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PHOSPHOENOLPYRUVATE CARBOXYLASE FROM THE ROOTS OF YELLOW LUPIN (LUPINUS LUTEUS)

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A crude preparation of PEP carboxylase (EC 4.1.1.31) from the yellow lupin roots exhibits the pH optimum of activity within the range of 7.4 - 8.6 and the temperature optimum at 32 - 40°C. Its $K_m$ for PEP is 0.1 mM, and $K_m$ for HCO$_3^-$ is 0.7 mM. The affinity of the enzyme towards Mg$^{2+}$ diminishes with the metal ion concentration. At the concentration of Mg$^{2+}$ below 0.5 mM $K_m$ for Mg$^{2+}$ is 0.07 mM and at the Mg$^{2+}$ concentration over 1.5 mM it rises to 0.47 mM. The Hill coefficients are 0.37 and 0.88, respectively. Among several compounds affecting the PEP carboxylase activity, such as organic acids, amino acids, and sugar phosphates, at physiological pH (7.0 and 7.8), malate shows the strongest inhibition of a competitive character, its $K_i$ being 2 mM. Also acidic amino acids strongly inhibit the enzyme activity, aspartate being more effective than glutamate. Glucose 6-phosphate and fructose 1,6-diphosphate markedly activate the enzyme. Both the inhibition by malate, aspartate and glutamate, and the activation by sugar phosphates rises considerably when pH is decreased from 7.8 to 7.0. Malonate scarcely affects the enzyme.

Phosphoenolpyruvate carboxylase (orthophosphate:oxaloacetate carboxylase, phosphorylating, EC 4.1.1.31) catalyses the reaction:

\[
\text{phosphoenolpyruvate} + \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{orthophosphate} + \text{oxaloacetate}
\]

Carboxylation of PEP is a highly exoergic process connected with a considerable decrease of free energy (Davies, 1979). This, as well as a high affinity of the enzyme towards carbon dioxide, makes the process of PEP $\beta$-carboxylation in the living cell irreversible. Several authors (Ting & Osmond, 1973b; Brulbert et al., 1979; Perriot et al., 1981) have described four forms of PEP carboxylase in higher plants, related to different types of metabolism.
The enzyme is regulated by many effectors. However, the purification of the enzyme from plants is frequently connected with a loss of allosteric properties and a substantial diminution of the activity (Wong & Davies, 1973). Even desalting of the enzyme preparation on Sephadex G-25 was followed by a 25% inactivation (Kluge et al., 1980). Therefore, although at first several trials at purification of PEP carboxylase were made and the enzyme has been purified to a high degree (Jones et al., 1978), especially from maize (Ueda & Sugiyama, 1976; Mukerji, 1977; O’Leary et al., 1981), more recent investigations have been performed with crude extracts (e.g. Outlaw & Kennedy, 1978; Barankiewicz et al., 1979; Winter, 1980; Briand et al., 1981; Holaday & Black, 1981; Winter et al., 1982).

There are various opinions on the localization of the enzyme and its role within different tissues of higher plants (Ting & Osmond, 1973b; Peterson & Evans, 1979; Perriot et al., 1981). The role of PEP carboxylase in green tissues is obvious, especially in C4 and crassulacean acid metabolism (CAM) plants. However, the function of the enzyme in non-photosynthesizing organs is obscure. Some authors suggest that the enzyme supplies malate as -COO− source for keeping constant pH of the cytoplasm (Davies, 1973, 1979) and produces NADPH for reductive purposes (Jackson & Coleman, 1959; Ting & Osmond, 1973a). Many authors propose that PEP carboxylase in roots and nodules of leguminous plants is related with ammonia assimilation (Lawrie & Wheeler, 1975a,b; Christeller et al., 1977; Peterson & Evans, 1979; Rawsthorne et al., 1980). On the basis of numerous investigations using 15N and 14C and assuming zero net carbon balance of the tricarboxylic acid cycle, it is nowadays accepted that an anaplerotic reaction must be involved in the carbon skeleton production, especially for C4 amino acids (Christeller et al., 1977). However, theoretical considerations indicate that in legumes PEP formed in glycolysis can hardly be the sole acceptor for dark CO2 fixation in C4 compound production (Schramm, 1982, 1983). According to present views (Lawrie & Wheeler, 1975a,b; Pate & Herridge, 1978; Rawsthorne et al., 1980), the amino acids which are first formed in root nodules are glutamate and glutamine, while aspartate and asparagine are produced by transaminations, asparagine being in many legumes the main product transported to the shoot. A particular carbon-nitrogen balance and the importance of a pathway leading to oxaloacetate, alternative to glycolysis, which provides carbon atoms for the synthesis of aspartate and asparagine, have been recently emphasized by Schramm (1982, 1983). Nevertheless, the activity of PEP carboxylase in legumes has been well documented and cannot be neglected.

To understand better the mechanism of dark carboxylation in non-photosynthesizing organs of legumes we made an attempt to investigate the properties of PEP carboxylase from the roots of yellow lupin (Lupinus luteus L).
MATERIALS AND METHODS

Chemicals. The reagents used were: Tris, sucrose, dithiothreitol, PEP-Na₃, NADH, bovine serum albumin, pyruvate, isocitrate, aspartate, glutamate, D-glucose 6-phosphate, D-fructose 1,6-bisphosphate, and malic dehydrogenase from Sigma (U.S.A.); Polyclar AT from Serva (F.R.G.); malate from LKB (Sweden); succinate and malonate from Reachim (U.S.S.R.). Other reagents were of Polish production.

Plant material. Yellow lupin (Lupinus luteus L) var. Jantart, two-month-old, field grown, was used for the investigation. The experiments were performed with roots deprived of the nodules.

Enzyme extraction. The lupin roots were homogenized with three parts (w/v) of the isolation buffer by grinding in a mortar with quartz sand. The isolation buffer was composed of 100 mM-Tris/HCl (pH 7.8), 1 mM-MgCl₂, 500 mM-sucrose, 5 mM-dithiothreitol, and 1% Polyclar AT. The homogenate was centrifuged for 25 min at 10 000 g. The pellet was discarded and the supernatant was made 25% (v/v) with glycerol to stabilize the enzyme. The measurements were performed within 48 h from the preparation.

Determination of the enzyme activity. The enzyme was determined spectrophotometrically by measuring the decrease of 340 nm absorbance of NADH, in the reaction catalysed by malate dehydrogenase (EC 1.1.1.37), which is stoichiometrically coupled with PEP carboxylation:

\[
\text{Phosphoenolpyruvate} + \text{HCO}_3^- \xrightarrow{\text{PEP carboxylase}} \text{oxaloacetate} + \text{P}_i
\]

\[
\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{malate dehydrogenase}} \text{malate} + \text{NAD}^+
\]

The rate of the reaction was expressed in nanomoles of oxidized NADH per minute and milligram protein. The incubation mixture contained: 100 mM-Tris/HCl (pH 7.8), 10 mM-MgCl₂, 5 mM-NaHCO₃, 1 mM-PEP-Na₃, and 0.1 mM-NADH. The reaction was initiated by addition of 0.01 ml of the enzyme preparation. No exogenous malate dehydrogenase was added, because no change of the overall activity was observed when desalted commercial malate dehydrogenase from pig heart was added. This evidenced that the content of malate dehydrogenase in the crude extract from the yellow lupin roots was in excess with respect to PEP carboxylase.

Protein determination. Protein concentration was determined by the method of Lowry et al. (1951) against a standard plot made with bovine serum albumin, fraction V, fatty acid-free.

RESULTS

Enzyme properties

pH dependence of PEP carboxylase was examined in the isolation buffer in the range between 6.7 and 8.6 (Fig. 1). A high activity of the
enzyme was observed in pH range of 7.4 - 8.6. Further investigations were carried out at pH 7.8 as possibly the closest to the physiological one.

Temperature optimum of the enzyme preparation was in the range of 32 - 40°C (Fig. 2). Above 40°C the activity of the enzyme decreased rapidly.

The dependence of the reaction rate versus PEP concentration follows the Michaelis-Menten plot (Fig. 3). The Hill coefficient $n_H = 0.96$ suggests

**Fig. 1.** Influence of pH on PEP carboxylase activity in the isolation buffer: 100 mM-Tris/HCl, 1 mM-MgCl$_2$, 500 mM-sucrose, 5 mM-dithiothreitol and 1%, Polyclar AT.

**Fig. 2.** Influence of temperature on PEP carboxylase activity in the isolation buffer (cf Fig. 1).

**Fig. 3.** Dependence of the PEP carboxylation rate (expressed in mmoles of oxidized NADH x min$^{-1}$ x mg protein$^{-1}$) on PEP concentration, and double reciprocal and Hill plots (inserts). Incubation mixture: 100 mM-Tris/HCl, pH 7.8, 10 mM-MgCl$_2$, 5 mM-NaHCO$_3$, 0.1 mM-NADH.
one binding site for PEP. $K_m$ for PEP determined from the Lineweaver-Burk plot was 0.1 mM, and $K_m$ for HCO$_3^-$ (not shown) about 0.7 mM.

The dependence of the reaction rate on Mg$^{2+}$ concentration is expressed by a plot very close to hyperbolic (Fig. 4). However, the same dependence plotted according to Lineweaver-Burk is biphasic, and the same occurs when the dependence is expressed in the logarithmic scale (Fig. 4).

![Graph](image)

**Fig. 4.** Dependence of the PEP carboxylation rate on Mg$^{2+}$ concentration, and double reciprocal and Hill plots (inserts). For reaction conditions see Fig. 3. PEP-$\text{Na}_3$ concentration in the incubation mixture was 1 mM.

The affinity of the enzyme to Mg$^{2+}$ decreases with the increase of Mg$^{2+}$ concentration; however, the absolute activity of the enzyme increases. The Hill coefficient value also increases with the decrease of the Mg$^{2+}$ affinity towards the enzyme.

**Effect of organic acids**

The effect of several organic acids on the PEP carboxylase activity was measured at pH 7.0 and 7.8 (Fig. 5) at saturating concentrations of all components of the reaction. The activity of the enzyme without any effector was taken as 100%. Oxo acids (pyruvate and 2-oxoglutarate) activated the enzyme. Malate, succinate and citrate were inhibitory at both examined pH values. The effect of malonate depended on pH: at higher pH (7.8) it was a weak inhibitor, at lower pH (7.0), however, a weak activator of the enzyme. Malate appeared to be the strongest inhibitor; at 2.5 mM concentration it exerted 80% inhibition at pH 7.0, and 46% inhibition at pH 7.8. The inhibition was competitive with $K_i$ of about 2 mM at pH 7.8 (Fig. 6).
Fig. 5. Influence of organic acids on PEP carboxylase activity, expressed as percentage of the enzyme activity without the effector. The reaction was followed at saturating concentrations of the essential components. Abbreviations: PA, pyruvate; OG, 2-oxoglutarate; MN, malonate; CI, citrate; I-CI, isocitrate; SA, succinate; MA, malate.

Fig. 6. Double reciprocal plot of the inhibition of PEP carboxylase by malate. The numbers at the plots indicate malate concentration.

**Effect of amino acids**

The influence of the acidic amino acids: aspartate and glutamate in the range of 0 - 10 mM was examined at pH 7.0 and 7.8, at saturating concentrations of all other components of the reaction (Fig. 7). Both amino acids appeared to inhibit lupin root PEP carboxylase, aspartate being a stronger inhibitor than glutamate. The inhibition increased with the decrease of pH.
Fig. 7. Influence of acidic amino acids and sugar phosphates on PEP carboxylase activity, expressed as percentage of the enzyme activity without the effector. The reaction was measured at saturating concentrations of the essential components. Abbreviations: Glc-6-P, glucose-6-phosphate; Fru-1,6-bP, fructose-1,6-bisphosphate; Glu, glutamate; Asp, aspartate.

**Effect of sugar phosphates**

Glucose 6-phosphate and fructose 1,6-bisphosphate activated the enzyme within the concentration range of 0 - 5 mm at both pH 7.0 and 7.8. Decreasing pH stimulated the activation (Fig. 7, Table 1).

**Table 1**

*Influence of various effectors on the activity of PEP carboxylase from yellow lupin roots*

Concentration of the effectors was 2.5 mM.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Inhibition (%)</th>
<th>Activation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 7.0</td>
<td>pH 7.8</td>
</tr>
<tr>
<td>Malate</td>
<td>80</td>
<td>46</td>
</tr>
<tr>
<td>Succinate</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Citrate</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>Malonate</td>
<td>activation</td>
<td>4</td>
</tr>
<tr>
<td>Aspartate</td>
<td>46</td>
<td>14</td>
</tr>
<tr>
<td>Glutamate</td>
<td>29</td>
<td>11</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The PEP carboxylase preparation isolated from yellow lupin roots was fairly stable. The decrease of the activity of the enzyme stored without any stabilizer at 4°C was 20% after 48 h. Glycerol (25%, v/v) was used
successfully for stabilization. Bonugli & Davies (1977) have suggested that diminution of the enzyme activity produced by staining is connected with modification of the enzyme molecule. With regard to this, in the present investigation the crude enzyme preparation was used no later than 48 h after isolation.

The enzyme exhibited a broad pH optimum from 7.8 to above 8.6 which does not seem to confirm its role in stabilization of pH as suggested by Davies (1973), at least in lupin root tissue. For PEP carboxylase from CAM plants this optimum usually is in the range of pH 6-7, but for C4 plants is above 8.0. The velocity of carboxylation of PEP also depends on temperature. Wong & Davies (1973) observed that the plot of the reaction rate versus PEP concentration was hyperbolic at 30°C and sigmoidal at 45°C.

In the present study we found a hyperbolic dependence on PEP and HCO$_3^-$ concentrations. The values of $K_m$ for PEP of 0.1 mM, and for HCO$_3^-$ of 0.7 mM are in the range reported in the literature. Similar values for PEP were obtained for the enzyme from etiolated maize seedlings (Maruyama et al., 1966), potato tubers (Bonugli & Davies, 1977), and soybean root nodules (Peterson & Evans, 1979). Most of $K_m$ values for HCO$_3^-$ for plant material are within the range of 0.05 - 1.5 mM (Maruyama et al., 1966; Mukerji & Ting, 1971; Mizioirko et al., 1974; Mukerji, 1977).

The $K_m$ value for Mg$^{2+}$ increased with the concentration of the cofactor. At the same time the Hill coefficient increased, which reflects the increase in the number of binding sites for the cofactor. Similar results were obtained by Mukerji (1974). According to this author the enzyme binds to the Mg-PEP complex, the final product being most probably Mg-E-Mg-PEP or E-Mg-PEP. In Mukerji's (1974) opinion free Mg$^{2+}$ could act as an activator, especially at higher concentrations.

Bonugli & Davies (1977) have demonstrated that PEP carboxylase from potato tubers has allosteric features only in the physiological pH range, namely between 7.0 and 7.8. Therefore, we investigated the influence of effectors on the activity of PEP carboxylase from the yellow lupin roots at pH 7.0 and 7.8. Smith et al. (1979) showed the influence of pH on the level of activity and mode of action of the enzyme from oat coleoptile.

All examined organic acids and two acidic amino acids acted as inhibitors. Malate has often been reported as a potent inhibitor of PEP carboxylase, mostly competitive (Ting, 1968; Kluge & Osmond, 1972; Ting & Osmond, 1973a; Hill & Bown, 1978).

However, Raghavendra & Das (1976) described the inhibition of PEP carboxylase from cotton leaves by malate as non-competitive at low concentrations of the effector but competitive at higher concentrations. The same was shown by Pays et al. (1980) for PEP carboxylase from Bryophyllum. Mukerji & Ting (1971) have suggested that malate may be bound to two centres of the enzyme molecule. Winter (1980) found in CAM
plants the inhibition exerted by malate to be stronger at lower pH (7.5) than at higher (8.0), which fits to our results. Malonate, which was reported to inhibit significantly PEP carboxylase in C4 plants (Raghavendra & Das, 1975), and in blue-green algae (Colman & Coleman, 1978) having a similar type of photosynthesis, as well as in B. gracilis at pH 7.8 (Pays et al., 1980), in our experiment was scarcely active. In our opinion this fits to the role of malonate in legumes as acceptor for dark CO₂ fixation, alternative to PEP, as proposed by Schramm (1982, 1983).

The oxo acids: pyruvate and 2-oxoglutarate, in our investigation were activatory. The same acids, however, inhibited the activity of PEP carboxylase from maize leaves (Wong & Davies, 1973), the inhibition raising with the lowering of pH. The inhibition of PEP carboxylase activity by organic acids and acidic amino acids confirms the hypothesis of Wong & Davies (1973) of the feedback inhibition of the first reaction of the pathway leading to their biosynthesis.

The activation of PEP carboxylase by sugar phosphates was observed by several authors (e.g. Ting & Osmond, 1973c; Wong & Davies, 1973; Harvey et al., 1976; Uedan & Sugiyama, 1976; Bonugli & Davies, 1977). Both malate and glucose 6-phosphate, which are particularly potent effectors, were proposed to be important regulators of PEP carboxylase in vivo (Pays et al., 1980).

REFERENCES


KARBOKSYLAZA FOSFOENOLPOFIROGRONIANOWA (EC 4.1.1.31)

Z KORZENI ŁUBINU ŻÓLTEGO (*LUPINUS LUTEUS*)

Streszczenie

Karbonsylaza PEP (surowy preparat) z korzeni lubinu żółtego wykazuje optimum pH w zakresie od 7.4 do 8.6 i optimum temperaturowe w zakresie od 32 do 40°C. Kₘ dla PEP wynosi 0.1 mm, a Kₘ dla HCO₃⁻ 0.7 mm. Powinowactwo enzymu do jonów Mg²⁺ zależy od ich stężenia. W zakresie stężeń jonów do 0.5 mm KₘMg²⁺ wynosi 0.07 mm, powyżej 1.5 mm KₘMg²⁺ wzrasta do 0.47 mm. Współczynniki Hilla wynoszą odpowiednio 0.37 i 0.88. Spośród wielu efektorów aktywności karbonsylazy PEP, takich jak kwasy organiczne, kwasowe aminokwasy i fosforany cukrów, w fyzjologicznym pH (7.0 i 7.8), najsilniejszą inhibicję o charakterze kompetycyjnym wykazuje jabłczan: stała inhibicji K₁ wynosi 2 mm. Kwasowe aminokwasy również silnie hamują aktywność enzymu, przy czym asparaginian efektywnie od glutaminian. Natomiast Glc-6-P i Fru-1,6-b-P są aktywatorami enzymu. Inhibicja przez jabłczan, asparaginian i glutaminian oraz aktywacja przez fosforany cukrów jest wyraźnie wyższa w pH 7.0 niż 7.8. Wpływ malonianu na aktywność enzymu jest nikły.

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