Plant Carbonic Anhydrases

I. DISTRIBUTION OF TYPES AMONG SPECIES

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ABSTRACT

On the basis of polyacrylamide gradient gel electrophoresis of leaf extracts from 24 species of higher plants, two main forms of carbonic anhydrase (EC 4.2.1.1) were recognized; the “dicotyledon” type and the “monocotyledon” type. More than one band of enzyme was found on gels from most species, suggesting the possibility of carbonic anhydrase isoenzymes in higher plants.

Carbonic anhydrase (EC 4.2.1.1), the enzyme which catalyzes the reversible hydration of CO2, is widely distributed in animal tissues (12). In erythrocytes it facilitates hydration of metabolic CO2 at the tissue capillaries and subsequent dehydration in the lung or gill, as well as being concerned in transfer or accumulation of H+ or HCO3- in secretory organs (12). In many tissues (12) more than one type of carbonic anhydrase (e.g., isoenzymes type B and C in erythrocytes) has been found. Although all contain zinc and are about the same size (mol wt 30,000) they differ in their distribution in tissues and catalytic activity.

Carbonic anhydrase activity has also been demonstrated in extracts from land plants and algae (3, 5, 7, 22, 23). The enzyme is restricted mainly to green tissues (22) and, although no specific function for the enzyme is known, it has been implicated in photosynthesis (5–9, 23, 24). Tobin (21) has shown that the carbonic anhydrase from parsley differs from the animal enzymes in size (mol wt 180,000) as well as in other properties. The parsley enzyme contained 1 atom of zinc per subunit (mol wt 30,000), but other workers using different species have prepared enzymes which contained no zinc (11, 17).

The possibility that more than one type of carbonic anhydrase occurs in plants has not been previously investigated. The electrophoretic mobility of the enzyme in polyacrylamide gradient gels has been measured from 24 families of flowering plants. Broadly, the results indicate that there are two groups of carbonic anhydrases, one which appears to be restricted to the dicotyledons and another to the mono-

cotyledons. Evidence was also found which suggests that in each group there could be two isoenzymes.

MATERIALS AND METHODS

Plant Material. Young leaf tissue was taken from 21 species (see Table I) growing in the field during the southern hemisphere summer and autumn. In addition, 14- to 21-day-old seedlings of Pisum sativum L., Helianthus annuus L., and Triticum vulgare L. as well as leaves from mature plants of Capsicum annum L., grown in vermiculite or garden soil in a glasshouse, were used. Chlamydomonas reinhardii was grown in liquid medium (18) bubbled with air at 25°C and illuminated with 300 ft-c of white light on a reciprocating shaker.

Extraction and Assay of Carbonic Anhydrase. Generally 1 or 2 g of leaf tissue was homogenized in a mortar and pestle at 1°C with an equal weight of sand and 1 or 2 ml of buffer (0.1 M tris-borate, pH 8.3, containing 1 mM disodium-EDTA and 0.1 M 2-mercaptoethanol) per g of tissue. The extract was filtered through two layers of nylon mesh (60 µm hole size, Nycloth Co., Harris Park, N.S.W. Australia), and the carbonic anhydrase activity of 1 to 50 µl was measured in the colorimetric assay of Wilbur and Anderson (25) as used by Rickli et al. (16). Activity was expressed in enzyme units (7, 16) on a chlorophyll (mg chl) or fresh weight of leaf tissue basis. Chlorophyll was determined in the homogenates by the method of Arnon (1).

Chlamydomonas cultures were harvested and washed by centrifugation. Homogenates were prepared with the same buffer used for leaves by passage through a chilled French press with 703 kg per cm².

Polyacrylamide Gel Electrophoresis. Two different sized samples of 5 to 30 µl of the homogenates used for enzyme assay, containing between 2 and 12 carbonic anhydrase units of enzymic activity, were applied to flat bed gels containing a 4 to 27% gradient of polyacrylamide and subjected to electrophoresis in the Gradiopore apparatus (Townson and Mercer, Sydney, Australia) for 12 hr at 5°C with 75 v and using a pH 8.3 (at 5°C) 0.1 M tris-borate buffer containing 2 mM disodium-EDTA and 10 mM 2-mercaptoethanol. The separation of proteins under these conditions is based predominantly on differences in molecular size and shape. Carbonic anhydrase activity was detected by H⁺ production on the gels in the presence of CO2 using the method described previously (15). Briefly, the cut surface of the cooled (0°C) gel was flooded with bromocresol purple (0.1% w/v) in electrophoresis buffer containing 10 mM dithiothreitol. After 1 min, the surface was blotted free of excess liquid and gassed with pure CO2 through an inverted filter funnel. The gel was immediately frozen in solid CO2 (−70°C) followed by transfer to liquid N2 (−186°C).

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RESULTS

The levels of carbonic anhydrase activity and polyacrylamide gel electrophoretic patterns for 24 species of higher plants from 24 families are shown in Table I. Except for the members of the Amaranthaceae, all the major carbonic anhydrase bands in the dicotyledons migrated 3 to 4 cm, whereas all the monocotyledons showed more mobile enzyme bands, moving 5 to 6 cm into the gel. Other dicotyledons examined (members of the Apocynaceae, Labiateae, Caprifoliaceae, Araliaceae, Amaranthus) showed bands consistent with the general pattern. The only exceptions found were Amaranthus hypnoidus and Atriplex spongiosa. These plants, which have the C4-carboxylic acid pathway of photosynthesis, gave three major bands of enzyme activity (Table I).

DISCUSSION

Polyacrylamide gradient gels can be used to compare proteins on the basis of charge and molecular weight (13). In a previous study (15) bovine erythrocyte carbonic anhydrase, which has a mol wt of 30,000, migrated to about the same position as the bands from the monocotyledon species (Table I). The molecular weight of carbonic anhydrase isolated from the monocotyledon Tradescantia albiflora was estimated by gel filtration to be 42,000 ± 2,000 (2). In contrast the molecular weights of carbonic anhydrases isolated from the dicotyledons Petroselinum crispum (21), Spinacia oleracea (17) and Pismum sativum (2) were 180,000, 140,000, and 188,000 ± 8,000 respectively. The multiple bands of activity found by electrophoresis suggest that plant leaves contain isoenzymes of carbonic anhydrase. In those exceptions in which a single band was found (e.g., H. macrophylla, R. satius, and T. vulgare, Table I), the two enzymes may not have been separated. In primate erythrocytes and in many other animal tissues (e.g., 19, 20) isoenzymes of carbonic anhydrase have been recognized and separated. These isoenzymes which have a similar molecular weight and zinc content differ in isoelectric point, electrophoretic mobility, specific activity, and affinity for substrate (12, 16). In addition, Tashian (19) has found that they exhibit differing electrophoretic mobility between species and in some cases within a species (10, 20). A certain amount of interspecies variation in mobility was also found in the plant enzymes (e.g., compare the major bands of P. crispiium and H. annuus in Fig. 1). A number of tests to confirm that the two bands shown on the gels were not artifacts was carried out. The gel pattern was not altered when the tissue was extracted with pH 8.3 tris-borrate, tris- SO₄, tris-acetate, tris-glycine, or veronal-acetate. When an extract from P. sativum was subjected to electrophoresis with 0.1 M tris-acetate, pH 8.0, 50 mM tris-0.38 M glycine, pH 8.3, 90 mM veronal-acetate, pH 8.3, or 0.1 M tris-borate, pH 9.3, buffer, the same gel pattern was found as that using the usual buffer. 0.1 M tris-borate, pH 8.3. Addition of 2 mM disodium EDTA did not qualitatively alter the gel pattern and replacing 2-mercaptoethanol with dithiothreitol had no effect. In a number of experiments gel slabs were prerun and examined under ultraviolet light (λmax = 366 nm). The zones of carbonic anhydrase activity fluoresced bright yellow against a pink background and were stable for several hours. Photographic records were made using either Polaroid land film (type 107) with a Wratten 74 filter or Kodacolor film with a Wratten 2A filter. The method is very sensitive, detecting less than 0.1 unit of carbonic anhydrase activity. The patterns of bands shown (Table I) were drawn from measurements and photographs (e.g., Fig. 1) of the developed gels.

for 3 hr to remove any residue of the ammonium persulfate used to catalyze gelling, and then the buffer was replaced before the samples of leaf homogenate were applied without a change in enzyme pattern. Thus “multiple banding” of a single protein due to persulfate oxidation (4, 14) seems unlikely. In this respect also extr. cis from T. albiflora and P. sativum which were freshly prepared did not differ from extracts stored at 5°C for up to 2 weeks. Partially purified preparations from these two species were identical with the crude extracts (2). Clear acceptance of the existence of two native carbonic anhydrases in the same tissue and their description as isoenzymes awaits isolation and characterization of the bands found on polyacrylamide gels. It is interesting to note, however, that Everson (6) proposed two sites for carbonic anhydrase in spinach chloroplasts, one being inside the plastid and a second on the plastid surface. He suggested that there is need for catalyzed interconversion of CO2 and HCO3- at both sites during photosynthetic CO2 fixation. There is a possibility that a low molecular weight enzyme also exists in small amounts in dicotyledons. The more mobile (5 to 6 cm) active bands which were found only in some extracts (B. vulgaris, H. annuus, P. edulis, P. sativum in Table I) were however not observed consistently and often did not form definite bands but rather a more diffuse spot (e.g., P. crispiium in Fig. 1). The proportion of this component varied between extracts of a single species suggesting that it might be a breakdown product (although a carbonic anhydrase) from the native enzyme. Also there were variable traces of less mobile activity in extracts from some monocotyledons (e.g., T. albiflora or A. belladonna in Table I). These might have been condensation products of the low molecular weight native enzymes. It is likely in most species examined that carbonic anhydrases greater than about 1% of the total activity would be detected by the sensitive method employed.
Occasionally a trace of enzyme activity was detected at the origin of gels but in *A. hybridus* (Table I), *Atriplex spongiosa* (Atkins and Graham, unpublished result) and to some extent in *C. reinhardii* (Fig. 1) a significant portion of the total activity was located in this position. It seems likely that this enzyme did not enter the gel because it was associated with particulate material in the homogenate. We have found (Atkins and Graham, unpublished results) that if extracts from *Zea*

Table I. *Distribution of Type and Activity of Carbonic Anhydrase among Higher Plant Species*

Enzyme activity is expressed as units (E.U.) per mg chlorophyll (chl) or per g fresh weight (FW) of tissue. The migration of enzyme activity was into 7 cm polyacrylamide gradient gels as in Figure 1.

<table>
<thead>
<tr>
<th>Family</th>
<th>Species</th>
<th>E.U./g FW</th>
<th>E.U./mg Chl</th>
<th>Migration in Gel (cm)</th>
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<tbody>
<tr>
<td>MONOCOTYLEDONS</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ALISMACEAE</td>
<td>Sagittaria graminea Michx (Aerial leaf)</td>
<td>6320</td>
<td>6420</td>
<td></td>
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<tr>
<td>AMARYLLIDACEAE</td>
<td>Amaryllis belladonna L.</td>
<td>800</td>
<td>330</td>
<td></td>
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<tr>
<td>ARACEAE</td>
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<td>3040</td>
<td>3710</td>
<td></td>
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<tr>
<td>CANNACEAE</td>
<td>Canna indica L.</td>
<td>3200</td>
<td>3600</td>
<td></td>
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<tr>
<td>COMMELINACEAE</td>
<td>Tradescantia albiflora Kunth</td>
<td>3300</td>
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<tr>
<td>GRAMINEAE</td>
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<td>1400</td>
<td>980</td>
<td></td>
</tr>
<tr>
<td>IRIDACEAE</td>
<td>Dietes iridioides Sweet</td>
<td>2790</td>
<td>2740</td>
<td></td>
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<tr>
<td>LILIACEAE</td>
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<tr>
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<td>Cymbidium sp.</td>
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<td>4820</td>
<td></td>
</tr>
<tr>
<td>PALMAE</td>
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<td>7120</td>
<td>3790</td>
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<td>9040</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>3220</td>
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<tr>
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<td>5930</td>
<td></td>
</tr>
<tr>
<td>COMPOSITAE</td>
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<td>4000</td>
<td></td>
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<tr>
<td>CONVOLVULACEAE</td>
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<td>8630</td>
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<tr>
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<td>813</td>
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<td>3430</td>
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<td>SAXIFRAGACEAE</td>
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<td>2160</td>
<td>3550</td>
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<tr>
<td>SOLANACEAE</td>
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<td>6240</td>
<td></td>
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<tr>
<td>VERBENACEAE</td>
<td>Lantana camara L.</td>
<td>12000</td>
<td>13850</td>
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</table>
mays or A. hybridus are centrifuged at 20,000g (15 min) and the pellet treated with detergent (0.2% v/v Triton X-100), up to one-third of the carbonic anhydrase activity assayed in the crude extract may be found associated with membranes which sediment at the centrifugal force used. Wistrand and Rao (26) have shown a similar effect in homogenates from human lens and dog kidney tissues treated with Tween 80. While the "detergent-induced" increase in the activity of plant carbonic anhydrase was only modest it might account for part of the difference in activity found between C-4 and C-3 plants (7). For example, in the study by Everson and Slack (7) the crude extracts from both groups of plants were centrifuged and the resulting supernatant used for enzyme assay.

The electrophoretic pattern of carbonic anhydrases from the alga C. reinhardtii (Fig. 1) was quite different from all the higher plants tested. Bowes (3) has in fact noted that some of the properties of the enzymes in extracts from 12 species of marine algae were not the same as those of the parsley enzyme isolated by Tobin (21).

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