On the Molecular Mechanism of Maize Phosphoenolpyruvate Carboxylase Activation by Thiol Compounds

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ABSTRACT

Incubation of purified phosphoenolpyruvate carboxylase from Zea mays L. leaves with dithiothreitol resulted in an almost 2-fold increase in the enzyme activity. The activated enzyme showed the same affinity for its substrates and the same sensitivity with respect to malate and oxalacetate inhibition. The activation induced by dithiothreitol was reversed by diamide, an oxidant of vicinal di thiols, suggesting that the redox state of disulfide bonds of the enzyme may be important in the expression of the maximal catalytic activity.

Titrations of thiol groups before and after activation of maize phosphoenolpyruvate carboxylase by dithiothreitol shows an increase of the accessible groups from 8 to 12 suggesting that the reduction of two disulfide bonds accompanied the activation. The thiol groups replaced by dithiothreitol were available to reagents in non-denatured enzyme and two of them were reoxidized to a disulfide bond by diamide.

It is concluded that the mechanism of phosphoenolpyruvate carboxylase activation by dithiothreitol involves the net reduction of two disulfide bonds in the enzyme.

Phosphoenolpyruvate carboxylase catalyzes the reaction of PEP with HCO₃⁻ to form oxalacetate. The enzyme is widely distributed among a variety of organisms. In C₄ and Crassulacean plants, the carboxylase catalyzes the primary carbon fixation for the photosynthetic pathway in mesophyll cells, whereas in bacteria, algae, and C₃ plants, it plays an auxiliary role.

PEP carboxylase requires a divalent metal ion for activity, but the role in catalysis is still a matter of some controversy. Recently, Manetas and Gavallas suggested that the activity of PEPP carboxylase from C₄ and Crassulacean (13) plants might be regulated in vivo by a reversible redox change of sulphydryl groups. Moreover, incubation of partially purified PEP carboxylase from maize (4) and Amaranthus viridis (22) with DTT resulted in an almost 2-fold increase in the enzyme activity while p-iodosobenzoate, an oxidant of vicinal di thiols, induced a total inactivation of the enzyme (22) pointing out the role of di thiols in the activity of the enzyme.

In the present work, we have examined the mechanism of activation of PEP carboxylase purified from maize leaves by incubation of the enzyme with DTT at room temperature. The effects of substrates and modulators on the DTT-activated enzyme as well as the content of free and accessible thiol groups of the enzyme are described.

MATERIALS AND METHODS

Materials. PEP (monopotassium salt), DTT, diamide, iodoacetate, iodoacetic acid (sodium salt), NADH, cis-oxalacetic acid, porcine heart malic dehydrogenase, and N-ethylmaleimide were obtained from Sigma Chemical Co.

The N-[ethyl-2-¹H]maleimide (300 Ci/mol) was purchased from New England Nuclear, 2,5-diphenyloxazole and naphthalene were from Beckman, and 1,4-dioxane was from Baker Chemical Co. All other reagents were of analytical grade.

Enzyme Purification and Assay. PEP carboxylase was extracted from leaves harvested from Zea mays L. (Simple hybrid, 252) of about 1 month of age. The enzyme was purified according to O'Leary et al. (17) except that Sepharose 6B and sucrose density gradient centrifugation were done instead of Sephacryl S-200 and hydroxylapatite chromatography steps (7).

The fractions containing PEP carboxylase activity recovered from the DEAE-cellulose column were precipitated with 60% saturated (NH₄)₂SO₄, centrifuged at 15,000 g for 15 min, and redissolved in a minimal volume of buffer containing 50 mM Tris-HCl (pH 7.3), 0.1 mM EDTA, 10% glycerol, and 10 mM β-mercaptoethanol. This concentrated enzyme was chromatographed on a 2×80-cm Sepharose 6B column that had been equilibrated with the same buffer. Two-mL fractions were collected and those with PEP carboxylase activity were pooled and precipitated with (NH₄)₂SO₄ (60% saturation).

After centrifugation at 15,000 g for 15 min, the pellet was redissolved in a small volume of 50 mM Tris-HCl (pH 7.3), 1 mM EDTA, and 10 mM β-mercaptoethanol and desalted by Sephacryl G-50 column chromatography. Then the protein (5 mg/ml) was loaded on a linear sucrose gradient (10–30%) prepared in the same buffer. After centrifugation at 180,000 g for 135 min at 25°C in a Sorvall OTD-65 ultracentrifuge, the band of PEP carboxylase activity was collected and the sucrose eliminated by dialysis overnight at 4°C against 1,000 volumes of 50 mM Tris-HCl (pH 7.3), 1 mM EDTA, and 10 mM β-mercaptoethanol. Finally, the purified enzyme was precipitated with (NH₄)₂SO₄ at 60% saturation and stored in liquid N2. Under this condition, the enzyme was stable for at least 6 months. Purified enzyme had a specific activity of 22 to 25 μmol·min⁻¹·mg⁻¹.

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2 C. S. A. is a Member of the Investigator Career of the Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Argentina, and A. A. I. is a Fellow of the same institution.
3 Abbreviations: PEP, phosphoenolpyruvate; MOPS, 4-morpholinepropanesulphonic acid.
Table 1. Modulation of PEP Carboxylase Activity by Light and Thiol Compounds

PEP carboxylase was extracted from maize leaves maintained in darkness or preilluminated at 25°C for 30 min by a 150-w tungsten lamp through 2 cm of a 1% solution of CuSO4, as heat-absorbing filter, essentially as described in the text except that β-mercaptoethanol was omitted. The leaf (1 g) was rapidly cut into pieces and homogenized at 0°C for 45 s in a high speed homogenizer in 10 ml of 100 mM Tris-HCl (pH 7.3) and 4 mM EDTA. Cellular debris was eliminated by filtration through a nylon cloth and the filtrate centrifuged at 12,000g in an Eppendorf microcentrifuge for 30 s. The PEP carboxylase activity of the crude extract was determined in 50-μl aliquots of the supernatant. The PEP carboxylase purified and kept as described in the text was desalted by centrifugation through Sephadex G-50 column equilibrated with 50 mM MOPS-NaOH (pH 7) plus or minus 10 mM β-mercaptoethanol before the assay of the activity.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PEP Carboxylase Activity (μmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>0.19</td>
</tr>
<tr>
<td>4</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>24.6</td>
</tr>
<tr>
<td>6</td>
<td>12.6</td>
</tr>
<tr>
<td>7</td>
<td>23.3</td>
</tr>
</tbody>
</table>

Fig. 1. Activation of PEP carboxylase by DTT. PEP carboxylase was preincubated at 25°C with the concentration of DTT stated (figures on the slope, mM) as described in the text. At the indicated times, aliquots were withdrawn and assayed for activity.

Enzyme activity was determined spectrophotometrically at 30°C monitoring NADH oxidation at 340 nm in a UNICAM SP 1800B spectrophotometer by coupling the PEP carboxylase reaction with malic dehydrogenase. The standard assay medium contained 50 mM Tris-HCl (pH 8), 5 mM MgCl₂, 0.15 mM NADH, 10 mM NaHCO₃, 2 IU malic dehydrogenase, 4 mM PEP, and PEP carboxylase (4 μg of protein) in a total volume of 1 ml.

The enzyme activity was also assayed following oxalacetate formation at 272 nm using an ε of 910 m⁻¹ cm⁻¹ (8, 27). The assay medium was the same as the above described except that NADH and malic dehydrogenase were omitted.

Protein Measurement. Total protein concentration was determined by colorimetric method of Lowry et al. (11) or alternatively by the Coomassie Brilliant Blue binding method (23), using BSA as standard.

DTT Treatment of PEP Carboxylase. Enzyme (1 μM) was incubated with different concentrations of DTT in 50 mM MOPS-NaOH (pH 7) at 25°C. Aliquots were withdrawn at different times and diluted 1000-fold in the assay medium. Controls were similarly treated except that DTT was absent. The addition of DTT (1000-fold diluted) in the assay medium had no effect on the enzyme activity. In some experiments, the excess of DTT was removed from the preincubation medium according to Peneffsky (19).

Titration of Accessible and Free Sulfhydryl Groups of PEP Carboxylase. Radiochemical titration of sulfhydryl groups was performed by incubation of PEP carboxylase (0.5 mg of protein/ml) with 5 mM N-[³H]ethylmaleimide (9.1 × 10⁶ cpm/ml) for 2 h at 25°C in a medium (0.3 ml) containing 100 mM MOPS-NaOH (pH 7) and 1 mM EDTA. Then SDS (1%) or urea (8 M) was added and the mixture was further incubated for 2 h. Finally, the excess of N-[³H]ethylmaleimide was eliminated by two consecutive centrifugations in Sephadex G-50 fine columns (19) equilibrated with 100 mM MOPS-NaOH (pH 7) and 1 mM EDTA. Protein concentration and N-[³H]ethylmaleimide incorporation were determined in aliquots of the desalted protein.

Radioactivity incorporated was determined in a Beckman LS 8100 liquid scintillation counter using a mixture of 0.25% 2,5-diphenyloxazole, 10% naphthalene in dioxane as the scintillation cocktail.

A mol wt of 400,000 (29) was used to determine the molar concentration of PEP carboxylase.

RESULTS

The activity of PEP carboxylase in a crude extract that was rapidly obtained from illuminated maize leaves ('light' activity) was about 65% higher than the activity of the enzyme in crude
Table II. Titration of Accessible and Free Thiol Groups of PEP Carboxylase

PEP carboxylase was incubated at 25°C for 60 min with the reagents shown. The reagent was eliminated by centrifugation through Sephadex G-50 column before the following treatment and determination of activities. The number of thiol groups available in denatured (free groups) and nondenatured (accessible groups) PEP carboxylase was determined by N\textsuperscript{3}H]ethylenediamine incorporation as described in the text. Values shown are means from three experiments.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment of PEP Carboxylase</th>
<th>PEP Carboxylase Activity</th>
<th>Thiol Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \mu \text{mol min}^{-1} \text{mg}^{-1} \text{ protein} )</td>
<td>mol NEM/mol enzyme*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Accessible groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>12.9</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>DTT (50 mM)</td>
<td>25.7</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>2, then diamide (10 mM)</td>
<td>12.1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Free groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>SDS (1%)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>SDS (1%) plus DTT (50 mM)</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Urea (8 mM)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Urea (8 mM) plus DTT (50 mM)</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

*Values are rounded to the nearest whole number.

The dark activity was enhanced until the level of the activity from illuminated leaves when it was incubated with 50 mM DTT during 5 min at 25°C, while the light activity was not affected by a similar incubation with DTT.

A similar difference was observed when the activity of the purified PEP carboxylase was assayed after filtration through Sephadex G-50 column equilibrated with 50 mM MOPS-NaOH (pH 7) with or without 10 mM \( \beta \)-mercaptoethanol. In this case, the activity of the purified enzyme desalted in the absence of \( \beta \)-mercaptoethanol was stimulated by the incubation with 50 mM DTT (Table I).

Different rates of PEP carboxylase activity were obtained when the purified enzyme was incubated with various concentrations of DTT at 25°C (pH 7) in the absence of EDTA (Fig. 1). The activity of control enzyme was stable during the preincubation time (Fig. 1). Similarly, other thiol compounds such as \( \beta \)-mercaptoethanol, cysteine, DTT, and 2,3-dimercaptopropanol activated purified PEP carboxylase in a time-dependent manner (not shown).

The activity of PEP carboxylase obtained after incubation of the enzyme with DTT was 66 \pm 6% (in 12 experiments) higher than control activity. The presence of MgCl\textsubscript{2} did not alter the stimulation induced by thiol compounds (not shown).

The rate of PEP carboxylase activation appears to be dependent on the reducing conditions of the reaction medium. After removal of the reducing agent, the activity diminished to the initial level (Fig. 2A) and was reduced faster by the addition of diamide, a specific oxidant of vicinal diethiols (9), in the presence or absence of EDTA. On the other hand, EDTA stabilized the maximal PEP carboxylase activity (Fig. 2A) presumably by chelating traces of heavy metal present in the medium. Low concentrations of CuCl\textsubscript{2} or CdCl\textsubscript{2} totally inhibited PEP carboxylase activity (6).

These results clearly suggest that the activation of PEP carboxylase by DTT may be mediated by reduction of disulfide bonds. Diamide may be able to eliminate the activation by oxidation of the newly formed vicinal diethiols. Blocking these groups with
iodoacetamide or iodoacetate would prevent oxidation and reversion of activation. Figure 2B shows that this is indeed the case. The incubation of DTT-activated PEP carboxylase with iodoacetamide or iodoacetate resulted in a lower rate of loss of enzyme activity (Fig. 2B). Moreover, this loss of activity presumably is a consequence of the modification of some of the thiol groups of the enzyme (1, 14, 25, 26) which would be exposed to the reagent in both forms of it. Thus, the activity of PEP carboxylase may be modulated by the reversible reduction-oxidation of disulfide bonds.

Table II shows the titration of accessible and free thiol groups of the PEP carboxylase. Titration of accessible thiol of the enzyme by labeling with N\textsuperscript{35}Hethylmaleimide shows an increase from 8 to 12 thiols/mol of PEP carboxylase after activation by DTT (Table II, experiments 1 and 2). The subsequent treatment of the DTT-activated enzyme with diamide partially reversed the change induced by DTT (Table II, experiment 3), resulting in the disappearance of two of four new thiol groups exposed during the preincubation with DTT. Thus, the activation of the PEP carboxylase by DTT can be visualized as a consequence of the reduction of two disulfide bonds. Apparently, the oxidation to a disulfide bond of two of the four new thiols is sufficient to reverse activation.

The number of free sulphydryl groups of PEP carboxylase was determined by incorporation of N\textsuperscript{35}Hethylmaleimide to the enzyme denatured by 1% SDS or 8 M urea (Table II, experiments 4 and 6). Under these conditions, we found 12 free thiols/mol of the enzyme. However, when the PEP carboxylase was denatured in the presence of DTT, the number of thiol groups blocked by N\textsuperscript{35}Hethylmaleimide was 16 (Table II, experiments 5 and 7). These results suggest that the PEP carboxylase has a total of 16 thiol groups/mol, four of which involved in the formation of two disulfide bonds (cf. line 4 with lines 4 and 6, Table II). From the 12 free thiols, eight are accessible and the other four are not (cf. line 1 with lines 4 and 6, Table II).

The activation of PEP carboxylase by DTT does not affect the affinity of the enzyme for its substrates. The values for $K_m$ for PEP (0.4 mM), MgCl\textsubscript{2} (0.9 mM), and bicarbonate (0.6 mM) did not change upon activation of the enzyme by DTT while the $V_{max}$ was increased by a factor of 1.65.

Organic acids like malate and oxaloacetate were reported to be inhibitors of the PEP carboxylase (5, 10, 20, 21, 28). The effects of these negative modulators on the DTT-activated enzyme did not differ from those obtained for the native enzyme since they yielded similar $K_i$ values (malate, 5 mM; oxaloacetate, 0.35 mM).

Figure 3 shows that the DTT-activated enzyme could be activated by glucose-6-P, a positive modulator of PEP carboxylases (2). Although the degree of the activation induced by glucose-6-P is markedly different in control and activated enzymes, the concentration of glucose-6-P necessary to achieve 50% of maximal activation was 0.8 mM regardless of the state of the enzyme. This result suggests that the DTT and glucose-6-P effects are not additive.

**DISCUSSION**

PEP carboxylase activity from maize was stimulated upon exposure of the purified enzyme to DTT at room temperature (Fig. 1). The activation did not require divalent cation such as Mg\textsuperscript{2+} and was totally reversed by diamide (Fig. 2).

Titration of thiol groups of the PEP carboxylase shows that it has 16 groups per mol of enzyme. Four of them involved in the formation of two disulfide bonds and the other 12 as free thiols (Table II). From the 12 free thiols, eight were accessible and the other four hidden to sulphydryl reagents.

The number of accessible thiols per mol of PEP carboxylase increased from 8 to 12 after treatment with DTT (Table II), suggesting that the reduction of two disulfide bonds is associated with the stimulation of the enzyme. Moreover, the reoxidation of a DTT-exposed vicinal dithiol by diamide resulted in a loss of the activation (Table II).

The DTT-activated enzyme showed the same affinity for its substrates but the maximal activity increased by a factor of 1.65. On the other hand, the activated form of the enzyme had the same sensitivity with respect to the modulators, malate, oxaloacetate, and glucose-6-P, although a significant difference in the magnitude of the activation afforded by the latter on both forms of the enzyme was observed (Fig. 3). The relatively smaller activation produced by glucose-6-P on DTT-activated enzyme can be interpreted assuming that the allosteric site for this ligand is partially altered.

Changes in the redox state of disulfide bridges in PEP carboxylase may be relevant to the regulation of this enzymic activity in vivo. Of particular interest is the observation made by Slack (24) on the physiological activation by light of PEP carboxylase from *Amaranthus palmeri*. We found a similar activation by light in the activity of PEP carboxylase in crude extracts rapidly obtained from illuminated maize leaves (Table I). Moreover, the activity of PEP carboxylase in crude extracts obtained from leaves previously adapted to the dark was enhanced until the level of the activity of the illuminated leaves by a short incubation with DTT while the latter was not affected by a similar treatment (Table I). The near 2-fold stimulation by light reported by Slack (24) and by us (this paper) is coincident with the stimulation by DTT described for the purified enzyme when it was desalted in the absence of $\beta$-mercaptoethanol (Table I; Fig. 1). Thus, it is conceivable that the light stimulation of PEP carboxylase in vivo can occur through a mechanism involving reversible reduction of disulfide bridges.

**LITERATURE CITED**

THIOl ACTIVATION OF PEP CARBOXYLASE


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