Regulation of Phosphoenolpyruvate Carboxylase from *Crassula argentea*[1]

FURTHER EVIDENCE ON THE DIMER-TETRAMER INTERCONVERSION

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ABSTRACT

Phosphoenolpyruvate carboxylase in Crassulacean acid metabolism plants during the day exists in dimeric form the activity of which is strongly inhibited by malate. Enzyme purified from *Crassula* leaves collected during the day and stored at −70°C for 49 days shows a steady progression of change from dimer to tetramer, and this change in oligomeric state is accompanied by a decrease in the sensitivity of the enzyme to inhibition by malate. At 10 minutes preincubation of enzyme after 11 days storage—which is composed of an equilibrium mixture of dimer and tetramer—with malate causes most of the enzyme to be converted to dimer and increases the sensitivity of the enzyme to malate inhibition during assay. Preincubation with phosphoenolpyruvate shifts the equilibrium toward the tetrameric form and reduces the maximal inhibition produced by 5 millimolar malate to less than 20%. However, none of the treatments used resulted in shifting the oligomerization equilibrium completely in either direction. Thus the question of whether some covalent modification of the enzyme, such as phosphorylation, is required to permit complete changes in equilibrium remains open.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) is a key regulatory enzyme in photosynthetic carbon metabolism of C4 and CAM plants (4, 5). Recent studies on the regulation of this enzyme in CAM plants (9, 16, 17) indicated that the enzyme exists in two forms with differing affinities for the substrate PEP[3] and different sensitivities to malate inhibition, which appears to be part of the diurnal cycle in the functioning of CAM metabolism (5).

The day, malate sensitive, form of the enzyme seems to be unstable. A rapid loss in the ability of malate to inhibit PEP carboxylase has been observed (14, 15), and an increase in the total enzyme activity during enzyme isolation and storage has been reported (6). Addition of malate (14, 16), casein (16) or glycerol (6) stabilized the enzyme somewhat, although the mechanism by which changes occur in the enzyme during isolation and storage of the enzyme is still unknown.

Earlier studies showed that PEP carboxylase from *Crassula* leaves is an oligomeric enzyme with at least two interconvertible

enzyme forms, dimer and tetramer (17, 18). The enzyme dimer, which occurs in nature during the day, is sensitive to malate inhibition and the tetramer, which is the night form, is presumably functional in fixation of CO_2 during the night because of resistance to inhibition by malate.

In this communication we provide further evidence on the dimer-tetramer interconversion of PEP carboxylase from *Crassula* leaves during storage, the effect of the ligands malate and PEP on this process and the relationship of malate sensitivity to the aggregation state of the enzyme.

MATERIALS AND METHODS

Plant Material. The plants used (*Crassula argentea* Thunb.) were grown in a greenhouse as described previously (17).

Chemicals. The chemicals used in this study were identical with those described earlier (17, 18) and were of the highest purity commercially available.

Enzyme Assay. The activity of PEP carboxylase was measured at 25°C in 1.0 ml of 50 mM Mops (pH 7.2) containing 0.15 mM NADH, 5 mM $MgSO_4$, 10 mM $NaHCO_3$, 5 mM PEP, and 1 IU of malate dehydrogenase (from Sigma). The reaction was started by the addition of enzyme and the oxidation of NADH was followed at 340 nm in the cell compartment of a Beckman DU-50 spectrophotometer controlled at 25°C. Kinetic parameters were determined by fitting assays at varying concentrations of PEP to the Michaelis-Menten equation modified to provide estimates of the Hill number as well as V_m and K_m (17).

Enzyme Purification. The enzyme was purified from day leaves collected after 4 to 5 h of light. The purification procedure was a modification of the method previously described (18). All steps were performed at 0 to 4°C. Leaves were homogenized in 2 volumes of 0.05 M Hepes buffer (pH 7.2), with 1 mM EDTA, 1 mM DTT and 20% (v/v) glycerol. A Polytron homogenizer was run in an ice bath at full speed for two 15 s bursts with a 15 s cooling interval. After filtering through two layers of fine nylon mesh, the homogenate was centrifuged at 500g for 10 min and the supernatant liquid discarded. The pellet was extracted with the extraction buffer containing 2% Triton X-114 but omitting glycerol, following the method of Pryde (10) for solubilizing membrane-associated proteins. After centrifugation, the material soluble in Triton X-114 was fractionated by ammonium sulfate precipitation, with the fraction precipitating between 35 and 60% ammonium sulfate saturation collected by centrifugation at 16,000g for 15 min and suspended in the extraction buffer without either Triton or glycerol. The preparation was then desalted using a 2 × 20 cm Sephadex G-25 column and applied to either a DEAE-cellulose (DE-52) column or a Fractogel TSK DEAE 650 M (from EM Science) column (1 × 28 cm) in the same buffer. The PEPC was eluted from this column using a

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[3] Abbreviations: PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase.

gradient of NaCl from 0 to 0.2 M in this buffer. The enzyme, which was free of competing activities, was stored at −70°C in 0.05 M Hepes with 1 mM DTT and 1 mM EDTA.

HPLC Size-Exclusion Chromatography. A Spherogel-TSK 4000sw column (7.5 × 300 mM) supplied by Beckman, was used at 24°C with a Beckman HPLC system consisting of a model 110 A pump, a model 420 controller, and a model 160 detector. The flow rate was 1 ml/min and 0.5 ml fractions were collected. Equilibration and elution was with 50 mM Tris-Mes at pH 6.8. Recording and integration of the protein profile was carried out with a Shimadzu model CR3A printing integrator. Activity profiles are shown with observed rates in each fraction connected by a smoothed line.

Molecular Weight Standards. Calibration of the HPLC system was carried out under the same conditions using thyroglobulin ($M_r = 670,000$) and γ-globulin ($M_r = 158,000$) from Bio-Rad and ferritin ($M_r = 450,000$) and catalase ($M_r = 240,000$) from Pharmacia. The equation derived by fitting the log mol wt of the standards against elution volume using a linear regression procedure is Log mol wt = 6.94 − (0.183 × elution volume [ml]) with a correlation coefficient, $r = 0.996$. Using this equation and the elution volume of the dimer and tetramer of PEPC, the indicated mol wt of the two enzyme forms are 219,000 and 415,000, respectively.

RESULTS

Aggregation State of PEP Carboxylase in Storage. Recent reports have indicated that factors such as ionic strength, temperature and various metabolites can affect the quaternary structure of PEP carboxylase from C_4 (11, 12) and CAM (18) plants. We have found that simple storage at low temperature is also sufficient to induce changes in the aggregation state of the CAM enzyme. This is illustrated in the activity profiles from TSK-4000 size exclusion column HPLC chromatograms shown in Figure 1. The aggregation state of the enzyme purified after a mid-day harvest changed from predominantly dimer with a mol wt of 219,000 a week after preparation to primarily tetramer after 49 d storage at −70°C. Storage thus has resulted in progressive enzyme aggregation to a larger form (tetramer), like the one found in *Crassula* leaves at night (18), with a mol wt of 415,000. The protein profiles from the same chromatograms (not shown) also demonstrate the appearance of a tetramer peak in the PEP carboxylase preparation after storage. PEP isotherms show a large difference in sensitivity to inhibition by 5 mM malate of
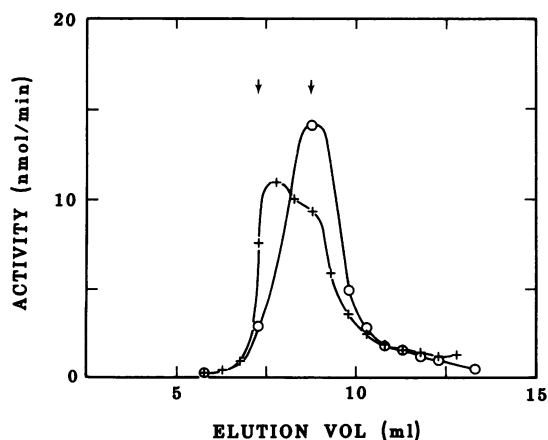
the fresh PEP carboxylase preparation and that which had been stored 49 d. Malate strongly inhibited the activity of the fresh enzyme preparation, causing a 66% inhibition of $V_{max(PEP)}$. The same treatment of the stored PEP carboxylase preparation resulted in only 17% inhibition of V_{max}. The tetramer after 49 d storage shows relatively little inhibition of V_{max} and also illustrates the competitive inhibition of malate. The ($K_{m(PEP)}$ for control = 0.09 ± 0.02 mM, with 5 mM malate, $K_{m(PEP)}$ = 1.20 ± 0.03 mM) like that observed previously (13). For the fresh enzyme a similar effect of malate on $K_{m(PEP)}$ was observed (K_m for control = 0.07 ± 0.01 mM, K_m, for malate-treated = 0.92 ± 0.03 mM). In both cases malate causes *ca* a 13-fold increase in K_m.

These results are in agreement with the observation that CAM PEP carboxylase extracted during the day may lose its initial sensitivity to malate inhibition after extraction (14, 15) and show that even extraction with glycerol and storage at −70°C may slow, but do not prevent this change.

A more detailed HPLC study of the time-course of the conversion of enzyme dimer to the tetramer during storage at −70°C is given in Figure 2. It is apparent that the dimer is gradually converted to the tetrameric form during storage. Integrating the areas under the dimer and tetramer peaks permits calculating the ratio of the two forms and shows that the ratio of the tetrameric enzyme to dimer plotted *versus* storage time produces a straight line with a positive slope. The successive chromatograms run while the oligomeric equilibrium is shifting from dimer to tetramer make possible an estimation of the relative specific activities of the two forms of PEPC using the replicate protein and activity scans over time. The specific activity (μmol/min/mg) of the tetramer = 2.2 ± 0.4, while for the dimer it is 1.3 ± 0.03. This 39% decrease in specific activity is in agreement with the changes in specific activity observed as a result of the conversion of tetramer to dimer by preincubation with malate (13).

Ligand Effects on Dimer-Tetramer Interconversion. We have shown recently that the inhibitor malate and the substrate PEP are capable of altering the sensitivity of the enzyme to malate (13). This is now extended to demonstration that this change in sensitivity is accompanied by changes in oligomeric state. After 11 d storage at −70°C the CAM PEPC existed as a dimer-tetramer mixture—see Figure 2 (+) and the legend for Figure 4. When a dimer-tetramer mixture—see Figure 2 (+) and the legend for
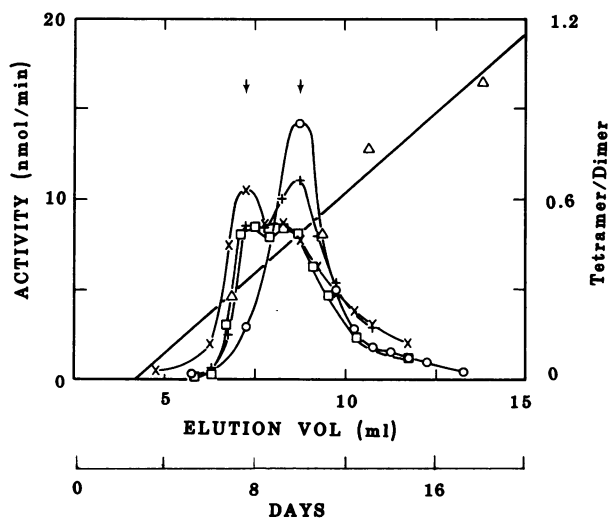

FIG. 1. Activity profile of PEPC stored for 7 d and after 49 d storage at −70°C. HPLC chromatography on a TSK 4000 size exclusion column. Note change from an equilibrium consisting primarily of the dimeric form of the enzyme (O) to one in which the tetramer predominates at the end of the storage period (+).

FIG. 2. Progressive aggregation of CAM PEPC stored at −70°C. Activity profile from HPLC chromatograms run as in Figure 1. (O) Stored 7 d; (+) stored 11 d; (□) stored 13 d; (×) stored 18 d. (△) Ratio of tetramer/dimer integrated from activity profiles plotted against days of storage at −70°C.
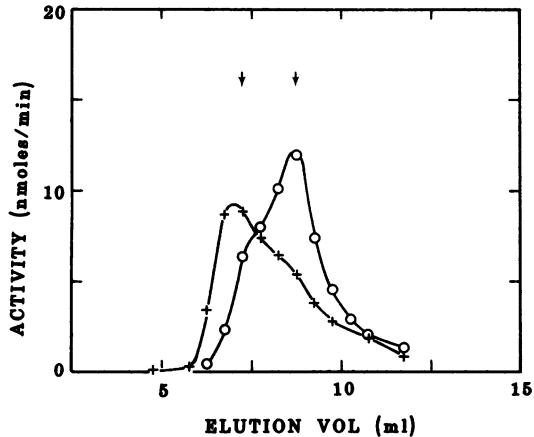
FIG. 3. Activity profiles from HPLC chromatograms of PEPC stored at −70°C for 11 d (see Figure 2 [+]), then preincubated 10 min with 5 mM malate (O) or 10 min with 33 mM PEP (+).
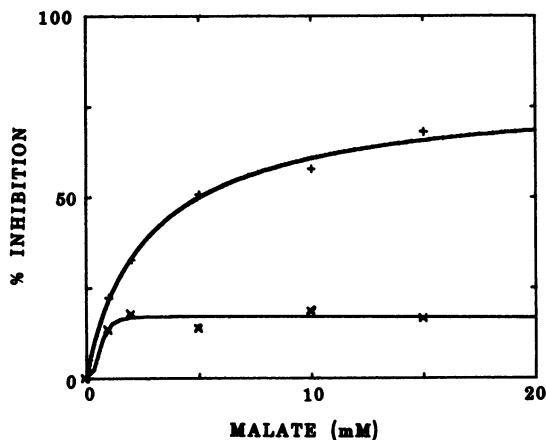

FIG. 4. Malate isotherms of PEPC isolated from peak fractions of Figure 3. Preincubated with 5 mM malate (+), maximal inhibition = 80%, $K_{i(mal)}$ = 2.86 mM or preincubated with 33 mM PEP (×) maximal inhibition = 17.2%, $K_{i(mal)}$ = 0.66 mM. For the control enzyme before preincubation treatments maximal inhibition = 43% and $K_{i(mal)}$ = 1.71 mM.

Figure 4. When this PEPC is preincubated with 5 mM malate for 10 min at pH 6.8 and 0°C the inhibition by malate of the activity of enzyme is enhanced—see Figure 4 (+)—and the oligomeric equilibrium is shifted toward the dimeric form—see Figure 3 (o). The same enzyme when preincubated with 33 mM PEP showed a lower level of malate inhibition at varying concentrations of malate—Figure 4 (×)—and a shift in the equilibrium toward the tetrameric form—Figure 3 (+). This juxtaposition of changes in aggregation state with shifts in sensitivity to malate inhibition clearly demonstrates the association of these phenomena.

DISCUSSION

The present study has provided further evidence that PEP carboxylase from *Crassula* leaves exists as both dimer and tetramer, that during storage at −70°C the dimeric form showed a strong tendency to aggregate to the tetramer and this aggregation was associated with less sensitivity to inhibition by malate. The dimer-tetramer interconversion was reversible and the equilibrium could be shifted toward the dimeric/malate-sensitive form by preincubation with malate, or toward the tetrameric/malate-insensitive form by preincubation with the substrate PEP. A recent report (8) attributes such a storage change as well as

diurnal changes in sensitivity to malate to phosphorylation of the enzyme and to the interconversion of different sized subunits in PEPC from *Bryophyllum*. We have earlier reported (13) some evidence that factors favoring phosphorylation appear to increase the resistance of *Crassula* PEPC to malate inhibition, but we find that this enzyme has only a single subunit (M_r = 100,000) (17) regardless of its oligomeric state or sensitivity to malate.

The present studies show that the oligomeric equilibrium of PEPC can be shifted toward either dimer (by malate) or tetramer (by PEP) without any obvious requirement for a covalent modification of the enzyme. On the other hand, the shift in molecular size is not complete with either of these treatments so the possibility of phosphorylation as a means of regulating this enzyme recently suggested by Nimmo *et al.* (7, 8) and Brulfert *et al.* (1) must be considered. These workers proposed that the daily change of PEP carboxylase properties in CAM metabolism could be due to phosphorylation-dephosphorylation of the enzyme. Budde and Chollet (2) also reported that PEP carboxylase from *Zea mays* was phosphorylated *in vitro* and their study of the enzyme aggregation state indicated that the day enzyme would dissociate when diluted, whereas the enzyme collected at night could not be dissociated. The question of whether modification of the enzyme by phosphorylation-dephosphorylation causes the change in the oligomeric status of the enzyme *in vivo* remains open. It seems clear that the enzyme is phosphorylated, although the evidence so far presented does not appear to demonstrate a complete association of the phosphorylation state of the enzyme with it's sensitivity to malate.

Huber *et al.* (3) have reported that the maize leaf PEPC exists in the cell as a tetramer of a single subunit and that subtle changes can influence both enzyme activity and sensitivity to malate inhibition. Some unpublished studies of the C_4 PEPC in our laboratory suggest that the maize enzyme may be regulated quite differently from the *Crassula* enzyme.

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