Communication

Role of Cysteine in Activation and Allosteric Regulation of Maize Phosphoenolpyruvate Carboxylase

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ABSTRACT

The effect of 5-5'-dithiobis-2-nitrobenzoate (DTNB) on the kinetic parameters and structure of phosphoenolpyruvate carboxylase purified from maize (Zea mays L.) has been studied. The $V_{max}$ is found to be independent of the presence of this thiol reagent. The $K_m$ is increased upon oxidation of cysteines by DTNB. At a substrate concentration higher than $K_m$ (3.1 millimolar Mgphosphoenolpyruvate), a significant reversible decrease of the activity is observed. Malate has little effect in preventing the modification of these cysteines. The $V$ type inhibition by malate was also studied at a saturating phosphoenolpyruvate level (9.3 millimolar Mgphosphoenolpyruvate). In the presence of 50 micromolar DTNB, up to 60% inhibition is caused by 15 millimolar malate; however, in the presence of both 50 micromolar DTNB and 50 millimolar dithiothreitol (DTT) this inhibition is reduced to 20%. The presence of DTT alone increases the size of the phosphoenolpyruvate carboxylase molecule as determined by light scattering. The activity at nonsaturating substrate concentration is increased by 36% in the presence of DTT. The oligomerization equilibrium between the dimer and the tetrameric form of the enzyme is affected by cysteine. The $K_m$ for the substrate, the sensitivity toward malate, and the size of the enzyme are found to be modified upon incubation in the presence of DTT.

MATERIALS AND METHODS

Chemicals

The trisodium salt of PEP was obtained from Sigma, as was the disodium salt of Glc-6-P. NADH was supplied by Boehringer-Mannheim. All chemicals were of the highest quality commercially available.

Enzymes

Lactic dehydrogenase from hog muscle and malate dehydrogenase from pig heart were purchased from Boehringer-Mannheim.

Enzyme Purification

The PEPC was purified as described earlier (15) from leaves of maize (Zea mays L.) grown in the greenhouse. Assays were carried out using the method previously described using 50 mM Aces, various concentrations of PEP as described later, 5 mM Mg$^{2+}$, 5 mM HCO$_3^-$, 0.2 mM NADH, 1 unit each of malate dehydrogenase, and lactic dehydrogenase in 1 mL volume at pH 7.0 and run at 25°C (15).

Light Scattering

Light scattering measurements were performed at 530 nm, using a SPEX fluorimeter. The measuring chamber was thermostated with a water bath and measurements were made at 25°C.

Sample Preparation

All samples and buffers were filtered through a 0.22 μm membrane (Millipore Millex-GV4) directly into the 1 cm light path cuvette, which was sealed with Parafilm to prevent the introduction of dust. The sample was allowed to equilibrate 10 min at the chamber temperature before beginning the measurements.

Kinetic Data Analysis

The kinetic data were analyzed using a modified form of the Michaelis-Menten equation (2).

SH Titration

The reactivity of enzyme thiol groups toward DTNB was measured at 25°C in a 50 mM Hepes buffer, pH 7. The reaction...
was started by adding a small amount of DTNB to the enzyme solution, and was monitored by following the absorbance at 412 nm, using an ε value equal to 14,100 M⁻¹ cm⁻¹. Controls were made in the absence of the enzyme: DTNB was stable for the period of measurement.

RESULTS

Oxidation of Critical SH Groups by DTNB

When maize PEPC is incubated with 50 μM DTNB, the activity of the enzyme at 3.1 mM MgPEP decreases with time (Fig. 1). After 40 min, it reaches a plateau (not shown). When the reaction is monitored at 412 nm, we observe the titration of up to five sulphydryl groups by a monomeric subunit. The overall titration is slow, and no particular class of cysteines seems to be selected (no rapidly titrated cysteine is observed, for example).

As described in Table I, up to 16.7 sulphydryls can be titrated per monomer of PEPC, in the presence of 5 mM urea. The urea-treated enzyme is completely inactive. No protection against DTNB inactivation is given by either PEP or Glc-6-P (plus or minus Mg²⁺). L-Malate gives about 20% protection against the DTNB inactivation as shown in Table I. The same number of sulphydryls seems to be titrated. About half of the activity of the enzyme is lost after the titration of five sulphydryl groups by DTNB.

Reversibility of the Deactivation Process

PEPC loses activity at 3.1 mM MgPEP when reacted with DTNB (Fig. 1). To know whether the effect observed was due to a decrease of the affinity of the enzyme for its substrate or to a decrease in its intrinsic velocity, the enzyme was incubated 40 min in the presence of buffer, with or without 50 μM DTNB. Then, a PEP isotherm was run. The DTNB-treated enzyme was then incubated 30 min in the presence of 50 mM DTT, and another isotherm was run.

Table I. Lack of Protection against DTNB by Different Ligands

<table>
<thead>
<tr>
<th>Treatment</th>
<th>−SH Titrated/Monomer</th>
<th>Residual Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no DTNB)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>DTNB 50 μM</td>
<td>5.05</td>
<td>54</td>
</tr>
<tr>
<td>+ 5 mM urea</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td>+ MgPEP</td>
<td>5.05</td>
<td>52</td>
</tr>
<tr>
<td>+ MgGlc-6-P</td>
<td>5.05</td>
<td>46</td>
</tr>
<tr>
<td>+ Malate</td>
<td>5.05</td>
<td>64</td>
</tr>
</tbody>
</table>

The results are presented in Table II. The Vₘₐₓ of the reaction remains almost unchanged regardless of the treatment. The Kₘ is increased nearly threefold for the DTNB-treated enzyme, and is reduced 1.7-fold when the DTNB-treated enzyme receives DTT. The Hill number is essentially unchanged by any of the treatments.

Relation between the Size, Activity, and Sulphydryl Groups of the Enzyme

To know whether the oligomeric structure of the enzyme was a function of the status of some cysteine groups, we measured the intensity of the light scattered by PEPC at 25°C in 50 mM Aces buffer at pH 7.0 in the absence and in the presence of 25 mM DTT. Activity was determined in parallel at 3.1 mM MgPEP. Here the presence of DTT caused the light scattering to double (21,000 to 45,000 emission units) while the activity increased by one-third (132%). From this, it is clear that both the light scattered by the enzyme and the velocity of the reaction are increased in the presence of DTT.

Malate Sensitivity

The malate sensitivity of the enzyme at saturating levels of PEP was examined as a function of the state of cysteines. As seen in Figure 2, the DTNB-treated enzyme is very malate sensitive (up to 60% inhibition). When the DTNB-treated enzyme was preincubated a further 10 min with 50 mM DTT, the inhibition was reduced to 20%.
Several cysteines, at least 1 to 5, are involved in the activity of PEPC. The $K_m$ of DTNB-modified PEPC for MgPEP increases from 1.6 to 4.5 mM. Subsequent addition of 50 mM DTT reduces the $K_m$ to 2.7 mM MgPEP. When incubated in the presence of 25 mM DTT, the size of the native enzyme as determined by light scattering increases. This increase in size is associated with an increase of the activity at nonsaturating substrate concentration.

As the quaternary structure of the enzyme appears to be affected by the status of these reactive cysteines, it was of interest to examine the malate sensitivity of the enzyme after the DTNB treatment. In this case, the enzyme is strongly inhibited (60%) by 15 mM malate. But if the same enzyme is additionally treated with DTT, malate inhibition decreases to 20%. Thus modification of cysteines changes such important characteristics of the enzyme as $K_m$, oligomerization state, and sensitivity to malate.

This work confirms the earlier observation that the oligomerization state of PEPC is crucial to regulation of this enzyme (16, 19). It confirms also that some important cysteines are involved in the activity (5, 6, 9, 11) and also in the oligomerization of maize PEPC (13). We believe this is the first attempt to link activity, malate sensitivity, and oligomerization state with the status of cysteine residues in the enzyme.

This work illustrates the importance of the status of thiols to critical aspects of the regulation of PEPC from maize. It is clear that this situation is similar to that described in the case of the reductive activation of some enzymes of the Calvin cycle (10). The possibility that treatments intended for other purposes, such as phosphorylation of the enzyme (8), may affect cysteines critical to regulation must be considered.

Because phosphorylation may change the net charges of the enzyme (12) and perhaps its conformation, then facilitate or prevent aggregation, critical amino acids being exposed to the solvent. This is important at alkaline pH values at which endogenous heavy metals have been found to bind to cysteine (6, 15), and at which the enzyme is very unstable (4, 15). A factor often ignored is that malate is a "mixed" inhibitor of PEPC, so that for information on the competitive inhibition—which appears to be the variable one involved in regulation (14)—it is important to have both substrate and effector at saturating levels. Activity measurements such as these, if they are run at low levels of PEP and malate, are likely to lead to erroneous conclusions regarding the effects on PEPC activity and sensitivity to malate.

The results shown here illustrate that even at a "normal" pH such as 7, there are dangers in concluding from relatively small changes in $K_m$(PEP) or $K_m$(malate) that phosphorylation is controlling the activity of PEPC in the cell. The effects of thiol reagents as shown here indicate that, where conclusions regarding the role of phosphorylation have been drawn from assays using substrate levels less than $K_m$ and a small amount of malate (8), such conclusions must be questioned.

Data currently available give some support to the possibility that both phosphorylation and aggregation/disaggregation may be primarily responsible for the regulation of PEPC. The data presented here raise the further question of whether regulation may be primarily under the control of the oxidation state of certain cysteine groups. Perhaps all of these possibilities have some role in PEPC regulation and perhaps others not yet identified may be participants as well. It seems clear at this point that, of the variety of apparent factors now seen to be involved in PEPC regulation, not all can be primary causes, and perhaps the focus for the near future on these questions should be on the identification of primary and secondary participants in the process. The involvement of thioredoxin in the regulation of PEPC will be an attractive hypothesis for further works, although thioredoxin from C₃ plants has been tried without effect (8).

LITERATURE CITED

Cysteine in Regulation of PEPC


