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Effects of phosphate on the activity, stability and regulatory properties of phosphoenolpyruvate carboxylase from the C_4 plant *Cynodon dactylon*

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Abstract

The extraction of phosphoenolpyruvate carboxylase, PEPC (EC 4.1.1.31) from leaves of *Cynodon dactylon* (L.) Pers. with phosphate buffer (pH 7.4, 105 mM) was advantageous in comparison to the usual extraction with Tris-HCl buffer (pH 7.4, 100 mM); a higher activity was obtained, which was most evident at low substrate (phosphoenolpyruvate) concentrations. The PEPC activity was stable under dilution or in storage for at least 48 h at room temperature. The effects of phosphate buffer were not due to inhibition of phosphatase(s) action during the extraction, since they were also observed when the phosphates were added after the extraction with Tris-HCl. The phosphate-extracted enzyme was less responsive to both L-malate inhibition and activation by glucose-6-phosphate. The effects of phosphates might be due to preferential exclusion from the enzymic protein domain and, therefore, to a confinement of the enzyme to a fraction of the total volume.

Additional key words: glucose-6-phosphate; glycerol; L-malate; pH; phosphatase; Tris-HCl buffer.

Introduction

Leaf PEPC (EC 4.1.1.31) of C_4- and CAM-plants catalyzes the fixation of CO_2 on phosphoenolpyruvate (PEP) to form oxaloacetate and phosphate (P_i) (O'Leary 1982, Andreo *et al.* 1987), which is the first step of the C_4-pathway (Hatch 1987) and Crassulacean acid metabolism (Kluge and Ting 1978), respectively. The activity of PEPC is modulated by the dark/light cycle (Karabourniotis *et al.* 1983, 1985, Huber and Sugiyama 1986), through a reversible phosphorylation (for references see Jiao and Chollet 1991) or/and a dimer/tetramer interconversion (Wu *et al.* 1990). The activity of PEPC and its sensitivity towards malate is unstable in storage (Hatch and

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Oliver 1978, Winter 1981, Selinioti *et al.* 1987), suggesting that changes in its properties may happen even during extraction. Several attemps have been made to avoid them, using very short extraction times (Doncaster and Leegood 1987), additives like glycerol (Manetas 1982, Karabourniotis *et al.* 1983, Jiao and Chollet 1988), L-malate (Huber *et al.* 1986, Jiao and Chollet 1988), a protease inhibitor (McNaughton *et al.* 1989), or osmotic rupture of mesophyll protoplasts directly in the assay cocktail (Petropoulou *et al.* 1990, Devi and Raghavendra 1992). Organic buffers of the desired pH are routinely used both in extraction and assay media, with Tris-HCl being the most common among them. The involvement of P_i as substrate or as a product of different enzymatic reactions is widely spread between the pathways existing in a plant cell (Iglesias *et al.* 1993). However, the active enzyme is phosphorylated (Jiao and Chollet 1991) and inorganic phosphate in the assay medium activates PEPC (Meyer *et al.* 1989, Podestá *et al.* 1990). We thought, therefore, that an extraction medium buffered with inorganic phosphate salts could either suppress the action of any protein phosphatase(s) by product inhibition or activate PEPC, and we tested these possibilities.

Materials and methods

Cynodon dactylon (L.) Pers. (Bermuda grass) was used as the main experimental plant. Results with *Zea mays* L. were similar, but are not shown. Plants were grown in pots under natural summer conditions. Fully expanded leaves were cut in small pieces and mixed thoroughly before use in paralled extractions with Tris-HCl and phosphate buffers. Leaf tissues were ground at room temperature in a pre-chilled mortar with purified sea sand and the proper extraction medium. Either 100 mM Tris-HCl or 105 mM phosphate buffer (KH_2PO_4/Na_2HPO_4), both at pH 7.4, were used as buffers, plus 1 mM EDTA, 10 mM $MgCl_2$, 2 % m/v polyvinylpyrrolidone PVP (m.m. 10 000), a small amount (*ca.*100 mg) of insoluble PVP, and 20 % v/v glycerol. The latter was omitted in some extractions (see Results). Routinely 0.2 g leaf tissue was extracted with 5 cm^3 extraction medium. However, to obtain extracts with higher protein concentrations, ratios from 0.4 g per 1.25 cm^3 to 0.2 g per 5 cm^3 were also used. The extracts were centrifuged for 6 min at 15 000 × *g* and the clear supernatant was used as crude enzymic source. Extractions with other organic buffers (Hepes-KOH, Aces-KOH, Mops-KOH, Tricine-KOH) were also made; in all cases the extracted PEPC activity did not differ substantially from that obtained with Tris-HCl extraction. Thus, the comparisons between the effects of Tris-HCl and phosphate buffer are also valid for all the organic buffers tested. Similarly, extractions with phosphate buffers at pH 7.2, 7.4, 7.7 (plus $MgCl_2$) and 8.0 (minus $MgCl_2$) gave comparable levels of PEPC activity.

The assays for PEPC activity were run at 30 °C in 3 cm^3 final volume of 100 mM Tris-HCl, pH 7.2 or 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH, 4.5 units of malate dehydrogenase (pig heart, *Sigma*), and PEP, L-malate and glucose-6-phosphate (Glc-6-P) as specified. All stock solutions were made in the same buffer and readjusted to the proper pH (7.2 or 8.0), when necessary. The reaction was

started with the addition of 10 mm^3 enzymic extract (Angelopoulos *et al.* 1988) and the rate was measured by the decrease in absorbance at 340 nm (oxidation of NADH). Protein measurements were made with the Folin phenol reagent (Lowry *et al.* 1951).

Results

Phosphate *vs.* Tris-HCl as extraction buffers: The activity of PEPC extracted with phosphate buffer was considerably higher than that obtained after extraction with Tris-HCl (Fig. 1). At both assay pH values, rate curves from the Tris-HCl extract were more sigmoid than the ones from the phosphate extract and, therefore, the activating effect of the phosphate buffer was more evident at low substrate (PEP) concentrations. However, the magnitude of activation by phosphate buffer and the

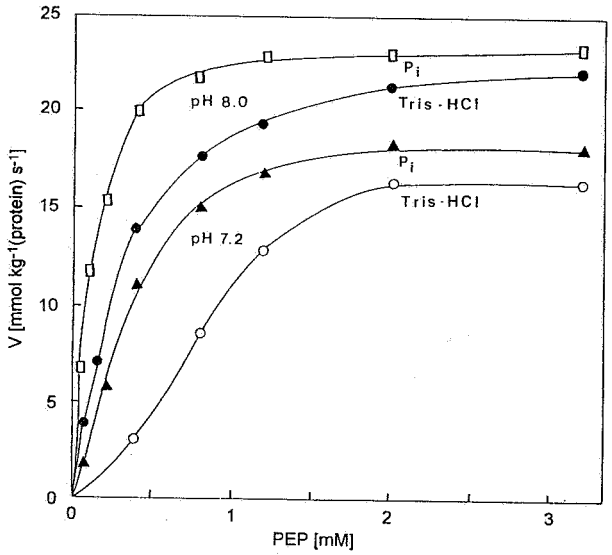

Fig. 1. Rate curves, at pH 7.2 and 8.0, of PEPC extracted with Tris-HCl (100 mM) and phosphate (105 mM) buffers.

total PEPC activity extracted varied considerably among the numerous extractions made over a period of several months, indicating an interference by factors related to the season or/and the nutritional status of the plants. A concentration dependence for inorganic phosphate on PEPC extracted activity (PEP at 0.4 mM, pH 7.2) (Fig. 2*A*) showed that lower concentrations than 50 mM phosphates did not cause activation, whereas from 70 to 150 mM maximal activation was observed. Subsequent experiments with different ratios of extraction medium to fresh mass of leaf tissue revealed that the percent activation by phosphate buffer was inversely related to the concentration of proteins in the extract, *i.e.*, the higher the concentration of proteins in the extract the lower the activation by phosphate buffer by Tris-HCl buffer (Fig. 2*B*). The reduced recovery of activity as a function of protein concentration

originated from decreased activity (due to dilution) in Tris-HCl buffer (results not shown). The differences in total protein extracted by the two buffers were also negligible. The amount of phosphates transferred to the 3 cm^3 assay medium with the 10 mm^3 of the phosphate extract had only a negligible activating effect on the reaction rate, as ascertained by the addition of that amount of phosphates in assays of the Tris-extracted activity.

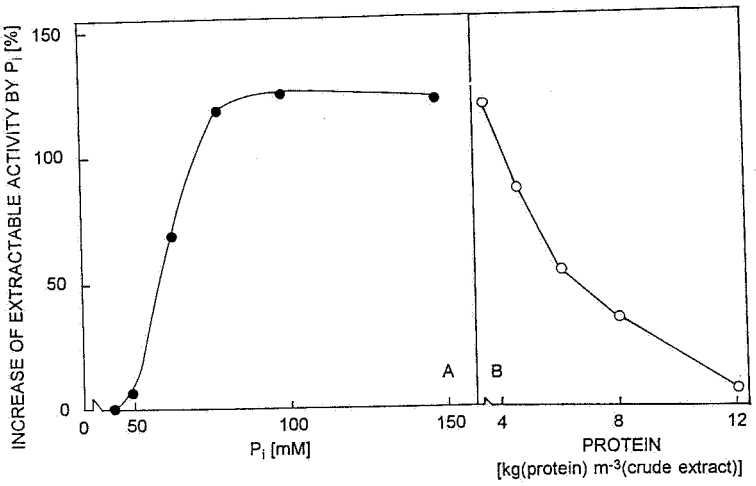

Fig. 2. Per cent increase of P_i-extracted PEPC activity, over that extracted with Tris-HCl, *vs.* concentration of P_i (*A*) or proteins (*B*) in the extraction buffer (PEP at 0.4 mM, pH 7.2). The concentration of protein in *A* was 4 kg(protein) m^{-3} crude extract, of P_i in *B* was 105 mM.

Obviously, the activating effect of the phosphate extraction buffer could be due either to inhibition of protein phosphatase(s) during extraction or to a direct effect on the enzyme molecule (see Introduction). To resolve this dilemma we mixed, in a 1:1 ratio, a double strength phosphate buffer (210 mM) with Tris-HCl extracts, so that the final concentration of phosphate salts was equal to that used in extraction (105 mM). Comparisons of the activities obtained with Tris-extracts, P_i-extracts and Tris-extracts suplemented with phosphates after extraction (Table 1) showed that the activating effect of the phosphate buffer was not due to inhibition of protein phosphatases, but it was rather exerted directly on the enzymic molecule.

Table 1. Effect of phosphates, added after the extraction with Tris-HCl buffer, on phosphoenolpyruvate carboxylase (PEPC) activity. Double strength phosphate buffer (P_i×2) was used, so that the final concentration of phosphates was the same as in extraction with P_i (105 mM). The values given are relative (means ± S.D., 3 replications) to the initial activity.

PEP [mM]	Initial extractions		Dilution 1:1 with	
	Tris-HCl	P_i	Tris-HCl	P_i
0.4	100.0 ± 4.6	259.0 ± 18.4	108.7 ± 5.2	250.6 ± 24.0
4.0	100.0 ± 3.2	137.0 ± 3.7	106.4 ± 1.4	128.4 ± 40.0

Stabilization of PEPC by phosphate buffer: In crude or desalted leaf extracts the activity of PEPC usually declines with time even in the presence of glycerol, which is routinely used as a stabilizer (Manetas 1982, Karabourniotis *et al.* 1983, Jiao and Chollet 1988). With phosphate extraction buffer, however, this decline was not observed, at least for 48 h at room temperature; instead, a small but reproducible activation took place (Fig. 3*A*, line *a*). Phosphate buffer conferred considerable stability even without glycerol (Fig. 3*A*, line *b*); it was a better stabilizer than Tris-HCl plus glycerol (Fig. 3*A*, line *c*), but inferior to phosphate plus glycerol. Obviously, the phosphates can only partially substitute for glycerol, so that the combination of the two stabilizers appears to be the proper medium when stability of the PEPC activity in storage is the primary concern. When both stabilizers were absent, the decline of activity was extremely fast (Fig. 3*A*, line *d*).

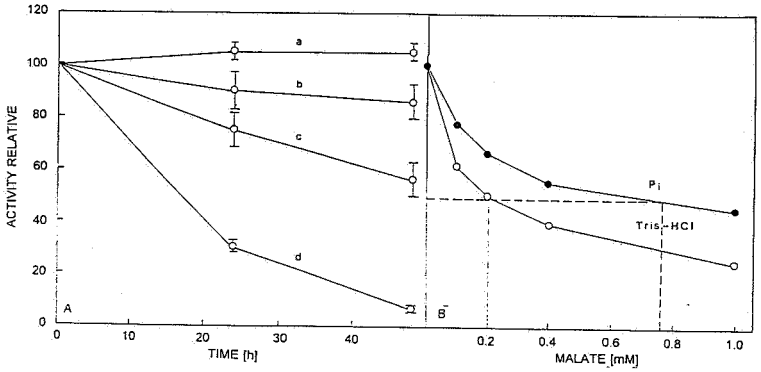

Fig. 3. (*A*) Changes of PEPC activity with time at room temperature when extracted with (*a*) phosphate buffer, (*b*) phosphate buffer minus glycerol, (*c*) Tris-HCl buffer, and (*d*) Tris-HCl buffer minus glycerol. Assays at 4 mM PEP, pH 7.2, means ± S.D. from 3 replications. (*B*) L-Malate inhibition of PEPC activity extracted with phosphate and Tris-HCl buffers (PEP at 0.6 mM, pH 7.2).

The activity of PEPC diminishes upon dilution (Huber and Sugiyama 1986, Selinioti *et al.* 1987, Stamatakis *et al.* 1988, Wu *et al.* 1990). As shown in Table 2, phosphates plus glycerol stabilized the enzyme also against dilution, whereas phosphate buffer minus glycerol was somewhat inferior only after 1 h of storage at room temperature. Both the above treatments, however, were much better stabilizers than Tris-HCl plus glycerol or Tris-HCl alone.

Table 2. Effect of 1:19 dilution on the activity of PEPC extracted with phosphate or Tris-HCl buffers. The values given are relative (means ± S.D. from 3 replications) to the initial activity.

Extraction buffer	Glycerol	Initial activity	Activity after dilution t = 0 h	t = 1 h
Phosphate	+	100.0 ± 3.9	96.7 ± 3.1	97.5 ± 4.0
	-	100.0 ± 4.6	96.3 ± 4.9	93.7 ± 4.8
Tris-HCl	+	100.0 ± 4.1	82.3 ± 4.5	76.6 ± 2.6
	-	100.0 ± 3.2	29.4 ± 3.2	5.9 ± 2.8

Effects of phosphate buffer on PEPC regulation: Though a large number of metabolites affect PEPC activity *in vitro* (O'Leary 1982, Meyer *et al.* 1989), only Glc-6-P and triose phosphates act as activators (Ting and Osmond 1973, Doncaster and Leegood 1987, Wedding *et al.* 1989) and L-malate as inhibitor (Huber and Edwards 1975, Wedding *et al.* 1990). The effects of P_i extraction on the kinetics and stability of PEPC suggested a shifting of the dimer/tetramer equilibrium towards the tetramer (see Discussion). Taking into account the existing evidence that the dimer is more sensitive to L-malate inhibition (Wu and Wedding 1985, 1987, Ngam-Ek *et al.* 1989, Meyer *et al.* 1991) and also that Glc-6-P induces aggregation of PEPC dimers to tetramers (Wagner *et al.* 1987, Angelopoulos *et al.* 1988, Meyer *et al.* 1991, Willeford and Wedding 1992), the prediction could be made that the P_i-extracted enzyme would be less responsive to both L-malate and Glc-6-P.

As expected, the P_i-extracted enzyme was much more resistant to L-malate inhibition than the one extracted with Tris-HCl (Fig. 3*B*); the L-malate concentrations for 50 % inhibition, at pH 7.2 and 0.6 mM PEP, were 0.75 and 0.20 mM, respectively. In addition, when the Tris-HCl extract was incubated with double strength phosphate buffer (1:1 ratio) the L-malate concentration for 50 % inhibition was changed from 0.25 to 0.45 mM.

The response to Glc-6-P (5 mM) was also weaker with the P_i-extracted enzyme (Fig. 4). At pH 7.2 and 0.4 mM PEP there was a 160 % activation with the Tris-extract and only 45 % with the P_i-extract. The respective activations at 3.2 mM PEP were 11 and 3 %, respectively.

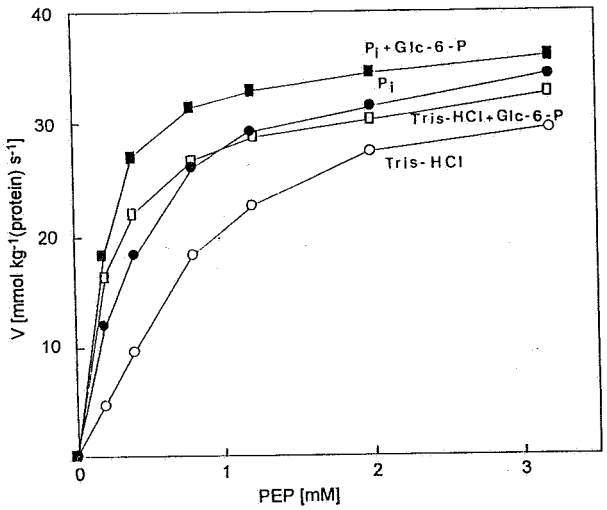

Fig. 4. Rate curves of PEPC activity extracted with phosphate (P_i) and Tris-HCl buffers, as affected by Glc-6-P (5 mM) in the assay medium of pH 7.2.

Discussion

Our results show that the use of phosphate buffer for the extraction of PEPC offers several advantages: it prevents the inactivation of the enzyme during storage (Fig. 3*A*) or under dilution (Table 2) and augments its activity at low substrate concentrations (Fig. 1). It is, therefore, the buffer of choice when stability of the extracted PEPC is the prime concern. An activating effect of phosphates, when added in the assay cocktail for PEPC, has been reported (Meyer *et al.* 1989, Podestá *et al.* 1990). In both papers the results suggest that the phosphate is binding at the Glc-6-P activation site. In our experiments, however, extractions with 50 mM phosphate buffer did not activate PEPC (Fig. 2*A*), therefore, the modes of phosphate action in extraction and in the assay are apparently different.

The inverse relationship between protein concentration in the extract and extent of activation at low PEP (Fig. 2*B*) is an important clue for the interpretation of the above effects of phosphate buffer. Taking into account that the tetramer of PEPC is the most active form, particularly at low PEP concentrations (Wagner *et al.* 1987, Wu *et al.* 1990) and that dilution disaggregates this form into dimers (Wu *et al.* 1990, Meyer *et al.* 1991, Willeford and Wedding 1992), the inference can be made that phosphates, at the high concentration used, are preferentially excluded from the protein domain (Timasheff 1992) and, as it is the case with glycerol and other organic cosolutes (Selinioti *et al.* 1987, Stamatakis *et al.* 1988), confine the protein to a fraction of the total volume. This procedure favours protein-protein interactions that shift the dimer/tetramer equilibrium of PEPC towards the tetramer. This forced aggregation may eventually lead to protein precipitation and, indeed, phosphate (HPO_4) is the most active anion in the Hofmeister series for salting-out proteins.

Based on the above hypothesis we predicted that the P_i-extracted PEPC should be more resistant to L-malate inhibition and less responsive to activation by Glc-6-P. Our results (Fig. 3*B* and Fig. 4) are consistent with these predictions and strengthen the hypothesis that the presence of phosphates at high concentrations in the medium favours the formation of the tetramer of PEPC.

The resistance of PEPC to malate inhibition has been connected to protease action during extraction, since chymostatin, a protease inhibitor, preserves the sensitivity of the enzyme towards L-malate (McNaughton *et al.* 1989). However, another possible interpretation for the chymostatin effect has been offered recently (Salahas *et al.* 1994) and, even more, the fact that malate resistance can be induced by the addition of phosphates after the extraction with Tris-HCl suggests that malate sensitivity of PEPC may not be a simple matter of truncation near its N-terminus by protease action.

No physiological significance can be attributed to the observed effects of phosphates on PEPC, since high concentrations (>70 mM) are needed for activation and stabilization of the enzyme during extraction or in storage. However, the use of phosphate buffer for extraction, storage and probably for purification of PEPC is advantageous and the same may be true for other oligomeric enzymes that are inactivated *in vitro* by dilution.

The crucial question concerning the quaternary structure of this enzyme *in vivo* remains virtually unanswered and, for the present, only intelligent guesses can be made. It suffices to say that *in vivo* regulation by dimer/tetramer interconversion (Wu and Wedding 1985, Wu *et al.* 1990, Meyer *et al.* 1991), under the influence of several factors, is not incompatible with regulation by reversible protein phosphorylation (Jiao and Chollet 1991), if phosphorylation shifts the dimer/tetramer equilibrium towards the tetramer.

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