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# Maize Leaf Phosphoenolpyruvate Carboxylase[1]

## Oligomeric State and Activity in the Presence of Glycerol

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### ABSTRACT

**Maize (*Zea mays* L.) leaf phosphoenolpyruvate (PEP) carboxylase activity at subsaturating levels of PEP was increased by the inclusion of glycerol (20%, v/v) in the assay medium. The extent of activation was dependent on $H^+$ concentration, being more marked at pH 7 (with activities 100% higher than in aqueous medium) than at pH 8 (20% activation). The determination of the substrate concentration necessary to achieve half-maximal enzyme activity ($S_{0.5}$) (PEP) and maximal velocity (*V*) between pH 6.9 and 8.2 showed a uniform decrease in $S_{0.5}$ in the presence of glycerol over the entire pH range tested, and only a slight decrease in *V* at pH values near 8. Including NaCl (100 millimolar) in the glycerol containing assay medium resulted in additional activation, mainly due to an increase in *V* over the entire range of pH. Glucose-6-phosphate (5 millimolar) activated both the native and the glycerol-treated enzyme almost to the same extent, at pH 7 and 1 millimolar PEP. Inhibition by 5 millimolar malate at pH 7 and subsaturating PEP was considerably lower in the presence of glycerol than in an aqueous medium (8% against 25%, respectively). Size-exclusion high performance liquid chromatography in aqueous buffer revealed the existence of an equilibrium between the tetrameric and dimeric enzyme forms, which is displaced to the tetramer as the pH was increased from 7 to 8. In the presence of glycerol, only the 400 kilodalton tetrameric form was observed at pH 7 or 8. However, dissociation into dimers by NaCl could not be prevented by the polyol. We conclude that the control of the aggregation state by the metabolic status of the cell could be one regulatory mechanism of PEP carboxylase.**

The $C_4$ pathway of carbon fixation is a highly specialized metabolism, the main effect of which is to increase the concentration of $CO_2$ in the bundle sheath cells (7), therefore optimizing the function of the ribulose-1,5-bisphosphate carboxylase/oxygenase in the direction of carboxylation, and decreasing energy losses by photorespiration.

An important enzyme in this metabolism is PEPC[2] (EC 4.1.1.31), an oligomeric protein often described as a tetramer of 400 kD (2, 29), which catalyzes the $\beta$-carboxylation of PEP by $HCO_3^-$ to yield oxaloacetate and Pi.

Insight into many of its regulatory properties (2, 20) has been provided from studies on the influence on enzymic activity of several metabolites (4, 10), the level of illumination (12, 15, 17), and covalent modification by phosphorylation (3, 16, 19). With respect to the molecular structure of the $C_4$ plant enzyme, only recently some reports have presented evidence on the possible spatial arrangement of the carboxylase molecule (1, 25, 27). Further evidence comes from the dissociation observed after chemical modification of histidine or thiol groups (28) or after exposure to increasing ionic strength (26). However, the physiological significance of these findings remains to be proven, since the conditions existing during these *in vitro* experiments, and particularly the protein concentration, are far different from those existing within the plant cell (6, 11).

It has been reported by several authors that the marked instability of PEPC activity in aqueous extraction or assay media could be overcome by the addition of glycerol (18, 23, 29). The action of this compound, when present as a cosolute, as a stabilizer of protein structure and enzyme activity has been studied extensively by Gekko and Timasheff (8, 9). They proposed that the presence of the organic cosolute acts by preventing protein unfolding and by increasing protein-protein interaction, which results in the formation of aggregates.

Despite the efforts to describe the behavior of PEPC activity in binary solvents, a detailed study on the molecular aspects of PEPC in such media is still lacking. Also, in view of the recent report that an increased ionic strength dissociates PEPC (under some conditions irreversibly and with complete loss of activity (26), it raises the question as to how can the enzyme survive a harsh salting-out fractionation procedure during purification.

In this paper we characterize the activity changes and stabilization brought about by glycerol and correlate them with the oligomeric state of the carboxylase in such conditions, as determined by SEHPLC.

## MATERIALS AND METHODS

### Plant Material

PEPC was extracted and purified from leaves of 6-week-old maize (*Zea mays* L.) plants as described previously (14). The enzyme was more than 95% pure as judged by SDS-PAGE.

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[2] Abbreviations: PEPC, phospho*enol*pyruvate carboxylase; PEP, phosphoenolpyruvate; SEHPLC, size-exclusion high performance liquid chromatography; $S_{0.5}$, substrate concentration necessary to achieve half-maximal enzyme activity; DEPC, diethylpyrocarbonate; $R_s$, cooperativity index; *V*, maximal velocity.

### Activity Assay

Enzymic activity was measured at 30°C in a Hitachi 150-20 double beam spectrophotometer, by following NADH oxidation at 340 nm in an assay medium (1 mL final volume) containing Tricine-Mops (25 mM, each) brought to the corresponding pH with NaOH, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.150 mM NADH, 4 IU malic dehydrogenase, and PEP, PEPC, or other additions as indicated in the figure legends. The reaction was initiated by the addition of PEPC.

### SE-HPLC Experiments

The mol wt determinations were performed on a TSK 3000 SW column (570 × 9 mm), connected to a Waters Associated, Inc. HPLC system consisting of a M-6000 A chromatography pump and a U6K valve loop injector. All runs were made at a flow rate of 1 ml·min$^{-1}$ and 25°C. The buffers consisted of a mixture of Tricine-Mops (25 mM, each) brought to pH 7.0 or 8.0 with NaOH and, where indicated, addition of glycerol or glycerol plus NaCl (20%, v/v, and 100 mM, respectively). The column was calibrated with spinach coupling factor 1 ($M_r$ 400,000), catalase ($M_r$ 240,000), yeast alcohol dehydrogenase ($M_r$ 150,000), yeast hexokinase ($M_r$ 104,000), and bovine serum albumin ($M_r$ 66,000 monomer and 132,000 dimer). At both pH-values, no differences were observed in the calibration curves between those obtained with aqueous buffers and those containing 20% glycerol. The void volume, $V_o$, was determined with blue dextran. The logarithm of $M_r$ was linearly correlated with $K_{AV}$, defined as $K_{AV} = (V_E - V_o)/(V_T - V_o)$, where $V_E$ and $V_T$ are the elution and packed bed volumes, respectively. Elution profiles were recorded in a Gilson holochrome spectrophotometer, following absorbance changes at 280 nm.

The solutions used in these experiments were filtered through Millipore HA, 0.45 μm-pore size filters and degassed under vacuum.

### Protein Assay

Protein concentration was determined by the dye-binding method as described by Sedmak and Grossberg (22), using bovine serum albumin as standard.

### Materials

PEP (monocyclohexylammonium salt), NADH, bovine serum albumin, Tricine, Mops, pig heart malic dehydrogenase, glucose-6-phosphate, L-malate, and mol wt standards were from Sigma Chemical Co. All other reagents were of analytical quality.

## RESULTS

### Effect of Glycerol on PEPC Activity

The addition of glycerol (20%, v/v) to the assay medium at pH 7.0, as shown in Figure 1, produced a marked increase in enzyme activity, up to 180% of the initial value. The activities ($\Delta A_{340}$/min) before and after addition of the organic cosolute were constant with respect to time, as indicated by

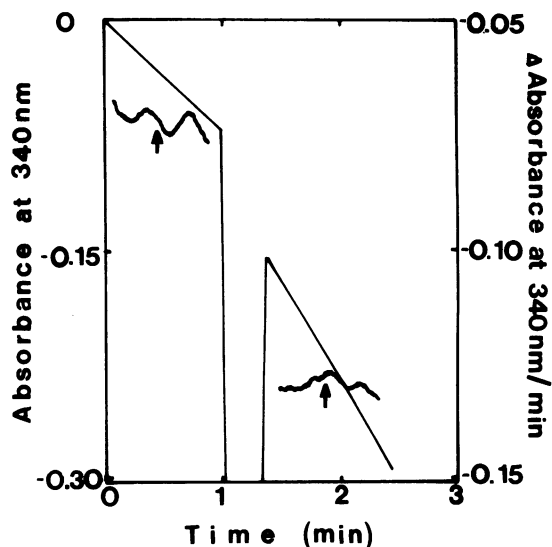


Figure 1. Effect of glycerol on PEPC activity. Activity was measured in a reaction medium at pH 7.0 with 1 mM PEP and 1 μg PEPC. The discontinuity in the main trace is due to the addition of 250 μL of 87%, v/v, glycerol, 0.2 μg PEPC and 1 μL of 200 mM PEP. The arrows indicate the first derivative trace.

the first derivative trace (Fig. 1), suggesting that a fast activating process was taking place. Similar results were obtained at pH 8.0 (not shown), though the activation was not so marked (120% of initial activity).

Preincubation of the enzyme with 20% glycerol in 50 mM Hepes (pH 7.0) 5 min prior to assay in an aqueous medium, resulted in no differences with respect to a sample preincubated without the cosolute, indicating the reversible nature of the activating effect.

The quantitation of the activating action at different concentrations of glycerol revealed a maximum of activity between 15 and 25%, v/v, of the polyol. Higher concentrations decreased the extent of the activation (not shown).

### Effect of Ligands on Glycerol-Activated PEPC

To further examine the behavior of PEP activity in the presence of glycerol, the response of the enzyme to the presence of two effectors of opposite characteristics was studied.

Table I shows the PEPC activity values in the presence of the activator glucose-6-P (4) or the inhibitor L-malate (2) at pH 7.0 in the presence or absence of 20% glycerol. The response of PEPC activity to glucose-6-P was almost the same in the absence of glycerol (75% activation, compare line d with line a in Table I) or in the presence of the polyol (65% activation, compare line c with line b in Table I). Final activities in the presence of glycerol and glucose-6-P differed by about 9% depending on the order of the addition (compare line c with line e in Table I). The inhibition caused by L-malate was not the same if the effector was added before or after glycerol. A 25% inhibition was observed when malate was added to the native enzyme (line f related to line a in Table I), in contrast with 8% in the presence of the cosolute (line h against line b, Table I), suggesting that the change elicited by malate is such that it cannot be overcome by

**Table I.** *Influence of Glucose-6-P and Malate on the Response of PEPC Activity to Glycerol at pH 7.0*

Activity (expressed in μmol/min/mg protein) was assayed at pH 7 in the presence of 1 mM PEP and 1 μg PEPC, during 0.8 min, and then the indicated additions were made.

| Condition | Activity[a] |
|---|---|
| (a) Native enzyme | 6.0 (100) |
| (b) a + 20% glycerol | 12.0 (200) |
| (c) b + 5 mM glucose-6-P | 19.8 (330) |
| (d) a + 5 mM glucose-6-P | 10.5 (175) |
| (e) d + 20% glycerol | 21.7 (361) |
| (f) a + 5 mM malate | 4.5 (75) |
| (g) f + 20% glycerol | 8.8 (147) |
| (h) b + 5 mM malate | 11.1 (184) |

[a] Numerals in parentheses represent the percent of activity with respect to the native enzyme in the absence of effectors (line a).

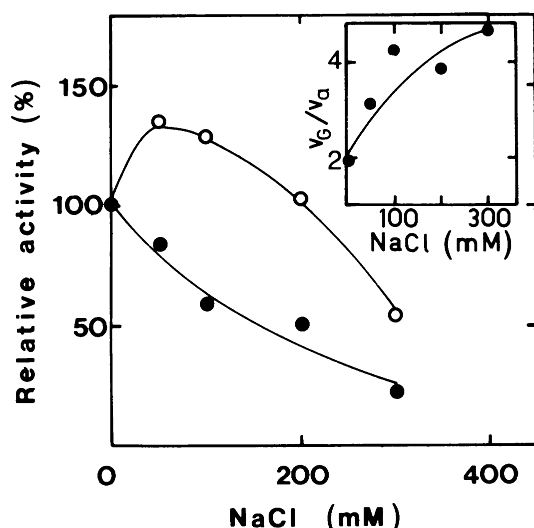


**Figure 2.** NaCl effect on PEPC activity. Activity (expressed as percent of that measured in the absence of NaCl) was assayed at pH 7.0 with 1 mM PEP and 1 μg PEPC in the presence (○) or absence (●) of 20%, v/v, glycerol and increasing concentrations of NaCl. Without NaCl, PEPC specific activity was 6.45 and 12.5 μmol·min⁻¹·mg⁻¹, in the absence and presence of glycerol, respectively. Inset: Ratio of activities in the presence ($V_G$) and absence ($V_a$) of glycerol as a function of NaCl concentration in the assay medium.

glycerol and, conversely, the cosolute protects the enzyme against such a change when added prior to the inhibitor.

#### PEPC Activity in Water-Glycerol-NaCl Mixtures

It has long been known that PEPC has a marked sensitivity to NaCl (18, 21, 26), and it has been shown that a dissociation process accompanies the loss of activity in the maize enzyme (26). Manetas *et al.* (18), working with the carboxylase of two $C_4$-halophytes, *Cynodon dactylon* and *Salsola soda*, observed a recovery of activity in NaCl-inhibited enzyme by the compatible solutes betaine and proline.

When the maize enzyme was subjected to a combined salt-glycerol treatment, a NaCl-concentration-dependent response was observed. Figure 2 shows a plot of relative activity as a function of NaCl concentration in the assay medium at pH 7.0. The increasing inhibitory effect of NaCl observed in the aqueous medium contrasts with the activation observed when 20% glycerol was also present, up to a salt concentration of 150 mM. Further increases in salt concentration were inhibitory, though the activity values were always higher in the binary solvent mixture (inset, Fig. 2).

On the other hand, at pH 8.0 the activity in the presence of NaCl and glycerol decreased with time to complete inactivation, but the presence of the organic cosolute partially protected against such an effect: the half-time for inactivation by 400 mM NaCl increased from 2 min in aqueous medium to 20 min when glycerol was also present.

#### Changes in the PEPC Kinetic Properties

Our experiments showed a hyperbolic response of PEPC activity to PEP concentration at pH 8.0, while a marked deviation toward sigmoidicity was observed at pH 7.0. This can be seen in Figure 3, where the response to glycerol is also plotted. The cooperativity index ($R_S$) was calculated from this graph, according to Dixon and Webb (5), and values of $R_S$ of 13 and 83 were obtained at pH 7, in the absence and presence of glycerol, respectively. These values show that the strong positive cooperativity of PEPC was eliminated by the presence of the polyol ($R_S$ equals 81 for an enzyme displaying hyperbolic kinetics) (5). The same features were obtained when the data were analyzed in a Hill plot (not shown); an $n_H$-value of 2 was obtained for the aqueous control, while the addition of 20% glycerol lowered this value to 1.

#### Effect of pH

To gain further insight into the interactions of PEPC with glycerol, the variation of the kinetic parameters of the carboxylase with pH, in the range 6.9 to 8.2, was investigated. In Figure 4A the logarithm of $V$, obtained form double reciprocal plots, was plotted against pH. A trace curving downward at acidic pH resulted for the aqueous control, in agreement with

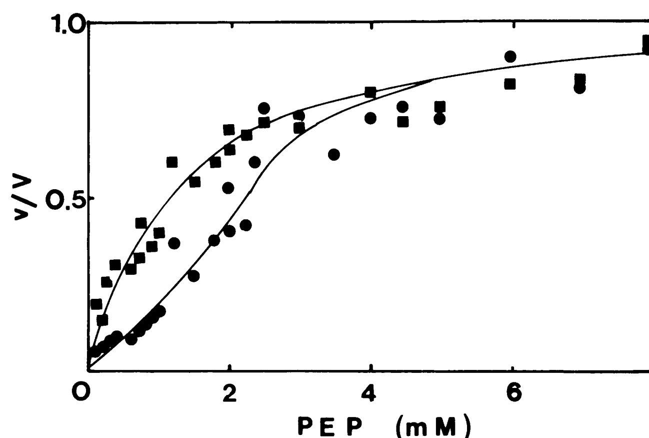


**Figure 3.** Saturation kinetics of PEPC activity with respect to PEP. Activity was measured in the absence of (●) or presence (■) of 20% v/v glycerol, as described in "Materials and Methods." The ordinate represents the relative velocity, v/V. V was determined form double reciprocal plots as described in (5).

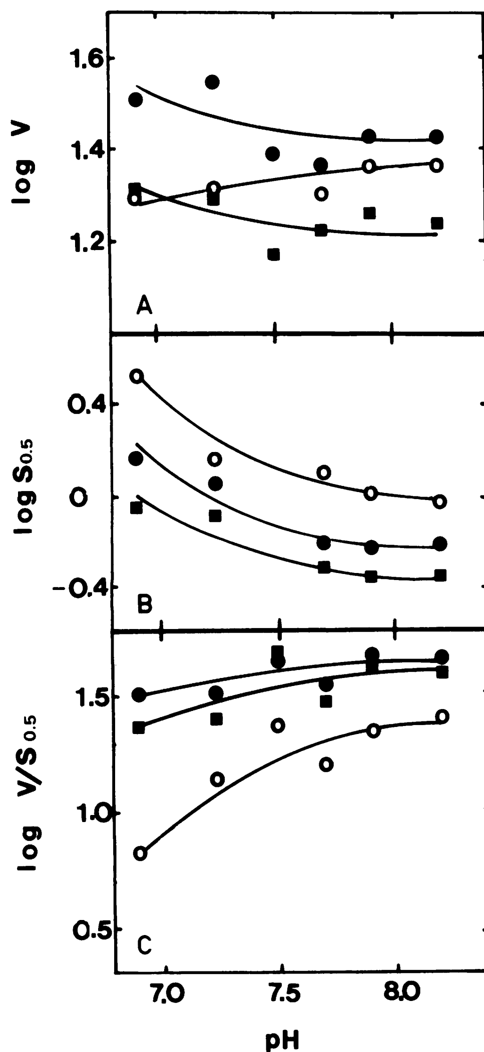


**Figure 4.** Effect of pH on the kinetic properties of PEPC. Enzyme activity was measured as described under "Materials and Methods" at the stated pH values. Activity in the presence of NaCl was tested by the addition of 20 μL of a 5 M solution of the salt to the glycerol containing medium. The symbols represent the activity measurements in aqueous assay medium (○), 20% v/v glycerol (■), or glycerol plus 100 mM NaCl (●).

previous reports (14), while the presence of glycerol produced the inverse effect, *i.e.* an upward curvature as pH decreased. The presence of 100 mM NaCl together with 20% glycerol increased log V in the entire pH-range tested. It must be noted that NaCl alone diminished $V$ as the pH decreased (see Ref. 26). On the other hand, glycerol and glycerol plus NaCl, in the amounts stated above, lowered the values of $S_{0.5}$ in the pH range tested (Fig. 4B), but did not modify the shape of the curve. Figure 4C shows that the ratio of $V/S_{0.5}$ was affected by pH in such a way that it became almost constant when 20% glycerol (with or without 100 mM NaCl) was present.

### Effect of Glycerol on the Quaternary Structure

When PEPC was subjected to SE-HPLC in aqueous buffer, a main $A_{280}$ peak corresponding to 400 kD and a shoulder at 200 kD were observed at pH 8.0 (Fig. 5A), whereas at pH 7.0 two well defined peaks at 400 and 200 kD appeared (Fig. 5C). The elution profiles of samples preincubated for 30 min in 20% glycerol, and run in the same buffer with the cosolvent, are shown in Figure 5, B and D. Whereas the shoulder at 200 kD was less marked at pH 8.0, the most striking change with glycerol can be seen at pH 7.0, where only the 400 kD form was present. The same result, *i.e.* a single $A_{280}$ peak at 400 kD, was obtained by injecting native (nonpreincubated) enzyme and running it in buffer containing glycerol at both pH values (not shown). Oligomeric forms larger than 400 kD could not be detected under any of these conditions after Sepharose-6B chromatography (not shown; see also ref. 26).

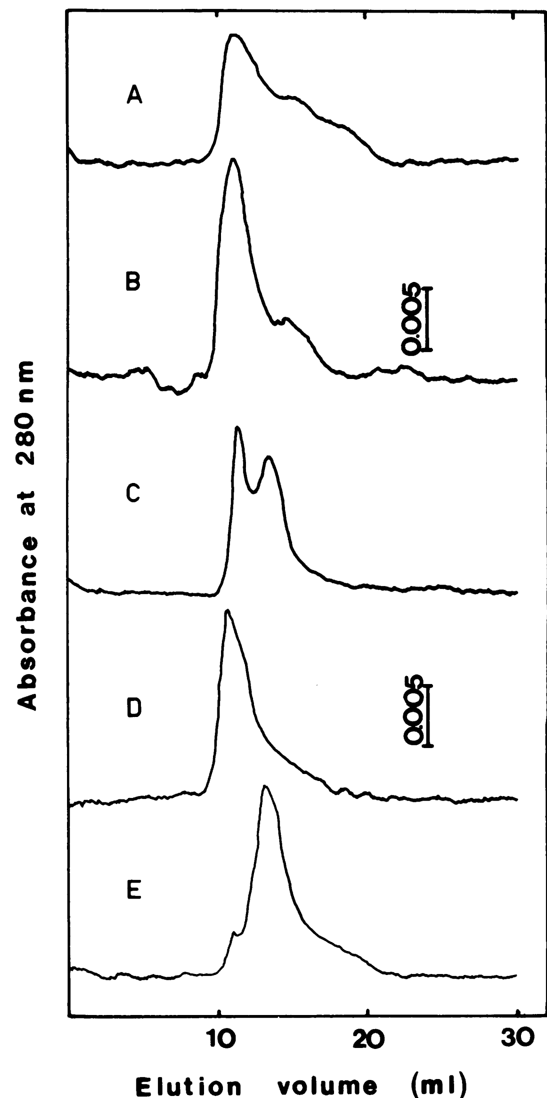


**Figure 5.** SEHPLC elution profiles of PEPC. The enzyme (20–30 μg in 200 μL) was injected and run in aqueous (A and C), 20% glycerol (B and D), or glycerol plus 100 mM NaCl containing buffers (E) as described in "Materials and Methods," at the following pH values: pH 8 (A and B), or pH 7 (C, D, and E). Pretreatments of the enzyme were as follows: none (A and C); 30 min in 20% v/v glycerol at pH 7.0 or 8.0 (D and B, respectively); 30 min in 20% v/v glycerol-100 mM NaCl at pH 7.0 (E).

When 100 mM NaCl was present in the running buffer with glycerol, only the dimeric form of 200 kD with a small peak at 400 kD was seen at pH 7.0 (Fig. 5E), that is, glycerol did not prevent PEPC from salt-induced dissociation. However, when a sample that was preincubated for 30 min with 100 mM NaCl in aqueous buffer at pH 7.0 (a condition that induces complete dimerization of PEPC [26]) was chromatographed in 20% glycerol, only one peak at 400 kD was observed (not shown).

## DISCUSSION

It is known that PEPC activity displays an allosteric behavior for the binding of PEP (at neutral pH) (29) and L-malate (with $n_H$ values increasing with decreasing pH) (10), and that this cooperativity is abolished by raising pH or by the addition of glycerol, glycine, or glucose-6-P with respect to PEP (4, 29), or by the presence of glucose-6-P with respect to L-malate (10). Experimental evidence providing an explanation at the molecular level for such observations has not yet been presented. It is also known that thiol and histidine modification (28), and ionic strength (26) dissociate the enzyme with drastic change in enzymic activity. It has been proposed that the effect of malate could be elicited through changes in the quaternary structure (13). Manetas *et al.* (18) have shown that the organic cosolutes betaine and proline protect PEPC from NaCl inhibition, increase the affinity of the enzyme for PEP, and stabilize the activity at low PEP levels. Selinioti *et al.* (23) showed the existence of a strong dependence between PEPC concentration and its stability in crude or partially purified extracts, and they extended the studies with cosolutes, using also glycerol and polyethyleneglycol. They explained the stabilizing effect of these compounds through the 'exclusion volume' theory. According to Gekko and Timasheff (8, 9), glycerol is excluded from the domain of the protein in solution, and this exclusion (and the chemical potential of glycerol) increases on denaturation. The preferential hydration of the protein would be, at least in part, due to enhanced solvent ordering, and would result in a more compact molecule. Also, solvent ordering induced by glycerol could act stabilizing hydrophobic interactions, thus promoting protein self-association. However, the proposal that PEPC stabilization by glycerol could be the result of enhanced subunit association has not been proved experimentally. It is also unclear at present whether the reversible dissociation processes which affect maize PEPC activity could be of importance for *in vivo* enzyme regulation.

The theory predicts that if a dimer-tetramer equilibrium exists, and the affinity for PEP is greater in the tetrameric form, a positive cooperativity is expected, which should decrease as protein concentration increases (5). This last feature can be mimicked by the presence of a compatible organic cosolute, as discussed above. Such changes in the kinetic behavior of PEPC were effectively observed when glycerol was present during assay (Fig. 4). Moreover, both the 400 and 200 kD forms were observed when native PEPC is subjected to SE-HPLC in aqueous buffer (pH 7 or 8), but only the 400 kD peak appears in 20% glycerol at pH 7. This effect, well demonstrated at pH 7.0, is accompanied by a substantial increase in enzyme activity. It should be noted that upon injecting 10 times the amount of protein than in these experiments, only the 400 kD peak could be observed at pH 7.0 (not shown; see also ref. 26), demonstrating the protein concentration dependence of the oligomeric state (also see 23).

The hyperbolic kinetics observed at pH 8.0 for the native or glycerol-treated enzyme was correlated with the much less activation produced by the polyol and with only a slight change in the SEHPLC profile, which consists mainly of the 400 kD peak at this pH.

Examination of the values of $S_{0.5}$ (PEP) and V through the range of pH 6.9 to 8.2 (Fig. 3) also provided information about the activation afforded by glycerol. Analyzing first the data near pH 7.0, the main change glycerol-induced is in $S_{0.5}$ (approximately threefold lower), while V remains approximately the same. This feature also fits a model in which two different forms of PEPC were present in aqueous solutions, each catalyzing the same reaction at the same velocity but with different substrate affinities. These experiments also indicate that substrate concentration has much to do with the molecular form present. Since at low substrate v tends to $(V/S_{0.5})S$, the greater change at pH 7.0 in the logarithm of $V/S_{0.5}$ is an evidence that glycerol action is preferentially exerted at low PEP.

At alkaline pH, V was slightly diminished by the presence of glycerol (70% of that of the aqueous control). This could be the consequence of a nonspecific effect on the rate of breakdown of the enzyme-substrate complex or to the presence of less active or inactive high mol wt aggregates. The detection of such aggregates by HPLC was hindered by the resolution of the column (300 kD), but its existence cannot be ruled out, since aggregation upon aging of PEPC, together with a loss of activity has been reported (29). Finally, the shape of the semilog plot of *V* against pH is altered by the addition of glycerol, indicating a shift in the pK of residues involved in the enzyme-substrates complex. Iglesias and Andreo (14) have postulated that these residues could be the essential histidine groups modified by DEPC, and it is of interest to compare these results with those of Walker *et al.* (28), who showed that chemical modification by DEPC promotes dissociation of PEPC.

A first conclusion arising from our experiments is that the presence of glycerol optimizes the efficiency of the use of PEP as substrate by PEPC in a broad pH range, and that such an effect could be achieved by the stabilization of the quaternary structure in its most active (tetrameric) form. One of the reasons could be the change in the pK of ionizable groups involved either in catalysis and/or maintenance of the structure.

The presence of NaCl together with glycerol resulted in additional activation when the salt concentration was less than 150 mM (Fig. 2). The principal effect was on V (Fig. 4), *i.e.* at saturating PEP, since $V/S_{0.5}$ was not much changed by the salt (with respect to glycerol alone). An activating effect of NaCl in aqueous solutions has been reported for the PEPC from two $C_4$-halophytes by Shomer-Ilan *et al.* (24), who attributed it to spatial changes in the carboxylase. The maize enzyme is always inhibited by NaCl in aqueous solutions, but the extent of the inhibition is dependent on PEP concentration (26). This dependence on the presence of the substrate is

also reflected by the fact that during SE-HPLC in a NaCl-glycerol buffer at pH 7.0 complete dissociation into dimers is observed, in the same manner as in an aqueous solution of NaCl (26).

Although unclear at the present stage, the role of NaCl in the change of PEPC activity in mixed solvents can be attributed to a nonspecific increase in the rate of breakdown of the enzyme-substrate complex or, more likely, to dissociation of inactive, larger than 400 kD, PEPC aggregates. The presence of the substrates and $Mg^{2+}$ and glycerol would be crucial for the maintenance of the quaternary structure in the presence of the salt, avoiding dimerization.

The allosteric activator glucose-6-P protects PEPC against NaCl inhibition (26), probably preventing it from dissociation. When its effect was assayed in the presence or absence of glycerol, the extent of activation obtained was slightly higher in the native enzyme. This result indicates that glucose-6-P affects the molecular structure by other means than only affecting the aggregational state of the carboxylase. However, an indirect effect of glucose-6-P due to the lowering of the $S_{0.5}$ for this substrate cannot be disregarded. The glycerol-induced activation, which is additive with respect to the effect of glucose-6-P (Table I), should be compared with the activating effect of thiol reduction, which is not (15).

L-Malate, on the other hand, acted in a different manner with respect to glycerol-treated or native PEPC, with final activities in the presence of both effectors depending on the order of addition (Table I). This experiment was performed at pH 7.0, where the $K_I$ for the inhibitor is 13-fold lower than at pH 8.0 (10) and, moreover, the competitive pattern of inhibition restricts the binding of PEP to the carboxylase. A possible explanation is that the dimeric form, which exists in aqueous solution at pH 7, is more susceptible to malate inhibition and at the same time is stabilized by the hydroxy-acid, thus rendering the dimer insensitive to the action of glycerol.

Although further studies are needed, we believe that the evidence presented here supports the view that PEPC could be an enzyme regulated substantially, if not mainly, by association-dissociation processes. We have shown that a glycerol-induced increase in protein-protein interactions, as is expected to occur within the plant cell, is not sufficient entirely to prevent NaCl-induced dissociation or to shift completely the equilibrium at pH 8 (Fig. 5). The equilibrium between different forms may be governed by substrate disposition, metabolite concentrations, light status, and ionic strength within the cytoplasm, as well as by the pH. Another factor that needs to be considered is the state of phosphorylation of the enzyme, which role in enzyme activity and/or regulation is not yet completely elucidated (16).

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