Catalytic Activity of Maize Leaf Phosphoenolpyruvate Carboxylase in Relation to Oligomerization

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ABSTRACT

The relationship between the state of oligomerization and activity of purified maize leaf phosphoenolpyruvate carboxylase using size exclusion high performance liquid chromatography was examined. Maximum activities of 35 to 38 micromoles per minute per milligram protein were found when 100% of the enzyme was in its tetrameric form. The effects of the sulphydryl group modifiers CuCl2 and ρ-chloromercuribenzoate on enzyme inhibition and the state of aggregation of the protein complex were examined. Aggregation of the enzyme is temperature and pH sensitive with low temperature and high pH favoring depolymerization. Stability of the tetrameric form is largely dependent upon histidyl residues, and to some extent this explains the biphasic response of enzyme activity to changes in MgCl2 concentrations. Modification of the tetramer’s histidyl residues by the inhibitor diethylpyrocarbonate (0.125 millimolar) results in its dissociation to the dimeric form and loss of activity. Subsequent treatment with 0.4 molar hydroxylamine results in reassociation to the tetramer and restoration of enzymic activity.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) is found in a variety of organisms and catalyzes the irreversible carboxylation of PEP² to form oxalacetate. In C₄ and CAM plants PEP carboxylase catalyzes the initial carboxylation of atmospheric CO₂ photosynthesis.

PEP carboxylase requires a divalent metal cation for catalysis (17), for which a precise mechanism is still unknown (16, 18). This enzyme is also unique in that it does not contain biotin, but uses HCO₃⁻ in place of CO₂ as a substrate (4).

In C₄ and CAM plants, PEP carboxylase appears to be highly regulated. When isolated from these species, it exhibits allosteric activation in the presence of glucose-6-P (3, 18). This activation may favor utilization of PEP for PEP carboxylase rather than for gluconeogenesis. Similarly, PEP carboxylase is inhibited by two C₄ dicarboxylic acids, malate and oxalacetate, which indicates the possible existence of a negative feedback mechanism (7, 12, 19, 25).

Previous reports have shown that thiol (1, 8, 9, 13, 14, 22–24), histidine (11), and arginine (10) residues are essential for PEP carboxylase activity. Evidence that thiol groups are essential for PEP carboxylase activity has been provided by studies using various inhibitors such as diamiide, PCMB, CuCl₂, CdCl₂, and NEM (8, 9, 23).

We have used size-exclusion HPLC to examine the effects of thiol and histidine modifiers on maize leaf PEP carboxylase activity in relation to changes in the oligomeric assembly of the enzyme’s subunits. Evidence is also presented which suggests that the degree of biphasic behavior of PEP carboxylase previously reported (17) with increasing concentrations of MgCl₂ is the result of histidine residue modification.

MATERIALS AND METHODS

Plant Material and Partial Purification of PEP Carboxylase. Zea mays was grown in trays containing a commercial premix (55% peat moss, 30% pumice, and 15% soil). The plants were harvested after 3 weeks of growth in a controlled growth chamber with a temperature and photoperiod regime of 27/22°C and 14/10 h, respectively. Light was provided by a combination of fluorescent and incandescent lamps, resulting in a photosynthetic photon flux density of 350 to 400 μE·m⁻²·s⁻¹. Plants were watered and fertilized every 2 d.

PEP carboxylase was extracted from maize leaves according to the method of Uden and Sugiyama (26) using 25 mM Mops (pH 6.8) in place of the Tris buffer. The final 70% saturated (NH₄)₂SO₄ precipitate was desalted using a Sephadex G-25 column before loading on a DEAE-cellulose column. The DEAE column was first eluted with 80 mM, then 120 mM KCl in 25 mM Mops (pH 6.8). The fractions containing high PEP carboxylase activity were combined and precipitated with 60% saturated (NH₄)₂SO₄. The protein precipitate was collected by centrifugation at 15,000 × g for 15 min. The resulting pellet was redisolved in a minimal volume of buffer containing 10 mM Mops (pH 6.8), 25% (v/v) glycerol, 10 mM MgCl₂, 2 mM DTT, and stored at −20°C.

As protein was needed, 2 ml aliquots (approximately 2000 IU) of the enzyme from DEAE-cellulose preparations were desalted using a Sephadex G-25 column equilibrated with 10 mM Mops (pH 6.8). The protein fraction was further purified using size exclusion, high performance liquid chromatography (HPLC). The fractions containing the tetramer were collected and were 90 to 95% homogeneous as determined by gel electrophoresis. In the following discussion, this preparation will be referred to as the native protein. Details concerning the HPLC procedures are presented later.

Enzyme Activity. Enzyme activity was determined spectrophotometrically by monitoring NADH oxidation at 340 nm by coupling the PEP carboxylase reaction to NADH-malate dehydrogenase at 25°C. The standard 3 ml assay medium contained 25 mM Tricine (pH 8.4), 4 mM MgCl₂, 0.2 mM NADH, 10 mM KHCO₃, 2 IU malate dehydrogenase, 3 mM PEP, and 5 μg of PEP carboxylase. Other additions to the assay will be referred to as needed.

Protein Measurement. Total protein concentration was determined according to Bradford (2), using BSA as the standard. The relative amount of the PEP carboxylase in tetrameric, dimeric, and monomeric forms was determined at 214 nm by adapting
the method of Scopes (21).

Modification of Thiol Residues by PCMB. Inactivation of PEP carboxylase by PCMB was accomplished by using the procedure of Stiborova and Leblova (23). The modifications were performed at the indicated temperatures in 1 ml volumes of either 25 mM Tricine (pH 8.4) or 25 mM Mops (pH 6.8), with the indicated PCMB concentrations and 150 μg of PEP carboxylase. At the end of the indicated preincubation periods, 0.1 ml of the reaction medium was removed and added to 2.9 ml of the assay medium and the activity measured. At the same time, another 0.1 ml of the modified protein was mixed with an equal volume of 20 mM sodium phosphate (pH 6.8), 100 mM Na2SO4, 20% (v/v) acetonitrile, and 5% (v/v) glycerol. A portion of this (0.1 ml) was then injected to the HPLC column and the chromatogram recorded. This same procedure was used for all protein chromatograms.

Modification of Histidine Residues by DEPC. Inactivation of PEP carboxylase by DEPC was performed at 25°C in 1 ml reaction volumes containing 25 mM Mops (pH 7.5) and 0.5 mg protein. The reaction was terminated at the indicated times by adding 10 mM histidine (pH 7.5), following which a 0.03 ml aliquot of the modified enzyme was added to 2.97 ml of the assay medium and the activity recorded.

Before the DEPC modified PEP carboxylase was monitored to determine the restoration of activity by hydroxylamine, the enzyme was first centrifuged through a Sephadex G-25 column according to the method of Saul and Don (20). The eluate containing the protein was then added to an equal volume of 0.8 M hydroxylamine (pH 7.5) and incubated at 25°C.

Size Exclusion HPLC Chromatography. The HPLC system consisted of a Shimadzu LC-4A liquid chromatograph with a 200 μl injection loop and a UV detector (Beckman 160). All chromatograms were taken at 214 nm unless otherwise indicated. Analysis was done at a flow rate of 18 ml/h and a chart speed of 20 cm/h. The HPLC column was a Bio-Sil TSK-400 (200 × 7.5 mm) purchased from Bio-Rad. This column has the theoretical capability to separate proteins between 5 and 1000 kD.

The recommended mobile phase was isocratic and consisted of 10 mM sodium phosphate (pH 6.8) and 50 mM Na2SO4. The mobile phase was filtered under vacuum through a 0.22 μm Millipore filter (type GS, 47 mm diameter). After filtration, the mobile phase was heated to 50°C and thoroughly degassed under vacuum. Additional degassing of the mobile phase was performed on a daily basis. The HPLC column temperature was maintained at 25°C using a thermostatically-controlled water jacket.

Recovery studies were carried out using commercially prepared protein standards as well as laboratory prepared PEP carboxylase first equilibrated with the mobile phase. The results were obtained by comparing the peak area of the samples injected and eluted through the column with the area obtained when a stainless steel capillary tube was used in place of the column. Recoveries were greater than 90%. Evaluations of the interconversions between the different forms of the enzyme were made by estimating the peak areas on the chromatograph of the tetramer, dimer, and monomer; and, in some cases, the degree of interconversion was compared with the change in enzyme activity.

Reagents. Acetonitrile (HPLC grade) was obtained through J. T. Baker. All other chemicals and protein standards were purchased from Sigma.

RESULTS AND DISCUSSION

Recently, we have demonstrated that tetrameric (400 kD aggregate), dimeric (200 kD), and monomeric (100 kD) forms of the maize leaf PEP carboxylase can be separated using SEHPLC and the hydrophobic, hydrophilic and ionic character of these forms were described (27). This finding made it possible to examine enzyme activity versus state of oligomerization under various conditions including modification of the PEP carboxylase protein during oxidation of thiol residues by PCMB and during alteration of histidine residues by DEPC, that inhibition by PCMB is more effective at pH 8.4 and 0 to 4°C (t0.5 = 7 min) than at 6.8 and 25°C (t0.5 = 12.6 min); and that loss of activity qualitatively follows the conversion of the tetramer to dimer.
Once the majority of the original protein is in the dimer-monomer form more than 90% of the activity is lost. Further evidence for the tetramer being the active form of maize PEP carboxylase was obtained with the cyclopropane analog of PEP (phosphocyclopropanol carboxylic acid), a potent inhibitor of PEP carboxylase (18). When this PEP analog complexed with the tetramer, an approximate 50% increase in absorption at 214 nm resulted. No increase in absorption could be seen in the accompanying dimer or monomer peaks during SEHPLC.

Similar experiments were performed using the thiol inhibitor, CuCl₂. The inhibition of PEP carboxylase by 15 μM CuCl₂ and its subsequent reactivation by EDTA has been shown by Iglesias and Andreo (8), although they did not examine the effect of CuCl₂ on the state of polymerization. In the present study, there were no changes in degree of polymerization of the enzyme during the time course of inhibition by 15 μM CuCl₂ and reactivation with 1 mM EDTA (data not shown). This suggests either that PCMB is complexing with thiol residues that are not available to Cu²⁺ or that the mercurial compound is complexing in a much different manner than the Cu cation, which causes a loss in the tetrameric form. Evidence for the latter interpretation comes from Webb (28). In the case of divalent cation inhibition...
(Cu, Cd, etc.) and reaction with thiol groups to form mercaptides, the metal ion can oxidize two SH-groups simultaneously to form a noncovalent metal complex; whereas with PCMB, the monovalent Hg⁺ ion must pair 1:1 with the thiol group. From the work of Iglesias and Andreo (8) using [³H]NEM, it has been shown that complete inhibition of PEP carboxylase was correlated with the modification of thiol groups, in which four per homotetramer are essential for activity. Perhaps Cu, which complexes twice as many thiol groups as PCMB on a molar basis, causes inhibition of catalysis, but without structural dissociation of the tetramer.

Modification of Histidine Residues by DEPC. It has been shown that the 100 kD subunit of PEP carboxylase contains seven histidine residues (11). However, only two of these seven histidine groups are needed for activity when the native enzyme is titrated with DEPC or reactivated with hydroxylamine. Inhibition of catalysis by preincubation with DEPC can be largely prevented when substrate levels of PEP and 10 mM MgCl₂ are included in the preincubation media (11). Figure 2A shows the time course of loss of activity following the preincubation of maize PEP carboxylase with two different concentrations of DEPC. The reactions were terminated at the indicated times by the addition of 10 mM histidine (see "Materials and Methods") and the remaining activity assayed. Samples of the protein were simultaneously prepared for HPLC analyses. Of particular interest is the difference the two concentrations of DEPC have on the state of aggregation of the enzyme. Figure 2 (B and C) shows chromatograms of the enzyme protein at the various times indicated during the inactivation periods shown in Figure 2A. Figure 2B shows that there was no detectable change in the native protein before (trace a) and after 60 and 120 s treatments with 0.125 mM DEPC (traces b and c). We have seen from Figure 2A that at these time points, there were approximately 45 and 60% losses in activity, respectively. The protein obtained after 120 s pretreatment with DEPC (trace c) was then treated with 0.125 mM PCMB for 10 min at 25°C and a complete transition to the monomer resulted (trace d). Figure 2C is a series of chromatograms of PEP carboxylase following treatment with 0.25 mM DEPC. Within 60 s the native enzyme (trace a) showed a transition to approximately 50% dimer (trace b). This corresponds to a decrease in activity of 70% (Fig. 2A). After 120 s, over 80% of the activity was lost, and this corresponds to a further increase in the fractions of dimer and monomer (trace c). After 5 min, the enzyme (trace d) had been totally converted to the monomer. It should be pointed out that the disappearance of the dimer was not due to changes in absorption at 214 nm caused by the DEPC treatment. Separate experiments were performed to observe absorption changes between 300 and 210 nm. Although there were considerable changes in absorption between 240 to 255 nm and at 280 nm, there were no corresponding fluctuations at 214 nm. These experiments indicated that hydroxylamine treatments did not result in anomalies at 214 nm (15).

Figure 3A shows that addition of either 10 mM MgCl₂ or 5 mM PEP, or a combination of both, can prevent the inhibition by 0.25 mM DEPC to various degrees. Glycine, a known activator of PEP carboxylase from maize, showed no significant protection against DEPC inactivation. The addition of 10 mM MgCl₂ extended the half-time of inactivation from approximately 35 to 60 s. The presence of the substrate PEP further increased the time for 50% inhibition to 150 s. In combination, the presence of 10 mM MgCl₂ and 5 mM PEP resulted in only a 15% inhibition by 0.25 mM DEPC after 150 s.

Figure 3B is a series of chromatograms showing the relative forms of PEP carboxylase after 150 s treatments with 0.25 mM DEPC, with or without Mg²⁺ and PEP. Exposure of the native enzyme (trace a) to 0.25 mM DEPC without MgCl₂ or PEP resulted in a complete conversion to the monomer (trace e). When the native enzyme was exposed to 0.25 mM DEPC in the presence of 5 mM PEP and 10 mM MgCl₂ (trace b), the enzyme was largely retained in the tetrameric form with a small amount of dimer (less than 20% based on peak area). This increase in dimer formation in comparison to the non-DEPC treated sample (trace a) paralleled a similar decrease in activity as shown in Figure 3A. When the native enzyme was exposed to DEPC and 5 mM PEP, about 50% of the enzyme was retained in the tetrameric form and 50% in dimer (trace c), and with this treatment, the enzyme lost about half of its activity (Fig. 3A). Addition of MgCl₂ with DPEPC treatment (trace d) was not as effective as PEP (trace c), in maintaining the enzyme in the tetrameric form and there was a corresponding greater loss of activity with MgCl₂ alone (Fig. 3A).
These results indicate that the substrate (PEP) and MgCl₂ maintain the enzyme in its tetrameric form and prevent inactivation of the enzyme by binding to the histidyl residues and preventing their modification by DEPC. This also suggests, indirectly, that endogenous metabolite levels may facilitate keeping the enzyme associated.

It is apparent that the addition of 10 mM histidine can prevent the further inhibition of PEP carboxylase by DEPC (Fig. 2A). It has also been shown that treatment of PEP carboxylase with 0.4 M hydroxylamine after modification with 0.125 mM DEPC reacti- vates the enzyme to its original level of activity (11).

It was noted earlier (Fig. 1) that the native purified enzyme existed as a tetramer/dimer mixture. Experiments were performed to determine if preincubating maize PEP carboxylase with 0.4 M hydroxylamine had any effect on the native protein’s activity and aggregation. Preincubating the native enzyme with 0.4 M hydroxylamine and 5 mM DTT for 40 to 60 min increased the activity approximately 1.5-fold (Fig. 4A). Subsequent experiments showed that the increase in activity was maximized within 20 min if 5 mM PEP and DTT were present during the hydroxylamine modification (Fig. 4A). The activation was dependent upon the presence of 0.4 M hydroxylamine, since DTT and PEP alone did not activate the enzyme. Protein samples were taken during these periods of reactivation and chromatographed. Figure 4, B and C, shows there was dimer present in the native protein (trace a) which was converted to tetramer by the time maximum activity was reached (60 min with DTT and 30 min with DTT and PEP). When sampled after 10 min of treatment with hydroxylamine and DTT, approximately 50% of the PEP carboxylase dimer was converted to tetramer (Fig. 4B, t₀), and the activity had increased by 15%. The hydroxylamine-treated sample with DTT plus PEP showed a similar conversion of dimer to tetramer and an increase in activity of 35% (Fig. 4C, t₀). Thus, there was not a direct correlation between increase in the tetramer and increase of activity. The 20 and 30 min time points (Fig. 4, B and C, t₂₀ and t₃₀) show additional decreases in dimer with increasing activity. The lack of a strict stoichiometry between dimer/tetramer conversion and change in activity may suggest that a specific conformational form of the tetramer is required for enzyme activity with subtle changes around the active site.

Effects of MgCl₂ on Kinetics. Metal ions, particularly Mg²⁺, are essential for PEP carboxylase activity. Several years ago, Mukerji (17) reported that Mg²⁺ binds at a regulatory site on the enzyme and serves as an activator. Also, biphasic kinetics were obtained with varying concentrations of Mg²⁺, although this has not always been observed in other studies (18). In the present study, we found that PEP carboxylase activity can exhibit biphasic kinetics at pH 7.2 and 8.3 with increasing concentrations of MgCl₂ (note the response is to total Mg rather than free Mg since some of the cation will be complexed by PEP and Tricine). The degree of biphasic behavior increased with the number of freeze-thaw cycles the enzyme had experienced. Experiments were performed to determine if the sulfhydryl and/or histidine groups play a role in Mg²⁺ activation and if the activation could be correlated with the state of aggregation of the enzyme. Figure 5A shows the activity response of maize PEP carboxylase to increasing concentrations of MgCl₂ at pH 8.4. The enzyme which had previously undergone five freeze-thaw cycles under liquid N₂ exhibited typical biphasic kinetics. When the enzyme was first modified with 0.4 M hydroxylamine for 60 min at 25°C (pH 7.5) following the freeze-thaw cycles, titrating with MgCl₂ resulted in a hyperbolic response (Fig. 5A) which was dependent upon the presence of 5 mM DTT (not shown). An increase in specific activity to 37 μmol-min⁻¹·mg⁻¹ protein also resulted from pretreatment with hydroxylamine. To further consider a possible role of histidine groups with respect to MgCl₂, the native enzyme (once thawed) was preincubated with 0.10 mM DEPC for 60 s and the reaction terminated with addition of 10 mM histidine. A portion of the enzyme was then treated with 0.4 M hydroxylamine as previously described. Another equal volume of the DEPC-treated protein was diluted with the same volume of 10 mM HCO₃⁻, 5 mM DTT, and 5 mM PEP without MgCl₂. Figure 5B shows the results of these treatments with respect to the enzyme activity. The hydroxylamine treated sample showed only slight biphasic behavior and, with 0.6 mM MgCl₂, its rates were saturated and approached those shown in Figure 5A of 36.5 μmol-min⁻¹·mg⁻¹ protein. The portion of the enzyme suspension not treated with hydroxylamine showed a definite strong biphasic response to MgCl₂. The activity first became saturated between 0.4 and 0.87 mM MgCl₂ and again at 1.2 mM MgCl₂. It can also be seen that its activity never reached the values of the hydroxylamine-treated enzyme but saturated at 27 to 28 μmol·min⁻¹·mg⁻¹ protein. An examination of the enzyme under these conditions using
SEHPLC did not reveal changes in the tetramer and dimer ratios which could account for the biphasic behavior. With the non-hydroxylamine treated protein, the percentage of dimer (50%) remained constant with varying concentrations of MgCl₂. The hydroxylamine-treated enzyme showed only the tetramer form as we have previously seen from Figure 4, B and C.

In summary, the state of aggregation of PEP carboxylase in vivo and the activity of its different forms is of considerable interest from a standpoint of regulation. It is generally considered that the holoenzyme is a tetramer of approximately 400 kD with homogenous dimer and monomer subunits (18). The results of the present study with the maize PEP carboxylase indicate that the tetramer is the active form, while the dimer has little or no activity and the monomer is inactive. Also, the results suggest there may be at least two populations of dimers and that one population is more likely to dissociate following treatment with PCMB (Fig. 1). In this respect, it is of interest that Hague and Sims (5) observed two bands of PEP carboxylase from maize leaves on SDS polyacrylamide gradient slab gels which may represent slightly different natural subunits of the holoenzyme. We have presented evidence that suggests the high stability of the maize PEP carboxylase at 25°C and lower pH values reported by Hatch and Oliver (6) may be due to an increased susceptibility of thiol groups to oxidation at lower temperatures and more alkaline pH values (Fig. 1, B and C). While PCMB treatment resulted in dissociation of the enzyme to dimers and monomers, treatment with the reducing agent DTT (5 mM) did not increase the state of aggregation.

Thiol groups are not the only essential residues controlling maize PEP carboxylase activity and aggregation. The histidine modifier DEPC similarly inhibits activity by dissociating the tetramer into the smaller 200 and 100 kD subunits. Our
work shows, however, that there exists a cooperative effect between thiol and histidine residues. Treatment with 0.125 mM DEPC results in conversion from tetramer to dimer with a concomitant decrease in activity which can be recovered with 0.4 mM hydroxylamine (Figs. 2A and 5B). At higher concentrations of DEPC (0.25 mM), the enzyme is irreversibly denatured as the result of the complete dissociation to the monomer (Fig. 2C). This difference between the two concentrations of DEPC may be the result of the modifier also reacting with sulfhydryl groups (15).

Protection against 0.25 mM DEPC inhibition of the enzymic activity can be obtained by including PEP and MgCl₂ into the reaction media (Fig. 3A). Corresponding HPLC chromatograms revealed that the combination of PEP and Mg²⁺ protected the enzyme complex from denaturing to its subunits. Under conditions of intermediate protection by either PEP or MgCl₂ alone, the degree of inhibition could be correlated with the degree of dissociation of the tetramer. These results, besides indicating that histidine residues are protected by PEP and MgCl₂, suggest that this substrate cofactor complex may control the state of aggregation of the enzyme (Fig. 3, A and B).

The substrate PEP, by binding to the active site, may stabilize the tetrameric form of the enzyme as seen during activation in the presence of hydroxylamine and DTT (Fig. 4A). The presence of 5 mM PEP decreased the activation time from 40 min to 20 min. It is also apparent from Figure 4, B and C, that this activation corresponded qualitatively to a decrease in dimer, and maximum activities were only realized when the enzyme aggregate was in the tetrameric form.

These results suggest that if optimal conditions for thiol and histidine residues on the enzyme can be maintained in vivo, activation of the enzyme in the light could be due to the presence of substrate alone. In the case of thiol groups, Iglesias and Andreo (9) have shown that PEP, malate, and oxalacetate all provide protection against NEM inhibition. Malate and oxalacetate will not prevent inhibition by DEPC whereas PEP does (11). While it is speculative at best, this implies that there are common groups of thiol and histidine residues needed for full activity and binding of the PEP trianion. This binding may involve Mg as well as histidine residues for stabilization of the tetramer, assuming the accompanying sulphydryl groups are reduced.

We found that by subjecting the enzyme to several freeze-thaw cycles, the biphasic response to MgCl₂ could be enhanced (Fig. 5). Qualitatively, at least, these changes could be duplicated by treatment with DEPC. However, we could not determine any changes in the tetramer/dimer ratio which correspond to the kinetics changes by either of the two treatments. Treatment with hydroxylamine resulted in a homogenous tetramer population and the enzyme's response to MgCl₂ became hyperbolic. This implies that in vivo, the enzyme probably does not exhibit these biphasic responses, but they may be due to artifacts of preparation and/or subsequent treatments.

An important finding of this work is that the tetramer is the active form of maize PEP carboxylase. As more dimeric forms, activity tends to decrease accordingly. It is interesting to note in this context that Wu and Wedding (29) have shown that PEP carboxylase isolated from the CAM plant Crassula argentea exists mainly as a tetramer in the dark period and as a dimer in the light. Under limiting levels of PEP, maximum activity occurs with the tetramer, and oligomerization may account for the dark activation of the enzyme. In contrast to these results with the CAM plant enzyme, the dimeric form of the enzyme of maize has little or no activity. Experiments are presently under way to determine the forms of PEP carboxylase which exist in vivo in the C₄ plant maize.

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