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Salt Responses of Carboxylation Enzymes from Species Differing in Salt Tolerance

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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 Na_4SO_4 depended on the source of the enzyme. Phosphoenolpyruvate carboxylase isolated from leaves of C_4 plants was extremely sensitive to inorganic salts, whereas the enzyme extracted from roots of C_4 plants or from both shoots and roots of C_3 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 CO_2 dark fixation.

Among C_4 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.

The activity of many enzymes is affected by addition of salts during assay (2). Some enzymes show marked inhibition by inorganic ion concentrations above 100 mM (4). Concentrations higher than 100 mM are frequently encountered in plant tissues during growth in saline environments (3). It has been proposed that salt-tolerant species are better able to regulate the cytoplasmic ionic status and thus avoid interactions between enzyme and salt, which may be responsible for salinity damage in other species (4). Recent studies suggest that some cytoplasmic compartments have higher ion concentrations than others (6). Chloroplasts of algal and higher plant cells contain high levels of salt (6, 7). Thus it was of interest to assess the salt sensitivity of some chloroplast enzymes.

This paper reports a comparative study of the two photosynthetic CO_2-fixing enzymes, phosphoenolpyruvate carboxylase and ribulose-1,5-diphosphate carboxylase (5). The data show that ribulose-1,5-diphosphate carboxylase was much less sensitive to salts than the phosphoenolpyruvate carboxylase and that the form of the latter enzyme isolated from green tissues of C_4 plants was more salt sensitive than the enzyme involved in dark CO_2 fixation.

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 $^{14}CO_2$ into oxaloacetate. The spectrophotometric assay was carried out in 3 ml, containing Bicine buffer, pH 7.8, 50 mM; $MgCl_2$, 2 mM; NADH, 83 μM; $NaHCO_3$, 2 mM; and crystalline malate dehydrogenase, 10 units, and was initiated by addition of phosphoenolpyruvate at 1 mM. The ^{14}C assay was carried out in 1 ml and contained, in mM: Bicine buffer, pH 7.8, 50; $MgCl_2$, 2; $NaH^{14}CO_3$, 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 ^{14}C into acid-stable compounds. The assay was carried out in 0.6 ml and contained, in mM: Bicine, pH 7.8, 50; $MgCl_2$, 2.0; $NaH^{14}CO_3$, 20; and ribulose-1,5-diphosphate, 0.2. The reaction was stopped by addition of 0.2 ml 4 N HCl. Aliquots of the ^{14}C 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 C_4 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 $^{14}CO_2$ 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 C_4 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 C_3 plants *Atriplex hastata* and *Phaseolus vulgaris* (Figs. 1 and 3). Other experiments showed that phosphoenolpyruvate carboxylase from leaves of several C_4 species behaved similarly to that extracted from leaves of *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 C_4 plants but causes only small shifts in the pH optimum. Thus, phosphoenolpyruvate carboxylase from the leaves of C_4 plants is very much more sensitive than the enzyme from leaves and roots of C_3 plants and from roots of C_4 plants (Fig. 3), confirming the data presented in Figure 1.

There are smaller, but noteworthy, differences between phosphoenolpyruvate carboxylases from different C_4 species. The enzyme from corn leaves is less NaCl sensitive than that

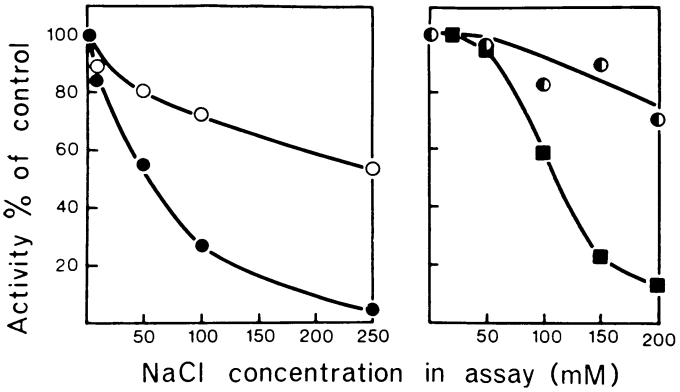

FIG. 1. NaCl spectrum for phosphoenolpyruvate carboxylase obtained from various tissues of plants differing in salt tolerance. Spectrophotometric assay. Leaves of *A. spongiosa* (●), a C_4 *Atriplex* species; leaves of *Atriplex hastata* (○), a C_3 *Atriplex* species; *Z. mays* shoots (■); *Z. mays* roots (◐).

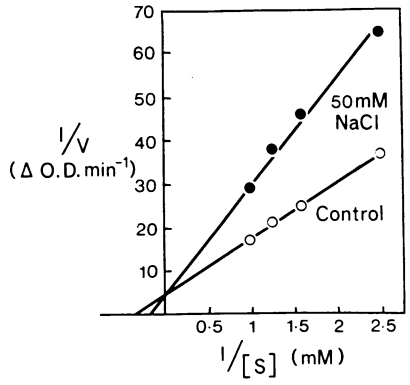

FIG. 2. Double reciprocal plot showing interaction between NaCl and phosphoenolpyruvate concentration during phosphoenolpyruvate carboxylase assay (spectrophotometric) in extracts of *A. spongiosa* leaves. Control assay (○) and 50 mM NaCl (●).

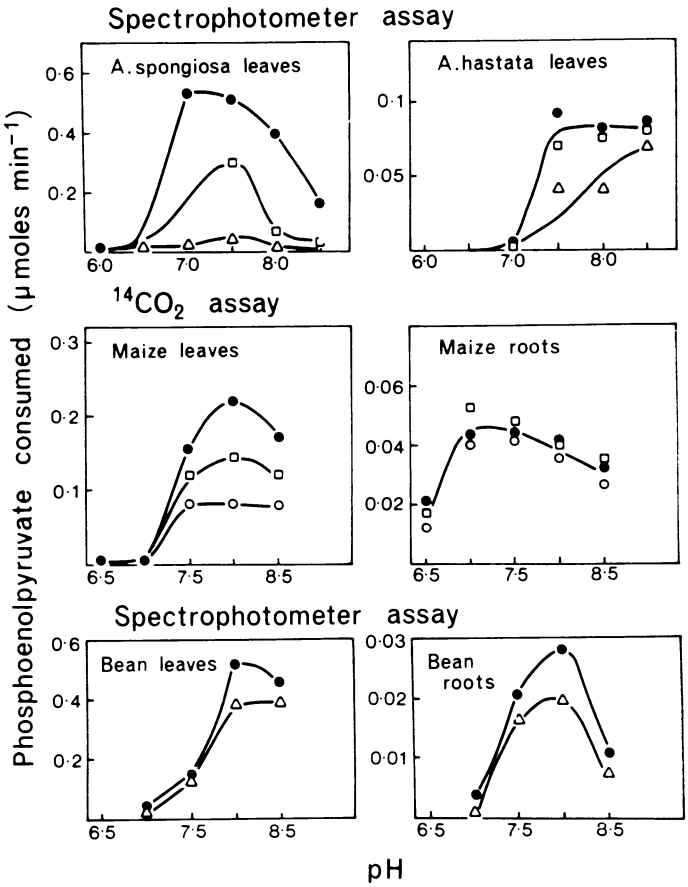

FIG. 3. pH response curve for phosphoenopyruvate carboxylase from various tissues of plants differing in salt tolerance, at different NaCl concentrations. Control, 0 NaCl (●); 50 mM NaCl (□); 100 mM NaCl (○); 150 mM NaCl (△).

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

$^{14}CO_2$ assay; activity expressed as percentage of rates at 0 NaCl concentration. Results are reported for pH giving optimal activity; differences between *Z. mays* and *A. spongiosa* were confirmed over a wide pH range.

NaCl	Shoots (pH 8.0)		Roots (pH 7.5)	
	A. spongiosa	*Z. mays*	*A. spongiosa*	*Z. mays*
mM	%			
50	29	62	61	96
100	15	39	35	81

from *A. spongiosa* leaves, and the same species difference holds for the roots (Table I). Even so, within the C_4 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 C_4 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).

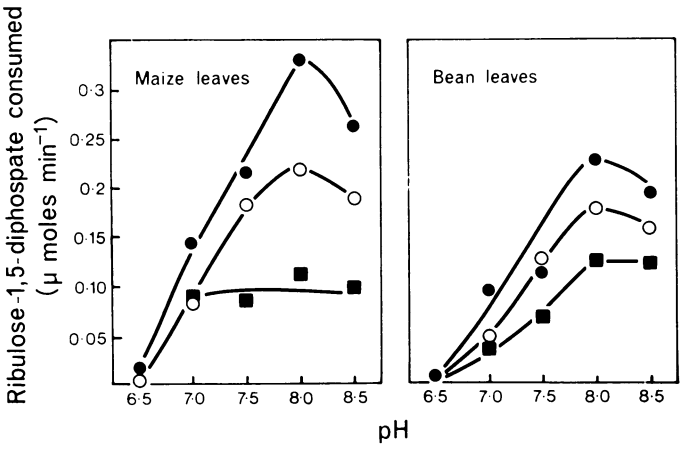

FIG. 4. pH response curve for ribulose-1,5-diphosphate carboxylase from leaves of *Z. mays* and *P. vulgaris* at different concentrations of NaCl. Control, 0 NaCl (●); 250 mM NaCl (○); and 400 mM NaCl (■).

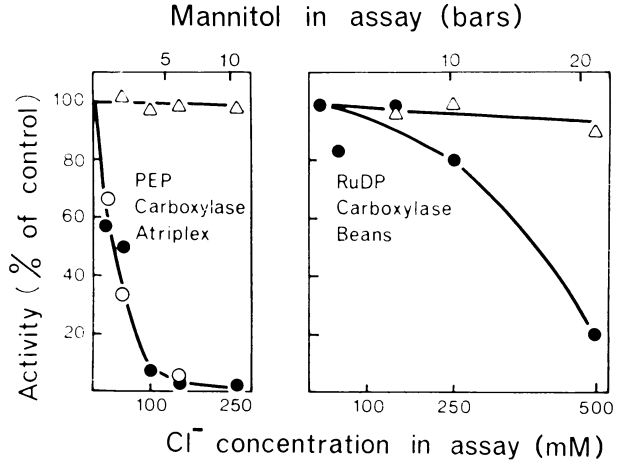

FIG. 5. Comparison of isosmotic concentrations of NaCl (●), KCl (○), and mannitol (△) on the activity of phosphoenolpyruvate carboxylase from *A. spongiosa* leaves and ribulose-1,5-diphosphate carboxylase from *P. vulgaris* leaves.

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

Specific activity is in μmoles substrate consumed/min·mg protein. *A. spongiosa* had been grown for 10 days in cultures containing high NaCl, and *P. vulgaris* had been grown for 6 days.

NaCl	Specific Activity	
	A. spongiosa	*P. vulgaris*
meq/liter		
1	310	13.6
5	291	
50		14.2
100	232	
200[1]		24.5
250	229	

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

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 Na_2SO_4 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 $^{14}CO_2$ failed to reveal any effect of salinity treatment on the distribution of ^{14}C among the products in *Atriplex* and *Z. mays* (Table III). There was no indication that salinity treatment decreased the proportion of ^{14}C incorporated into C_4 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 C_4 plants, compared with the same enzyme isolated from roots of C_4 plants or leaves and roots of C_3 plants. The extract of C_4 leaves would contain principally the photosynthetic carboxylase associated with mesophyll cells (5), but it may also contain another enzyme responsible for dark CO_2 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 C_3 and not for C_4 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 III. *The Effects of Salinity on the Products of 5-sec Photosynthetic $^{14}CO_2$ Fixation in Leaves of C_4 Plants*

Species	Treatment	Distribution of ^{14}C	
		C_4 acids	Sugar phosphates
		%	
Z. mays[1]	Control	87	6
	100 mM NaCl	84	8
Atriplex nummularia[2]	Control	70	20
	400 mM NaCl	73	7
	700 mM NaCl	73	19

[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, C_4 plants have 5- to 10-fold more phosphoenolpyruvate carboxylase activity than is required to support observed rates of CO_2 fixation. Similarly, C_3 plants have 2- to 3-fold more ribulose-1,5-diphosphate carboxylase than is required to support observed rates of CO_2 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 C_4 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 C_4 plants which retain the ability to form C_4 acids has proved unusually difficult. When techniques are available it will be of interest to examine the above alternatives in isolated chloroplasts.

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