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Differences in cold inactivation of phosphoenolpyruvate carboxylase among C_4 species: The effect of pH and of enzyme concentration

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Abstract

Among various C_4 plants we found a wide range in the level of inactivation of phosphoenolpyruvate carboxylase (PEPC) at low temperature (0 °C). The activity of the 2-fold diluted enzyme in crude leaf extracts after 60 min incubation (compared to zero time incubation) at pH 7.5, remained above 87 % at low temperatures for the species *Setaria verticillata*, *Portulaca oleracea,* and *Saccharum officinarum*, and between 11 and 17 % in the species *Cynodon dactylon* and *Atriplex halimus*. The enzyme exhibited intermediate levels of inactivation (42 to 58 %) for the species *Amaranthus* sp., *Zea mays*, *Salsola kali,* and *Digitaria sanguinalis*. The enzyme activity for *S. verticillata* was unaffected between pH 5.7 and 8.4 during incubation at room and low temperatures. Under similar conditions, the activity of the enzyme from *C. dactylon* was stable between pH 5.7 and 7.0 and decreased at pH above 7.0, but for *Z. mays* it was enhanced between pH 5.7 and 6.8 and decreased at pH above 7.0.

Additional key words: *Amaranthus* sp.; *Atriplex halimus*; *Cynodon dactylon*; *Digitaria sanguinalis*; *Portulaca oleracea*; *Saccharum officinarum*; *Salsola kali*; *Setaria verticillata*; *Zea mays*.

Introduction

PEPC (EC 4.1.1.31) primarily fixes carbon dioxide in plants that utilize C_4 and Crassulacean Acid Metabolism (CAM) pathways. This enzyme catalyzes the β-carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate, and is a homotetramer with a subunit of molecular mass of 100 kDa (Uedan and Sugiyama

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1976, Andreo *et al.* 1987, Stiborová 1988). Its activity is regulated by irradiation (Karabourniotis *et al.* 1983, Huber and Sugiyama 1986, Sailaja and Das 1995) through reversible phosphorylation (Jiao and Chollet 1988, Jiao *et al.* 1991). Also, PEPC activity in dilute solutions depends on its oligomerization state (Andreo *et al.* 1987, Podestá *et al.* 1990) which is affected *in vitro* by several metabolites, temperature, pH, and ionic strength (Wong and Davies 1973, Miziorko *et al.* 1974, Gonzalez *et al.* 1984, Andreo *et al.* 1987, Wagner *et al.* 1987, Shi *et al.* 1988, Stiborová 1988, Meyer *et al.* 1989).

PEPC from several plant sources is reversibly cold-inactivated (Hatch and Oliver 1978, Shi *et al.* 1981, Angelopoulos *et al.* 1988, Kleczkowski and Edwards 1990, 1991, Krall and Edwards 1993). The enzyme from *C. dactylon* is also progressively inactivated at alkaline pH, but it is partially reactivated during transition to neutral pH (Angelopoulos and Gavalas 1988). There are differences in cold stability of PEPC between two species of *Panicum* (Hatch and Oliver 1978, Krall and Edwards 1993) and between two ecotypes of *Echinochloa crus-galli* (Simon 1987).

The subject of this work is to give some insight to the mechanism of cold-inactivation of PEPC. To do that we studied the varying degrees of this phenomenon in nine C_4 plants as a function of the enzyme concentration. Based on this study, we chose three representative plants, a cold tolerant (*S. verticillata*), a cold sensitive (*C. dactylon*), and one with intermediate cold sensitivity (*Z. mays*), in order to test whether pH (5.5 to 8.5) has an additional effect on PEPC cold inactivation.

Materials and methods

Mature leaves were used from the perennial shrub *A. halimus* L. grown in NW Peloponnesus, from the annual C_4 plants *Amaranthus* sp., *C. dactylon* (L.) Pers., *S. verticillata* (L.) Beauv., and *Z. mays* L., that were grown in soil pots in a greenhouse, and from *P. oleracea* L., *D. sanguinalis* L., *S. officinarum* L., and *S. kali* L., that were grown in the fields.

The enzyme was extracted at different pH as follows: Leaf tissue (0.2 or 0.5 g) was mixed with 5 cm^3 of extraction buffers of 100 mM MES-KOH (for pH 5.8-6.7), 100 mM HEPES-KOH (for pH 6.9-8.1), and 100 mM Tricine-HCl (for pH above 8.1). All buffers were made in 1 mM EDTA, 5 mM $MgCl_2$, 5 mM 1,4-dithio-DL-threitol (DTT), and 3 % m/v polyvinyl pyrrolidone (PVP). The samples were mixed with 100 mg of solid PVP and ground in a mortar with purified sea sand. The mixture was centrifuged for 10 min at 15 000×*g*. The supernatant (crude extract), undiluted or diluted 2-fold with the corresponding extraction buffers, was used as source of PEPC. Desalted samples (through a 20×1 cm *Sephadex* G-25 column equilibrated with the corresponding extraction buffers) were tested as well, and gave results similar to the crude extracts (values not shown). All extraction operations were performed at room temperature. All incubations, as shown in figures and table, were for 60 min at 0 and 23 °C. We tested the activities of undiluted extracts *versus* time, and we found them to be relatively stable (with no more than ±10 % variation) for 4 h. This activity was used as "zero time" activity. For the 2-fold diluted samples, "zero

time" is defined as the starting incubation time after the dilution was made. The pH of the extraction/incubation buffers did not change at the low temperatures that were used for studying cold inactivation. Additional experimental conditions are described in the table and in figure legends.

The assay buffer used for determining PEPC activity consisted of 100 mM HEPES-KOH, pH 7.2, 1 mM $NaHCO_3$, 10 mM $MgCl_2$, 2.4 mM PEP, 0.14 mM NADH, and 5 units of malic dehydrogenase. The final assay volume was 1.5 cm^3, and the preincubated enzyme volume was up to 100 mm^3. The reaction was started with the addition of the enzyme to the assay buffer at 30 °C, and its progress was monitored by the absorbance decrease at 340 nm (NADH oxidation). The protein concentration was determined by the Folin phenol reagent (Lowry *et al.* 1951).

Results

The differences in extent of cold-inactivation of PEPC among C_4 species were studied in relation to undiluted extracts and in extracts diluted 1 : 2 with the HEPES-KOH extraction/incubation buffer, pH 7.5 (Table 1). When PEPC from all tested

Table 1. Influence of cold treatment on the activity of PEPC [unit m^{-3}] from various C_4 species. Crude extracts (undiluted or diluted 1 : 2) from each species were incubated (in 100 mM HEPES-KOH buffer, pH 7.5, containing 1 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT, 3 % m/v PVP) for 60 min at 0° and 23 °C, and the % residual activity was determined. For all incubations the "zero time" activity (see Materials and methods) is defined as 100 %. One unit of enzyme activity corresponds to 1 mmol CO_2 converted per s at 30 °C. The values given are means ± S.E. from three replications.

Species	PEPC activity	Protein [kg m^{-3}]	Dilution	% residual activity at 0 °C	23 °C
Cynodon dactylon	5.1 ± 0.3	0.52 ± 0.03		35 ± 2	97 ± 5
			1 : 2	17 ± 1	92 ± 5
Zea mays	4.9 ± 0.2	0.79 ± 0.04		66 ± 3	96 ± 5
			1 : 2	44 ± 2	90 ± 5
Setaria verticillata	5.6 ± 0.3	1.96 ± 0.05		107 ± 5	110 ± 6
			1 : 2	101 ± 5	103 ± 5
Digitaria sanguinalis	5.8 ± 0.3	0.89 ± 0.04		76 ± 4	104 ± 5
			1 : 2	58 ± 3	101 ± 5
Saccharum officinarum	6.5 ± 0.3	0.58 ± 0.03		91 ± 5	93 ± 5
			1 : 2	87 ± 4	86 ± 4
Salsola kali	5.5 ± 0.3	1.13 ± 0.06		69 ± 3	89 ± 4
			1 : 2	42 ± 2	85 ± 4
Amaranthus sp.	8.8 ± 0.4	1.32 ± 0.07		68 ± 3	106 ± 5
			1 : 2	55 ± 3	103 ± 5
Portulaca oleracea	6.2 ± 0.3	0.79 ± 0.04		92 ± 5	96 ± 5
			1 : 2	88 ± 4	92 ± 4
Atriplex halimus	6.3 ± 0.3	0.92 ± 0.05		18 ± 1	92 ± 4
			1 : 2	11 ± 1	80 ± 4

species was incubated at 23 °C for 60 min, there was little or no loss of PEPC activity in the undiluted and diluted crude extracts compared to zero time incubation. The activity of PEPC from *S. verticillata*, *S. officinarum*, and *P. oleracea* remained stable during incubation at 0 °C, *i.e.*, over 87 % of the initial activity. In contrast, the enzyme from *C. dactylon* and *A. halimus* was strongly inactivated at 0 °C, and the extent of cold inactivation of the undiluted and diluted enzyme extracts was between 11 and 35 %. The residual activity of the cold-inactivated PEPC from the remaining C_4 plants varied between 42 and 58 % in the diluted crude extracts. With the exception of *S. verticillata*, *S. officinarum*, and *P. oleracea*, the inactivating effect of cold treatment on PEPC was more profound in the diluted crude extracts from the other plants. The differences between the cold sensitive and insensitive plants can not be attributed to any *ab initio* differences of their enzyme concentrations, since, as Table 1 shows, the PEPC V_{max} activities of their undiluted extracts were similar.

The effect of pH on cold-inactivation of PEPC was studied in the C_4 species *S. verticillata*, *C. dactylon*, and *Z. mays*: During incubation at 0 and 23 °C for 60 min, activity of the enzyme from *S. verticillata* (Fig. 1*A,B*) remained stable between pH 5.8 and 8.4. It also remained stable at nonphysiological pH values up to 9.5 (values not shown). PEPC from *C. dactylon* (Fig. 1*C,D*) and *Z. mays* (Fig. 1*E,F*) gradually lost its activity at 23 °C as the alkalinity of the incubation medium increased. The PEPC activity of *Z. mays* at 0 °C tended to increase between pH 6.0 and 7.0, while the enzyme activity of *C. dactylon* remained constant over the same pH intervals. For pH greater than 7, the rate and the extent of PEPC inactivation in both plants was higher at 0 than at 23 °C.

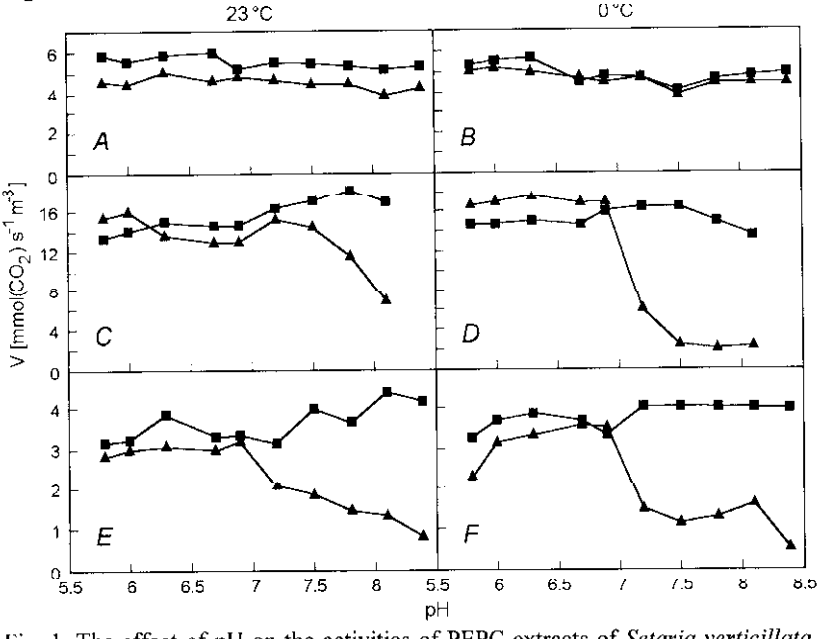

Fig. 1. The effect of pH on the activities of PEPC extracts of *Setaria verticillata* (*A, B*), *Cynodon dactylon* (*C, D*), and *Zea mays* (*E, F*) incubated at 23 (*A, C, E*) or 0 (*B, D, F*) °C. Activities after incubation time 0 (■) or 60 (▲) min.

Discussion

This study of PEPC cold inactivation in undiluted or 2-fold diluted crude extracts from different C_4 plants clearly shows that there are extreme differences in respect to the extent of cold inactivation between C_4 species, possibly due to diversity between the plant species (Table 1). Differences in cold inactivation of PEPC have also been observed among species of the same genus (Krall and Edwards 1993) or even between different ecotypic populations of the same species (Simon and Hatch 1994).

There was no substantial effect of pH on the activity of cold-treated or room temperature-incubated PEPC from *S. verticillata*, a representative of the tested cold tolerant plant species (Fig. 1*A,B*). Nevertheless, there was more profound effect of alkaline pH (above 7.0) on cold-treated than on room temperature-incubated PEPC extracts from the cold sensitive species *C. dactylon* and *Z. mays* (Fig. 1*C-F*). According to Wu *et al.* (1990), pH affects the structure and the catalytic properties of PEPC from C_4 and CAM plants. The enzyme looses gradually its activity at alkaline pH and at room temperature, and it regains part of or all activity when the pH becomes neutral or mildly acidic (Angelopoulos and Gavalas 1988). These activity changes may relate to changes in the oligomeric state of the enzyme. Alkaline pH seems to favour formation of the dimeric form (*i.e.*, depolymerization of the enzyme) which is less active than its native tetrameric form which is favoured at pH 6.8 to 7.2 (Wu *et al.* 1990). These are in agreement with the reported diminution of PEPC activity from *C. dactylon* upon dilution at room temperature and pH 7.4 (Salahas and Gavalas 1997). Cold-inactivation and/or dilution, with parallel dissociation of the oligomer to its monomers, can be explained either by weakening of the hydrophobic bonds at low temperatures (Oakenfull *et al.* 1977a, b) or/and by possible changes in the heat of ionization of the side-groups of amino acids (Bock and Frieden 1978, Jaenicke 1990). These postulations may be valid for PEPC since the maize enzyme monomers are highly hydrophobic, and the found ionization changes in the side-groups of histidine and cysteine in the maize enzyme may promote its depolymerization and polymerization at alkaline and acidic pH, respectively (Walker *et al.* 1986).

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