Salt Responses of Carboxylation Enzymes from Species Differing in Salt Tolerance

Received for publication April 5, 1971

C. B. OSMOND
Research School of Biological Sciences, Australian National University, Canberra 2601, Australia

H. GREENWAY
Department of Agronomy, Institute of Agriculture, University of Western Australia, Nedlands 6009, Australia

ABSTRACT

This paper reports effects of salts on in vitro activity of phosphoenolpyruvate carboxylase and ribulose-1,5-diphosphate carboxylase, isolated from species differing in salt tolerance. Inhibition of phosphoenolpyruvate carboxylase by the inorganic salts KCl, NaCl, and NaSO₄, depended on the source of the enzyme. Phosphoenolpyruvate carboxylase isolated from leaves of C₃ plants was extremely sensitive to inorganic salts, whereas the enzyme extracted from roots of C₃ plants or from both shoots and roots of C₄ plants was much less sensitive. Ribulose-1,5-diphosphate carboxylase was less salt-sensitive than the phosphoenolpyruvate carboxylases. Differences in salt sensitivity of carboxylases were observed over a wide pH range. The results suggest substantial physical-chemical differences between phosphoenolpyruvate carboxylases functioning in photosynthesis and in CO₂ dark fixation.

Among C₄ species, phosphoenolpyruvate carboxylase from halophytic species was more salt-sensitive than that from a salt-sensitive species. This anomaly, between in vitro response of enzymes and growth response of the plants, is briefly discussed.

MATERIALS AND METHODS

Leaf and root materials were grown, harvested, and extracted as described elsewhere (4, 10). Crude extracts did not interfere with the assay of crystalline ribulose-1,5-diphosphate carboxylase from spinach or partly purified phosphoenolpyruvate carboxylase from maize, used as internal standards. Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was assayed by the coupled spectrophotometric assay with excess malate dehydrogenase or by the incorporation of ¹⁴CO₂ into oxaloacetate. The spectrophotometric assay was carried out in 3 ml, containing Bicine buffer, pH 7.8, 50 mM; MgCl₂, 2 mM; NADH, 83 μM; NaHCO₃, 2 mM; and crystalline malate dehydrogenase, 10 units, and was initiated by addition of phosphoenolpyruvate at 1 mM. The ¹⁴C assay was carried out in 1 ml and contained, in mM: Bicine buffer, pH 7.8, 50; MgCl₂, 2; NaH¹⁴CO₃, 2; and phosphoenolpyruvate, 1. The reaction was stopped by addition of 1 ml of 1 N HCl saturated with 2,4-dinitrophenylhydrazine. This direct assay of oxaloacetate formation overcomes possible complications due to the salt sensitivity of malate dehydrogenase (12).

Ribulose-1,5-diphosphate carboxylase (EC 4.1.1.38) was assayed by the incorporation of ¹⁴C into acid-stable compounds. The assay was carried out in 0.6 ml and contained, in mM: Bicine, pH 7.8, 50; MgCl₂, 2.0; NaH¹⁴CO₃, 20; and ribulose-1,5-diphosphate, 0.2. The reaction was stopped by addition of 0.2 ml 4 N HCl. Aliquots of the ¹⁴C assays were evaporated, taken up in water, and then counted in dioxane scintillant.

In early experiments Bicine concentrations were kept at 10 mM, but later it was shown that 50 mM Bicine did not interfere with the response of enzymes to salts.

All assays were run at 30 C. Blanks contained all components except substrate. The various salts and mannitol were added to the buffer solutions prior to pH adjustment. Leaves of C₃ plants are a rich source of phosphoenolpyruvate carboxylase, and assays thus contained lower protein concentrations than those for other tissues. However, addition of inert protein (bovine serum albumin) did not modify the salt sensitivity of phosphoenolpyruvate carboxylase from different sources.

Leaves were exposed to ¹⁴CO₂ for 5 sec, and the products of fixation were analyzed as described elsewhere (10).

RESULTS

Figure 1 shows the activity of phosphoenolpyruvate carboxylase in extracts of roots and shoots of some plant species, as a function of NaCl concentration during assay. Extracts from leaves of C₃ plants such as Atriplex spongiosa and Zea mays showed much greater NaCl inhibition of this enzyme...
than extracts from roots of *Z. mays* and from leaves of the
*C₃* plants *Atriplex hastata* and *Phaseolus vulgaris* (Figs. 1 and 3). Other experiments showed that phosphoenolpyruvate carboxylase from leaves of several *C₃* species behaved similarly to that extracted from *A. spongiosa* and *Z. mays*.

Increased phosphoenolpyruvate concentrations decreased the NaCl inhibition of phosphoenolpyruvate carboxylase, and double reciprocal plots of rate against substrate concentration at different NaCl levels suggest a competitive type interaction between NaCl and phosphoenolpyruvate (for effects of 50 mM NaCl see Fig. 2).

One effect of high salt concentration on many enzymes is to displace the pH optimum, leading to an apparent stimulation or inhibition of activity under standard assay conditions (2). Figure 3 shows that pH shifts cannot account for the lowered activity of phosphoenolpyruvate carboxylases shown in Figure 1. Over a wide pH range, NaCl dramatically inhibits this enzyme isolated from leaves of *C₃* plants but causes only small shifts in the pH optimum. Thus, phosphoenolpyruvate carboxylase from the leaves of *C₃* plants is very much more sensitive than the enzyme from leaves and roots of *C₄* plants and from roots of *C₃* plants (Fig. 3), confirming the data presented in Figure 1.

There are smaller, but noteworthy, differences between phosphoenolpyruvate carboxylases from different *C₃* species. The enzyme from corn leaves is less NaCl sensitive than that

![Graph](image1)

**Fig. 1.** NaCl spectrum for phosphoenolpyruvate carboxylase obtained from various tissues of plants differing in salt tolerance. Spectrophotometric assay. Leaves of *A. spongiosa* (○), a *C₄* Atriplex species; leaves of *A. hastata* (●), a *C₃* Atriplex species; *Z. mays* shoots (●); *Z. mays* roots (●).

![Graph](image2)

**Fig. 2.** Double reciprocal plot showing interaction between NaCl and phosphoenolpyruvate concentration during phosphoenolpyruvate carboxylase assay (spectrophotometric) in extracts of *A. spongiosa* leaves. Control assay (○) and 50 mM NaCl (●).

![Graph](image3)

**Fig. 3.** pH response curve for phosphoenolpyruvate carboxylase from various tissues of plants differing in salt tolerance, at different NaCl concentrations. Control, 0 NaCl (○); 50 mM NaCl (●); 100 mM NaCl (○); 150 mM NaCl (●).

![Table](image4)

**Table I. Effect of NaCl Concentrations on the Activity of Phosphoenolpyruvate Carboxylase Isolated from Z. mays and *A. spongiosa***

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>Shoots (pH 8.0)</th>
<th>Roots (pH 7.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. spongiosa</em></td>
<td><em>Z. mays</em></td>
</tr>
<tr>
<td>50</td>
<td>29</td>
<td>62</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>39</td>
</tr>
</tbody>
</table>

from *A. spongiosa* leaves, and the same species difference holds for the roots (Table I). Even so, within the *C₃* species there remain prominent differences in NaCl sensitivity of phosphoenolpyruvate carboxylase from leaves and from roots. Ribulose-1,5-diphosphate carboxylase activity in extracts from leaves of all species was very much less sensitive to NaCl than was phosphoenolpyruvate carboxylase from leaves of *C₄* plants (cf. Fig. 4 with Figs. 1, 3, and 5). Ribulose-1,5-diphosphate carboxylase from *Z. mays* was inhibited considerably more by NaCl than the same enzyme from *P. vulgaris* (Fig. 4).
The above effects were not due to lower water potentials in assay systems with high levels of salts, for very high mannitol concentrations had little effect on phosphoenolpyruvate carboxylase and ribulose-1,5-diphosphate carboxylase activity (Fig. 5).

The response of phosphoenolpyruvate carboxylase to KCl and NaCl was essentially the same (Fig. 5), but equivalent concentrations of NaSO4 caused slightly less inhibition. Thus, the data obtained in the present experiments showed little specificity in effects of inorganic salts.

Seedlings were grown in saline cultures (A. spongiosa to 400 mM NaCl, Z. mays to 100 mM NaCl, and P. vulgaris to 50 mM) without significant effects of salt on the specific activity (Table II) or the NaCl sensitivity of phosphoenolpyruvate carboxylases. Short term photosynthesis in 14CO2 failed to reveal any effect of salinity treatment on the distribution of 14C among the products in Atriplex and Z. mays (Table III). There was no indication that salinity treatment decreased the proportion of 14C incorporated into C1 acids.

DISCUSSION

The phosphoenolpyruvate carboxylases isolated from different species and tissues appear to vary in physical properties, as indicated by pH optima and response to NaCl. A clear example of this difference is the much higher salt sensitivity of phosphoenolpyruvate carboxylase isolated from the leaves of C3 plants, compared with the same enzyme isolated from roots of C4 plants or leaves and roots of C4 plants. The extract of C4 leaves would contain principally the photosynthetic carboxylase associated with mesophyll cells (5), but it may also contain another enzyme responsible for dark CO2 fixation in these leaves. It is questionable whether the same isoenzyme of phosphoenolpyruvate carboxylase is involved in both processes. It would be interesting to determine the salt sensitivity of phosphoenolpyruvate carboxylase isoenzymes, particularly because isoenzymes of malate dehydrogenase show little difference in response to NaCl (13).

Whether salt inhibition of carboxylase activity is a significant process in vivo depends on the ionic activity in different cell compartments. The subsequent discussion will be confined to Cl- because this is the principal inorganic anion found in tissues of plants grown in natural, saline habitats. Data obtained by nonaqueous separation of chloroplasts are available only for C3 and not for C4 plants. For higher plants these techniques give estimates of 60 to 500 mM Cl- in the plastids (6). These values may be up to 10 times higher than those in the

Table II. The Effect of NaCl during Growth in Culture Solutions on the Specific Activity of Phosphoenolpyruvate Carboxylase

<table>
<thead>
<tr>
<th>NaCl (meq/liter)</th>
<th>Specific Activity</th>
<th>A. spongiosa</th>
<th>P. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>310</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>291</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>232</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>229</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>250</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 In this treatment several other enzymes, such as malate dehydrogenase, glucose 6-phosphate dehydrogenase, and isocitrate dehydrogenase, also increased in specific activity.

Table III. The Effects of Salinity on the Products of 5-sec Photosynthetic 14CO2 Fixation in Leaves of C4 Plants

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Distribution of 14C</th>
<th>C acids</th>
<th>Sugar phosphates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z. mays1</td>
<td>Control</td>
<td>87</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
<td>84</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Atriplex nummularia2</td>
<td>Control</td>
<td>70</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400 mM NaCl</td>
<td>73</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>700 mM NaCl</td>
<td>73</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

1 Grown 4 weeks on cultures containing high NaCl. Activity of ribulose-1,5-diphosphate carboxylase and phosphoenolpyruvate carboxylase was unaltered by salinity treatment.

2 Grown 23 weeks on cultures high in NaCl.
cytoplasm and two to three times higher than vacuolar ion concentrations (6). Approximate balance of cations and anions suggests that ionic activity is also close to these values. It may be significant that, under optimal conditions of substrate and pH, C₃ plants have 5- to 10-fold more phosphoenolpyruvate carboxylase activity than is required to support observed rates of CO₂ fixation. Similarly, C₄ plants have 2- to 3-fold more ribulose-1,5-diphosphate carboxylase than is required to support observed rates of CO₂ fixation. Thus, the activity of these enzymes is such that each could function adequately in chloroplasts containing high levels of inorganic anions.

It was surprising that the phosphoenolpyruvate carboxylase most sensitive to NaCl was extracted from salt-tolerant *Atriplex*. This apparent paradox may be resolved in at least three ways.

1) The activity of the carboxylases may not be limiting under any of the salt conditions encountered in vivo, due to a combination of high enzyme activity and high substrate levels.

2) Salt-tolerant species may regulate the ion concentration within cytoplasm and organelles by means of salt glands (7). The epidermal bladders of *Atriplex* may function in this way (9), and studies with *Limonium* suggest that gland activity may regulate chloroplast ion concentration (7).

3) The PEP-carboxylase of mesophyll cells in C₃ plants is loosely associated with the chloroplast (1, 11). It may be located in the peripheral reticulum, an elaboration of the inner membrane of the chloroplast envelope, or it may be a cytoplasmic enzyme (8). This carboxylase may thus be exposed to the lower cytoplasmic salt levels (6) and exhibit correspondingly greater sensitivity to salts than chloroplast enzymes. However, it is markedly more salt sensitive than the enzyme from maize roots, which is presumably a cytoplasmic enzyme.

The preparation of chloroplasts from the mesophyll cells of C₃ plants which retain the ability to form C₃ acids has proved unusually difficult. When techniques are available it will be of interest to examine the above alternatives in isolated chloroplasts.

Acknowledgments—This work was supported by grants to H. Greenway from the Soil Fertility Research Fund, the Nuffield Foundation, the University Research Grant Commission of the University of Western Australia, and the Australian Research Grant Commission. The skilful assistance of Mrs. A. Treffry and Misses B. Harris and M. Leach is gratefully acknowledged.

LITERATURE CITED