chloroplasts incapable of fixing CO_2 photosynthetically, does not allow a determination of whether part of this activity is associated with the cytoplasmic fraction.

Although the role of carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) in the photosynthetic process still remains to be elucidated, great interest in the potential importance of this subject has resulted in three views regarding its possible function. These have been discussed elsewhere by ZELITCH[2] and by GRAHAM AND REED[3].

At this stage, it seems important to determine quantitatively the amount of carbonic anhydrase activity present in isolated chloroplasts capable of fixing CO_2 at rapid rates. Another point, which could provide evidence about its function, would

be to determine the location of carbonic anhydrase within the chloroplast. Studies concerned with these questions are described in this paper.

MATERIALS AND METHODS

Preparation of chloroplasts

Chloroplasts were prepared from 10.0 g of greenhouse-grown spinach leaves (*Spinacia oleracea* L., var. Viroflay) and assayed for their CO_2 fixation rates by procedures already described[4,5]. When it was desired to damage these intact chloroplasts by removing their envelope membranes, chloroplast preparations were suspended at 4° for 5 min in Solution B[4] which was made hypotonic by decreasing the standard sorbitol concentration from 0.33 M. The membrane-stripped chloroplasts were then centrifuged (4°) at 15 000 × *g* for 5 min and samples of the pellet were taken for the two enzymatic extractions.

Preparation of chloroplast fractions

Intact chloroplasts were homogenized and their envelope membranes were isolated from this homogenate by differential centrifugation following the procedure of MACKENDER AND LEECH[6]. The stroma and lamellar membrane fractions were separated by differential centrifugation from intact chloroplasts that were sonically disrupted for 90 sec. The isolation was based on the procedure of PARK AND PON[7], except that 0.001 M Tris buffer (pH 7.8) was used in place of phosphate buffer.

Chlorophyll content

Chlorophyll was assayed by the method of BRUINSMA[8].

Enzyme extracts

Spinacia oleracea L. (var. Viroflay) was grown in soil in a greenhouse. When 3–4 weeks old, leaves were picked, washed, and ground as quickly as possible. Approx. 3–4 g of leaves were homogenized under N_2 for 15 sec in 20.0 ml of 0.025 M veronal buffer (pH 8.2) containing 0.001 M EDTA and 0.005 M 2-mercaptoethanol (4°). A "semimicro" jacketed (4°) Monel homogenizing vessel (attached to a Waring Blendor) was used. The resulting suspension was ground several times in a Ten Broeck homogenizer, and a sample was removed for chlorophyll determination. The suspension was then centrifuged at 15 000 × *g* (4°) for 5 min and the supernatant fraction was collected and immediately used for carbonic anhydrase activity determinations. This extraction procedure is a modification of the method of EVERSON AND SLACK[1]. Although the extract was green, the color did not interfere with the carbonic anhydrase assay since the extracts were greatly diluted.

Pellets of intact or damaged chloroplasts (half of the pellet obtained from 10.0 g leaves) were suspended under N_2 in a Ten Broeck homogenizer with 10.0 ml of 0.025 M veronal buffer containing 0.001 M EDTA and 0.005 M 2-mercaptoethanol. A sample was removed for the determination of chlorophyll and the remainder was centrifuged at 15 000 × *g* (4°) for 5 min. The resulting supernatant fraction was used for the carbonic anhydrase studies. When investigating ribulose diphosphate carboxylase activity, the chloroplasts were sonically ruptured[7] in Tris buffer (pH 7.8, 0.1 M) and

centrifuged at 15 000 × *g* (4°) for 10 min. The supernatant fraction was collected and assayed.

The envelope and lamellar membranes were ground in a Ten Broeck homogenizer with the buffers used, respectively, in the carbonic anhydrase and ribulose diphosphate carboxylase assays. The stroma fraction (supernatant) was used directly for assays after dilution with the appropriate buffers.

Carbonic anhydrase assay

The method of RICKLI *et al.*[9] was followed. This is based on the rate of change in pH resulting from the hydration of CO_2 observed with bromthymol blue at 0°. Activity units were calculated as 10 $(T_b/T_c - 1)$ where T_b is the time, in sec, of the uncatalyzed reaction and T_c, the time of the enzyme-catalyzed reaction.

Ribulose diphosphate carboxylase assay

The method of PAULSEN AND LANE[10] was utilized, based on the rate of $^{14}CO_2$ incorporation in the presence of ribulose diphosphate at 30°.

Variation of carbonic anhydrase activity with photoperiod

Greenhouse-grown spinach was transferred to a room kept at 16° and maintained under fluorescent lamps (1000 ft-candles). The normal day was 16 h of light and 8 h of darkness. The plants were allowed to adjust for 72 h prior to their experimental use. Samples were taken hourly.

RESULTS

Extraction of the carbonic anhydrase activity under the conditions described gave reproducible results. The buffering capacity was sufficient, and reextraction of the residue removed no more than an additional 5% of the carbonic anhydrase activity. Addition of known amounts of crystalline bovine erythrocyte carbonic

TABLE I

CARBONIC ANHYDRASE ACTIVITY IN SPINACH LEAVES AND CHLOROPLASTS

Each value represents a separate preparation isolated on a different day. The mean value of chloroplast activity at 0° represents over a 100-fold excess in the rate of HCO_3^- formation compared with the rate of CO_2 fixation per mg chlorophyll per h.

Expt. no.	*Carbonic anhydrase activity*	
	Whole leaf (units/mg chlorophyll)	*Chloroplast (% total leaf activity)*
I	4319	42.0
II	3498	59.0
III	4404	40.3
IV	2842	48.1
V	3927	35.2
VI	3960	44.6
VII	4973	40.9
Mean	3989 ± 684	44.3 ± 7.6

TABLE II

ENZYMATIC ACTIVITY OF FRACTIONATED AND DAMAGED CHLOROPLASTS

Values are the mean of two separate experiments. Total intact chloroplast activity of carbonic anhydrase was approximately 1700 units. Total intact chloroplast activity of ribulose diphosphate carboxylase was about 25% of the CO_2 fixation rate observed with the intact chloroplasts. Intact chloroplasts, prepared from greenhouse grown spinach, fix an average of 60 μmoles CO_2 per mg chlorophyll per h.

	Percentage of total intact chloroplast activity	
	Carbonic anhydrase	*Ribulose diphosphate carboxylase*
Chloroplast fractions		
Envelope membranes	N.D.*	N.D.*
Lamellar membranes	3.6	10.8
Stroma	95.4	84.9
*Damaged chloroplasts***		
0.10 M	89.3	84.0
0.05 M	86.1	66.6
0 M	54.4	52.8
H_2O	14.0	40.0

* Not detectable, over 90% of activity recoverable in supernatant fluid and pellet fractions remaining from the membrane isolation process.

** See MATERIALS AND METHODS, molarity values are for sorbitol concentration in Solution B. The concentration used for isolating intact chloroplasts was 0.33 M. H_2O means no Solution B and only distilled H_2O was used.

anhydrase (Worthington) to chloroplast samples were fully recovered. The carbonic anhydrase activity, expressed per mg chlorophyll, of spinach leaves was 3989 units (Table I). This figure is similar to the value of 3800 obtained by EVERSON AND SLACK[1].

Since the total leaf carbonic anhydrase activity is expressed on a chlorophyll concentration basis, and since chlorophyll is located in the chloroplast[11], the enzyme activity found in the chloroplasts can be expressed as a percentage of the total leaf carbonic anhydrase activity. The results of this determination (Table I) show that 44.3% of the total leaf carbonic anhydrase is present in the chloroplast.

Intact chloroplasts, chloroplasts damaged to varying degrees, and chloroplast fractions (*i.e.* the envelope membrane, the lamellar membranes, and the stroma) were each assayed separately for carbonic anhydrase. These results were compared with a marker enzyme, ribulose diphosphate carboxylase, which is entirely located in the stroma[7]. The results are shown in Table II. They indicate that carbonic anhydrase activity is found in the stroma of chloroplasts. Determinations of carbonic anhydrase in extracts of spinach leaves showed that there was no change in the carbonic anhydrase activity during continuous periods of light or darkness extending up to 30 h.

DISCUSSION

Leaf carbonic anhydrase was not believed to be associated with chloroplasts[12] until recently. In 1967 EVERSON AND SLACK[1] observed that up to 67% of the total leaf carbonic anhydrase was associated with chloroplasts isolated by the non-aqueous

method. Since such chloroplast preparations do not fix any CO_2, it seems appropriate to determine the distribution of carbonic anhydrase activity in chloroplasts isolated by a method which results in high rates of CO_2 fixation. ROSSI *et al.*[13] attempted such a study and found up to 20% of the enzyme activity in these chloroplasts. However, their chloroplast preparations were prepared in a medium containing sodium chloride. This failed to maintain the osmotic properties, and resulted in damaged chloroplasts that have lost their envelope membranes and some soluble stroma proteins[4]. Among these could be carbonic anhydrase, and indeed the present evidence (Table II) indicates that osmotically shocked chloroplasts do lose carbonic anhydrase activity.

The data obtained with chloroplasts that were 70–75% intact (envelope membranes present), showed that 44.3% of the total leaf carbonic anhydrase was present. If it is assumed that the remaining 25–30%, the damaged chloroplasts, had lost all of their carbonic anhydrase, this would provide a maximal value of 63% of the total leaf carbonic anhydrase as being chloroplast bound. Table II indicates that up to 86% of the chloroplast carbonic anhydrase was lost to the supernatant fluid in the most severely damaged chloroplasts, hence these values of 44 to 63% support the value of 67% found with non-functional chloroplasts isolated by the non-aqueous method[1]. These results imply that a large fraction of the carbonic anhydrase is present outside of the chloroplast, possibly in the cell cytoplasm.

An interesting parallel is that at least two forms of carbonic anhydrase have been found in man, monkey, horse, and rabbit[14]. Some evidence also exists that there are two forms of carbonic anhydrase in parsley leaves, although the second form may be a dimer[15].

Since carbonic anhydrase is present in the chloroplast, the question arises where within the chloroplast? Fractionation of chloroplasts into envelope membranes, lamellar membranes, and stroma show that carbonic anhydrase activity is localized in the stroma (Table II). Its distribution was similar to that of ribulose diphosphate carboxylase, a known constituent of the stroma[7].

Since free CO_2 is the species fixed by ribulose diphosphate carboxylase[16] and HCO_3^- is a more logical storage form of CO_2 because of its greater solubility[2], it is not surprising, therefore, to find carbonic anhydrase in the stroma. One could conceive of a reaction sequence between the two stroma enzymes whereby the carbonic anhydrase converts the storage form of CO_2, which is HCO_3^-, into the active form, CO_2, which is then carboxylated by the ribulose diphosphate carboxylase. If the two enzymes were in such close association, one might expect parallel losses of their activities as chloroplasts are increasingly damaged and more and more stroma material leaks out. This is confirmed in Table II.

The data do not rule out an alternate possibility. Even though carbonic anhydrase is found in the stroma fraction, it could be present in close proximity to the envelope membrane or even loosely attached to the inner side of the envelope membrane, so that it is extracted during the stroma isolation process. At this site, it could facilitate the transport of CO_2 across the envelope membrane. There is some support for this view, since carbonic anhydrase can increase CO_2 transport across artificial membranes[17,18].

With these two possibilities in mind, it is interesting to convert the present carbonic anhydrase units into absolute concentrations of CO_2 or HCO_3^-. Using the formula expressed by ROUGHTON AND BOOTH[19] and later modified by WAYGOOD[20],

the rate of the enzymatic hydration of CO_2 is equal to:

$$\left(\frac{T_b - T_c}{T_c - 1}\right) k_u[\text{av. } CO_2]$$

where T_b and T_c are the times of the uncatalyzed and catalyzed reactions (T_b = 90 to 100 sec, T_c about 20 sec), k_u is the velocity constant of the uncatalyzed reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$ (k_u = 0.0021 moles·l^{-1}·sec^{-1} and [av. CO_2] equals the average CO_2 concentration during the experiment ([av. CO_2] = 0.0197 moles). From these values, and assuming an average photosynthetic CO_2 fixation rate for plants in normal air of 200 μmoles CO_2 fixed per mg chlorophyll per h, it can be calculated that the carbonic anhydrase activity in chloroplasts (Table II) is capable of hydrating over 100 times the amount of CO_2 fixed by the plant. This rate is based on carbonic anhydrase assays at 0°, so this rate is a conservative estimate of the rate at normal temperatures. Since this hydrated form, H_2CO_3, spontaneously ionizes to $HCO_3^- + H^+$ it would be available in a form suitable for ion transport across the envelope membrane. However, the reversible dehydration rate from HCO_3^- is almost as rapid in the case of parsley leaf carbonic anhydrase[15], hence it could also provide enough CO_2 for fixation by ribulose diphosphate carboxylase.

Carbonic anhydrase appears to be located in the stroma, but its exact function in photosynthesis is still only speculative. Clarification of this point awaits further experimental evidence.

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