# Phosphoenolpyruvate Carboxylase from Maize Leaves. Studies Using β-Methylated Phosphoenolpyruvate Analogues as Inhibitors and Substrates*

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Phosphoenolpyruvate Carboxylase, Phosphoenolpyruvate Analogues, Reaction Mechanism, Maize Leaf

1. The phosphoenolpyruvate analogues phosphoenol-α-ketobutyrate and phosphoenol-α-ketoisovalerate are linear competitive inhibitors of maize leaf phosphoenolpyruvate carboxylase with respect to phosphoenolpyruvate. Phosphoenol-α-ketobutyrate is an excellent inhibitor ($K_i$: 18 μM in the presence of 5 mM $MgCl_2$). The inhibition constant for phosphoenol-α-ketoisovalerate is 0.38 mM under the same conditions. For both compounds, the inhibition is greater in the presence of $Mn^{2+}$ than with $Mg^{2+}$. 2. The analogues are dephosphorylated, but apparently not carboxylated, by the enzyme. For the reaction with phosphoenol-α-ketobutyrate, α-ketobutyrate and inorganic phosphate are the reaction products. Bicarbonate and a divalent cation are required for the dephosphorylation reaction. 3. The dephosphorylation reaction is activated by glucose-6-phosphate and the $V_{max}$ has the same pH dependence as that of the carboxylation of phosphoenolpyruvate. The $K_m$ for phosphoenol-α-ketobutyrate is reduced in the presence of 5 mM $MnCl_2$ (55 μM versus 140 μM with 5 mM $MgCl_2$). The $V_{max}$ is essentially the same in the presence of either $MgCl_2$ or $MnCl_2$. These results suggest that the dephosphorylation of the analogues occurs by a mechanism which is similar to that of the carboxylation of phosphoenolpyruvate, and that both reactions have a common rate-determining step.

## Introduction

P-enolpyruvate carboxylase (Orthophosphate: oxaloacetate carboxylyase (phosphorylating), E.C. 4.1.1.31) catalyzes the reaction: P-enolpyruvate + $HCO_3^- \rightarrow$ Oxaloacetate + $P_i$ [1, 2]. This enzyme has been found in bacteria, algae and plants, although its properties are different depending on the source [1–3]. The carboxylase is particularly important in the leaves of $C_4$ plants such as maize, where it is responsible for the primary $CO_2$ fixation reaction [2, 3].

A divalent cation is required for activity: $Mg^{2+}$ and $Mn^{2+}$ are the most effective ones [4, 5]. Several compounds which are analogues of P-enolpyruvate and contain a carboxyl and a phosphate group are competitive inhibitors with respect to P-enolpyruvate [2, 6–10]. The enzyme from $C_4$ plants is activated by glucose-6-phosphate [11].

The β-carboxylation of P-enolpyruvate, contrary to an earlier report [12], is currently thought to proceed by a stepwise mechanism in which the formation of carboxyphosphate and the enolate anion of pyruvate are intermediates [5, 13]. This hypothesis explains how the carbon atom of bicarbonate is activated to achieve carbon-carbon bond formation. The evidence which supports this least mechanism has been obtained through isotope effect studies [5], and the determination of the stereochemical course of the reaction at phosphorus using [(S)-$^{16}O^{17}O$] thiophosphoenolpyruvate as substrate in $H_2{}^{18}O$ [13]. It is also known that the addition of bicarbonate occurs on the *si* face of P-enolpyruvate [14].

Woods *et al.* [15] reported the synthesis of a number of homologues of P-enolpyruvate which were utilized to perform studies on the specificity and selectivity of several P-enolpyruvate utilizing enzymes [15–21]. Some of these analogues were also shown to be inhibitors of P-enolpyruvate carboxylase from *E. coli* [9, 22]. Recently, a report that the homologues P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate are dephosphorylated by the bacterial carboxylase has appeared [23]. This observation is highly important, since it gives information about the mechanism by which the carboxylation occurs.

We report here that P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate are competitive inhibitors, and that both analogues are dephosphorylated by the maize leaf P-enolpyruvate carboxylase. Kinetic

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studies using these analogues as substrates of the enzyme indicate that the dephosphorylation reaction has the same rate-determining step as the carboxylation of P-enolpyruvate.

## Materials and Methods

### *Enzyme purification*

P-enolpyruvate carboxylase was extracted and purified from *Zea mays* leaves as previously described [24]. The purified enzyme migrated as a single protein band in polyacrylamide gel electrophoresis and had a specific activity of 22–25 μmol $\cdot min^{-1} \cdot mg^{-1}$ when activated by thiol compounds (see [25]).

### *Assay of enzyme activity*

Enzyme activity was determined spectrophotometrically monitoring NADH oxidation at 340 nm and 30 °C in an UNICAM SP 1800 B spectrophotometer by coupling the P-enolpyruvate carboxylase reaction to malic dehydrogenase. The standard assay medium contained 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.15 mM NADH, 10 mM $NaHCO_3$, 2 IU malic dehydrogenase, 4 mM P-enolpyruvate and P-enolpyruvate carboxylase (4 μg protein), in a total volume of 1 ml. The production of α-ketobutyrate from P-enol-α-ketobutyrate was followed in a similar way, except that 10 IU lactic dehydrogenase replaced malic dehydrogenase, 2 mM P-enol-α-ketobutyrate replaced P-enolpyruvate and 25 μg of the enzyme was usually added to the assay mixture. Bicarbonate free assay medium at pH 7.2 was prepared by degassing the solution under low pressure and then bubbling with $CO_2$-free $N_2$ for several hours prior to the addition of the enzyme. This treatment significantly lowers bicarbonate levels (see [8]).

The phosphate liberation activity of the enzyme towards P-enolpyruvate or its analogues was determined in a reaction mixture containing 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 mM P-enolpyruvate (or its analogues) and enzyme (amounts indicated in the legends to figures), in a final volume of 1 ml. After incubation at 30 °C for the indicated times, 1 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. Inorganic phosphate was then determined as described by Taussky and Shorr [26]. The production of phosphate from P-enolpyruvate and P-enol-α-ketobutyrate at pH values from 6.5 to 9.0 was performed in MOPS-HEPES-borate buffer, 40 mM each, adjusted to the corresponding pH. In all cases, controls were made in which the enzyme was added after the addition of trichloroacetic acid; under these conditions no significant amounts of $P_i$ were produced.

The radiolabel assay of the carboxylase activity was conducted similar to the phosphate liberation assay, except that 10 μmol $NaH^{14}CO_3$ (specific radioactivity 1 mCi/mmol) were added. At different times 100 μl aliquots were removed and the reaction was stopped by the addition of an equal volume of 2,4-dinitrophenylhydrazine (1 mM in 0.1 N HCl). The mixture was evacuated overnight to remove the $CO_2$ and then aliquots were removed for counting in a Beckman LS 8100 liquid scintillation counter, using a mixture of 0.25% 2,5-diphenyloxazole and 10% naphthalene in 1,4-dioxane as scintillation cocktail.

### *Protein concentration*

Protein concentration was determined by the colorimetric method of Lowry *et al.* [27], or alternatively by the Coomassie Brilliant Blue dye binding method [28], using bovine serum albumin as standard.

### *Materials*

P-enol-α-ketobutyrate (*E-Z* mixture) and P-enol-α-ketoisovalerate (cyclohexylammonium salts) were gifts of Dr. A. E. Woods, Middle Tennessee State University. P-enolpyruvate (monopotassium salt), NADH, glucose-6-phosphate, porcine heart malic dehydrogenase and rabbit muscle lactic dehydrogenase were purchased from Sigma Chemical Co. [$^{14}$C]$NaHCO_3$ was obtained from Comisión Nacional de Energía Atómica (CNEA), Argentina. All other reagents were of analytical grade.

## Results and Discussion

### *P-enolpyruvate analogues as inhibitors*

The production of oxaloacetate from P-enolpyruvate and $HCO_3^-$ catalyzed by maize leaf P-enolpyruvate carboxylase was reversibly inhibited by the substrate analogues P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate. Both compounds exhibited linear competitive inhibition with respect to P-enolpyruvate at pH 8, and results for P-enol-α-ketobutyrate are shown in Fig. 1. The inhibition constants ($K_i$) for

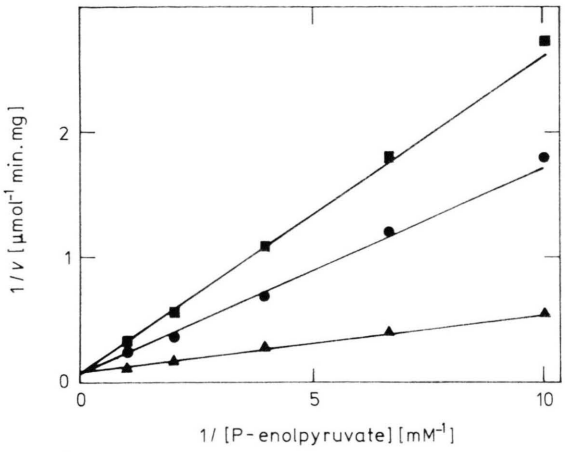


Fig. 1. Double-reciprocal plot of P-enolpyruvate carboxylase activity versus P-enolpyruvate concentration in the absence (▲) and in the presence of 50 μM (●) and 100 μM (■) P-enol-α-ketobutyrate at pH 8. Enzyme activity was measured by the coupled assay using malic dehydrogenase as described in the text.

P-enol-α-ketobutyrate, obtained from Dixon plots [29], were 18 μM and 9 μM in the presence of 5 mM $MgCl_2$ or 5 mM $MnCl_2$, respectively (Table I). The affinity of the enzyme for the inhibitor seems to be greater in the presence of $Mn^{2+}$ ions. A similar behaviour has been observed by other authors in the case of the competitive inhibitors 1-hydroxycyclopropane carboxylic acid phosphate [7], L-phospholactate [6, 7] and others, for both the maize and the spinach leaf enzyme [2]. The $K_i$ values obtained for P-enol-α-ketoisovalerate also showed the same correlation: $K_i = 0.38$ mM in the presence of $MgCl_2$ and 0.12 mM in the presence of $MnCl_2$ (Table I).

Table I. Inhibition constants ($K_i$) for the inhibition of maize P-enolpyruvate carboxylase by several P-enolpyruvate analogues with substitution at the β-position.

| Analogue | Divalent cation[a] | $K_i$ [μM] | Reference |
|---|---|---|---|
| P-enolbromopyruvate | $Mg^{2+}$ | 6.7–7.0 | 2, 8, 10 |
| | $Mn^{2+}$ | 26 – 30 | 2, 8 |
| P-enolfluoropyruvate | $Mg^{2+}$ | 85 –100 | 2, 10 |
| | $Mn^{2+}$ | 36 | 2 |
| P-enol-α-ketobutyrate | $Mg^{2+}$ | 18 | this study |
| | $Mn^{2+}$ | 9 | this study |
| P-enol-α-ketoisovalerate | $Mg^{2+}$ | 380 | this study |
| | $Mn^{2+}$ | 120 | this study |

[a] The inhibition constant was determined in the presence of the indicated divalent cation.

Katsuki, Izui and coworkers working with *E. coli* P-enolpyruvate carboxylase reported inhibition constants of 24 μM for P-enol-α-ketobutyrate and 0.89 mM for P-enol-α-ketoisovalerate [9, 23]. They also proposed that this enzyme has a hydrophobic pocket near the binding site of the methylene group of P-enolpyruvate, where an essential cysteine residue which is alkylated by bromopyruvate may be located [9]. The analogy observed between the inhibition parameters of the two compounds for the bacterial and the plant enzyme, suggests that this hydrophobic pocket may also exist in maize P-enolpyruvate carboxylase. Moreover, in the enzyme from maize there is an essential thiol group in the binding site of P-enolpyruvate [30] which seems to be alkylated by bromopyruvate [31].

Several P-enolpyruvate analogues with substitutions at the β-position have been tested as inhibitors of maize leaf P-enolpyruvate carboxylase. Their inhibition constants are listed in Table I. The case of P-enolbromopyruvate is significant since it suggests that the substitution of one hydrogen of the methylene group of P-enolpyruvate for a larger group improves the binding of the enol-phosphate to the enzyme. Our results with P-enol-α-ketobutyrate reinforce this hypothesis, since bromine and methyl groups are similar in size. Besides, the substitution for a smaller electronegative group such as fluorine yields a $K_i$ of 100 μM [10]. The comparison of the inhibition constants and the $K_m$ for P-enolpyruvate (see Table III) suggests that electronic, and to a greater extent, steric factors play an important role in the interaction of these compounds with the methylene binding site of the enzyme. The same behaviour was observed in P-enolpyruvate carboxykinase for P-enolpyruvate and its analogues [20, 32].

If the two hydrogens of the methylene group of P-enolpyruvate, are replaced by methyl groups, as occurs with P-enol-α-ketoisovalerate, the inhibition constant is increased by an order of magnitude. This effect would be most likely due to interference in the binding of one of the two methyl groups. If this is the case, the enzyme should show stereoselectivity between the two diastereoisomers of P-enol-α-ketobutyrate (*E*- and *Z*-P-enol-α-ketobutyrate). In this way, the observation of Woods *et al.* [15] that a mixture of (*E*-*Z*) P-enol-α-ketobutyrate highly inhibited pyruvate kinase while P-enol-α-ketoisovalerate did not, was followed by the finding that only *Z*-P-enol-α-ketobutyrate can bind tightly to this enzyme

[19, 20]. Further work with pure *E* and *Z* isomers is needed to clear this point in maize P-enolpyruvate carboxylase.

Among the competitive inhibitors of the maize leaf enzyme tested to date, P-enolbromopyruvate and 1-hydroxycyclopropane carboxylic acid phosphate were the most effective ($K_i$ for both approximately 7 μM) [2, 7, 8, 10]. It has been noted that potent inhibitors of the carboxylase from $C_4$ plants can be used as potential herbicides for these kind of plants [8]. P-enol-α-ketobutyrate is another excellent inhibitor that could be useful for this purpose.

*P-enolpyruvate analogues as substrates*

P-enolpyruvate carboxylase catalyzes the dephosphorylation of the two P-enolpyruvate analogues: P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate. When these compounds were incubated with the purified maize leaf enzyme in the presence of $Mg^{2+}$ ions and bicarbonate at pH 8, the production of inorganic phosphate in a time-dependent manner was observed (Fig. 2). The amounts of enzyme needed to achieve similar activities with P-enolpyruvate, P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate where 1:20:40, respectively. When $MgCl_2$ or the enzyme were omitted in the reaction medium, appreciable amounts of phosphate were not detected. Furthermore, the production of phosphate was linearly proportional to the amount of enzyme added (Fig. 2, inset). The enzymatic preparation was homogeneous (see Materials and Methods), and did not display any phosphatase activity towards β-glycerophosphate, ATP, P-glycolate or glucose-6-phosphate. Similar results were obtained with three separate enzyme preparations.

α-Ketobutyrate was identified as the product of the reaction catalyzed by *E. coli* P-enolpyruvate carboxylase when P-enol-α-ketobutyrate was used as substrate [23]. In view of this result, we tested the production of this α-ketoacid using lactic dehydrogenase and followed NADH oxidation spectrophotometrically. α-Ketobutyrate has been previously shown to be a good substrate for lactic dehydrogenase [17]. We found that a compound that was reduced by lactic dehydrogenase, most likely α-ketobutyrate, was produced from P-enol-α-ketobutyrate in the presence of maize P-enolpyruvate carboxylase and $MgCl_2$, and that $HCO_3^-$ was also required for this reaction to proceed (Fig. 3).

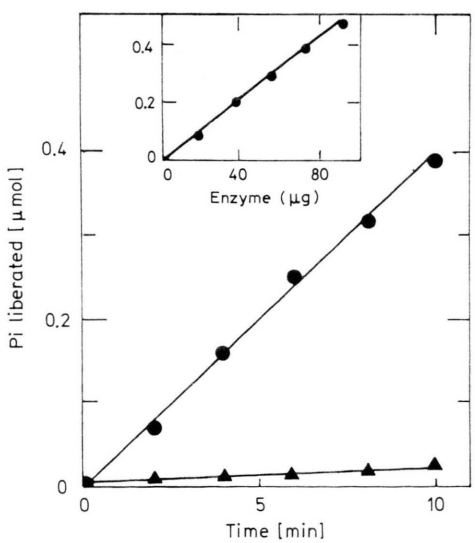


Fig. 2. Liberation of inorganic phosphate from P-enol-α-ketobutyrate by P-enolpyruvate carboxylase action as a function of time. The reaction was performed in the presence (●) and absence (▲) of 10 mM $MgCl_2$ and inorganic phosphate was determined as described in Materials and Methods. Seventy micrograms of enzyme were used. Inset, liberation of inorganic phosphate from P-enol-α-ketobutyrate by increasing amounts of P-enolpyruvate carboxylase; reaction time, 10 minutes.

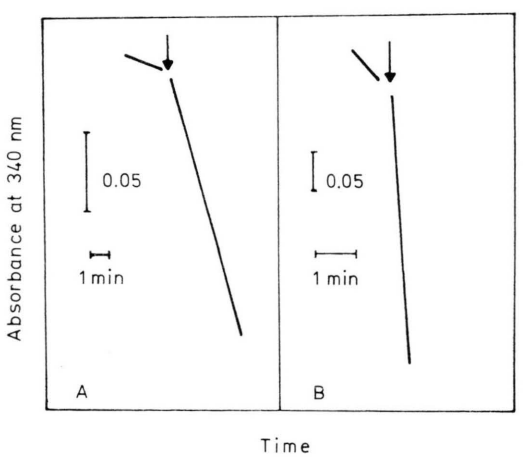


Fig. 3. Requirement of bicarbonate for the production of α-ketobutyrate from P-enol-α-ketobutyrate (A). The bicarbonate free reaction mixture was prepared and the production of α-ketobutyrate was measured at pH 7.2 with lactic dehydrogenase as coupling enzyme as described under Materials and Methods. The reaction was started by the addition of the enzyme. The arrow indicates the addition of 10 μl of 1 M $NaHCO_3$. (B) A control experiment with P-enolpyruvate as substrate, measuring oxaloacetate production as described in the text.

Table II. Stoichiometry of formation of inorganic phosphate and α-ketobutyrate from P-enol-α-ketobutyrate.

| Time [min] | $P_i$ [μmol/ml] | NADH oxidized [μmol/ml] | Ratio (NADH/$P_i$) |
|---|---|---|---|
| 10 | 0.126 | 0.113 | 0.90 |
| 20 | 0.207 | 0.210 | 1.01 |
| 30 | 0.297 | 0.306 | 1.03 |

Note. The reaction mixture contained all the components of the lactic dehydrogenase coupled assay, except that 0.32 mM NADH was used in a final volume of 3 ml. NADH oxidation was monitored continuously. At the times stated, 0.5 ml aliquots were removed and inorganic phosphate was determined as described.

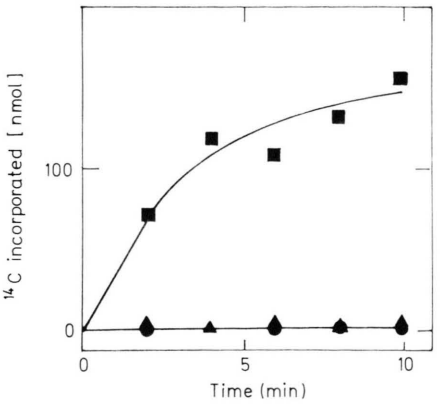


Fig. 4. Incorporation of $^{14}C$ into the products of the reaction of maize P-enolpyruvate carboxylase with P-enolpyruvate and its analogues. The reaction was carried out including [$^{14}C$]$NaHCO_3$ in the assay mixture as indicated under Materials and Methods. Other conditions were: (■) P-enolpyruvate 4 mM and 2 μg of enzyme; (▲) P-enol-α-ketobutyrate 2 mM and 50 μg of enzyme; (●) P-enol-α-ketoisovalerate 4 mM and 100 μg of enzyme.

Moreover, the production of phosphate showed a 1:1 relation with the oxidation of NADH (Table II). No radioactivity was incorporated into the reaction products (trapped as 2,4-dinitrophenylhydrazone derivatives) when [$^{14}C$]bicarbonate was included in the reaction medium (Fig. 4). Then, it seems that the reaction catalyzed by P-enolpyruvate carboxylase with P-enol-α-ketobutyrate as substrate is:

$$\text{P-enol-α-ketobutyrate} + H_2O \xrightarrow{Mg^{2+};\ HCO_3^-} \text{α-Ketobutyrate} + P_i.$$

Fujita *et al.* [23] presumed that P-enol-α-ketobutyrate cannot undergo a carboxylation owing to steric hindrance by its methyl group. This assumption seems logical, although P-enolbromopyruvate, a compound that should show similar steric interference, was shown to be carboxylated by the maize leaf carboxylase [8]. The argument that the carboxylated product methyloxaloacetate would be formed and immediately decarboxylated cannot be ruled out.

*Kinetics of P-enol-α-ketobutyrate dephosphorylation*

Further information about the interaction of P-enol-α-ketobutyrate with the enzyme was obtained by measuring the parameters $V_{max}$ and $K_m$ for its dephosphorylation by the carboxylase. Table III shows these results in the presence of 5 mM $MgCl_2$ or mM $MnCl_2$. The $K_m$ value is greater than the inhibition constant for P-enol-α-ketobutyrate. We do not know the reason for this discrepancy, but similar observations have been made with other enzymes [20]. The decrease in $V_{max}/K_m$ for P-enol-α-ketobutyrate, when compared with $V_{max}/K_m$ for P-enolpyruvate, seems to

Table III. Kinetic constants for P-enolpyruvate and P-enol-α-ketobutyrate as substrates for P-enolpyruvate carboxylase.

| Substrate | Divalent cation | $K_m$ [μM] | $V_{max}$ [μmol/min · mg] | $(V_{max}/K_m) \cdot 10^3$ |
|---|---|---|---|---|
| P-enolpyruvate | $Mg^{2+}$ | 550 | 15 | 27 |
| P-enolpyruvate | $Mn^{2+}$ | 110 | 2.8 | 25 |
| P-enol-α-ketobutyrate | $Mg^{2+}$ | 140 | 0.67 | 4.8 |
| P-enol-α-ketobutyrate | $Mn^{2+}$ | 55 | 0.55 | 10 |

Note. $K_m$ and $V_{max}$ were determined from a double-reciprocal plot. P-enolpyruvate carboxylase activity with P-enolpyruvate as substrate was measured with malic dehydrogenase as the coupling enzyme. When P-enol-α-ketobutyrate was the substrate, lactic dehydrogenase was used (see Materials and Methods). $Mg^{2+}$ and $Mn^{2+}$ were included as their chloride salts at a final concentration of 5 mM.

be due to an effect of $k_{cat}$, which can be explained by steric interference.

The effect of pH on the enzyme activity at saturating substrate concentrations for the dephosphorylation of P-enol-α-ketobutyrate was the same as that for the carboxylation of P-enolpyruvate (not shown). A slight decrease in activity below pH 7.5, and a constant activity from pH 7.5 to 9.0 was observed (see [5, 24]). The addition of 5 mM glucose-6-phosphate to the assay mixture at pH 8 with saturating levels of P-enolpyruvate or P-enol-α-ketobutyrate produced a similar effect (approximately 50% activation) with both reactions. The specific activity with P-enolpyruvate as substrate was increased from 15 to 23.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ when the assay was carried out with 5 mM $MgCl_2$. With P-enol-α-ketobutyrate, an increase from 0.67 to 0.99 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ was observed under the same conditions. The above results suggest that the rate-determining step is the same for the reaction with either P-enolpyruvate or P-enol-α-ketobutyrate.

The use of $MnCl_2$ in place of $MgCl_2$ reduced the $K_m$ for P-enol-α-ketobutyrate by a factor of 2.5. This effect was similar in magnitude to that seen for the inhibition constants for P-enol-α-ketobutyrate and P-enol-α-ketoisovalerate and for the $K_m$ for P-enolpyruvate. It should be noted that the $K_m$ for P-enolpyruvate was determined in the presence of 5 mM $MnCl_2$, which is an inhibitory concentration [4]. The parameters were obtained by extrapolation on a double-reciprocal plot at low P-enolpyruvate concentrations; at concentrations higher than 0.5 mM P-enolpyruvate the inhibition by $Mn^{2+}$ was relieved (unpublished observation). These facts explain the low value of $V_{max}$ obtained for P-enolpyruvate in the presence of $Mn^{2+}$. Since the relation $V_{max}/K_m$ is almost the same with both metal ions, it can be concluded that the decrease in $V_{max}$ is mainly due to the tighter binding of some intermediate or product to the catalytic site.

For the reaction with P-enol-α-ketobutyrate, the $V_{max}$ in the presence of 5 mM $MnCl_2$ was essentially the same of that with $MgCl_2$, suggesting that there is no inhibitory effect of $Mn^{2+}$ ions in this reaction. O'Leary *et al.* [5], using isotope effect studies proposed that the substitution of $Mg^{2+}$ by $Mn^{2+}$ has little effect on the rate of the first reaction step (the rate-determining one), and a larger effect on the rate of the second. The second step of the reaction with P-enolpyruvate as substrate would not take place in the case of P-enol-α-ketobutyrate, and so, the substitution would produce no effect on $V_{max}$.

In conclusion, the effects of glucose-6-phosphate and pH are consistent with a common rate-determin-

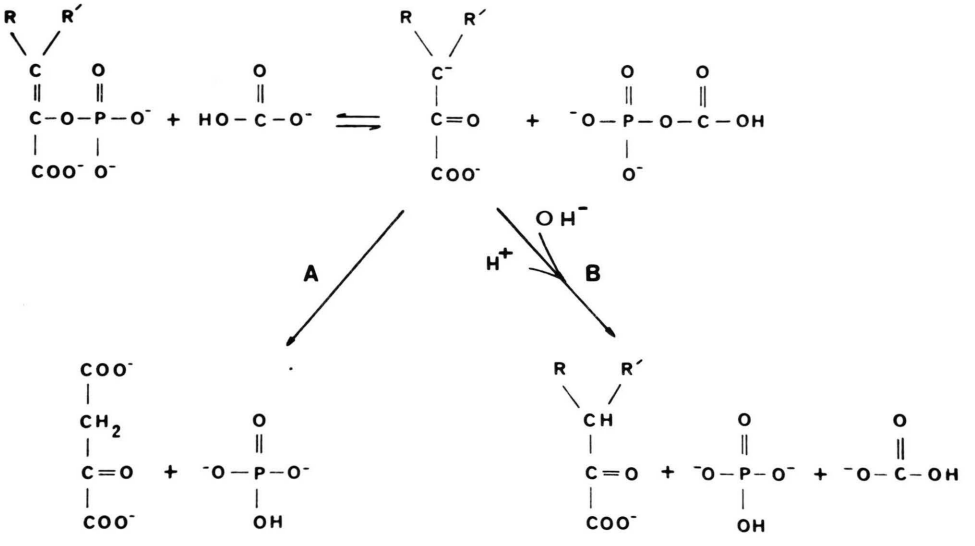


Fig. 5. Mechanism of the reactions catalyzed by P-enolpyruvate carboxylase: A: Reaction mechanism proposed for the carboxylation of P-enolpyruvate (R = R′ = H) [5]; the reaction with P-enolbromopyruvate probably follows the same mechanism [8]. B: Possible reaction mechanism for the dephosphorylation of P-enol-α-ketobutyrate (R = H; R′ = $CH_3$) and P-enol-α-ketoisovalerate (R = R′ = $CH_3$).

ing step for the reaction with either P-enolpyruvate or P-enol-α-ketobutyrate as substrates, indicating that both reactions occur by closely related mechanisms. The common rate-determining step would be the formation of carboxyphosphate and the enolate of the substrate. This mechanism is consistent with the isotope effect studies of O'Leary *et al.* [5]. The effect of $Mn^{2+}$ suggests that the second step is different for the two reactions. This step would be the carboxylation of the enolate in the case of P-enolpyruvate, and the addition of a proton from water together with hydrolysis of carboxyphosphate in the case of P-enol-α-ketobutyrate (Fig. 5).

The stereospecificity of the interaction of P-enol-α-ketobutyrate with several P-enolpyruvate utilizing enzymes has been studied [17, 19–21]. Similar studies with P-enolpyruvate carboxylase will be valuable to establish the arrangement of catalytic groups in the active site of this enzyme.

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[1] M. F. Utter and H. M. Kolenbrander, in The Enzymes, 3rd. ed. (P. D. Boyer, ed.), **Vol. 6,** pp. 117–168, Academic Press, New York 1972.
[2] M. H. O'Leary, Ann. Rev. Plant Physiol. **33,** 297–315 (1982).
[3] J. Coombs, in Encyclopedia of Plant Physiology, New Series (M. Gibbs and E. Latzko, eds.), **Vol. 6,** pp. 251–262, Springer Verlag, New York 1979.
[4] S. K. Mukerji, Arch. Biochem. Biophys. **182,** 352–359 (1977).
[5] M. H. O'Leary, J. E. Rife, and J. B. Slater, Biochemistry **20,** 7308–7314 (1981).
[6] H. M. Miziorko, T. Nowak, and A. S. Mildvan, Arch. Biochem. Biophys. **163,** 378–289 (1974).
[7] M. H. O'Leary, W. J. DeGooyer, T. M. Dougherty, and V. Anderson, Biochem. Biophys. Res. Commun. **100,** 1320–1325 (1981).
[8] M. H. O'Leary and E. Díaz, J. Biol. Chem. **257,** 14603–14605 (1982).
[9] K. Izui, Y. Matsuda, I. Kameshita, H. Katsuki, and A. E. Woods, J. Biochem. (Tokyo) **94,** 1789–1795 (1983).
[10] M. H. O'Leary, Physiol. Vég. **21,** 883–888 (1983).
[11] J. Coombs, C. W. Baldry, and C. Bucke, Planta **110,** 95–107 (1973).
[12] H. Maruyama, R. L. Easterday, H.-C. Chang, and M. D. Lane, J. Biol. Chem. **241,** 2405–2412 (1966).
[13] D. E. Hansen and J. R. Knowles, J. Biol. Chem. **257,** 14795–14798 (1982).
[14] I. A. Rose, E. L. O'Connell, P. Noce, M. F. Utter, H. G. Wood, J. M. Willard, T. G. Cooper, and M. Benziman, J. Biol. Chem. **244,** 6130–6133 (1969).
[15] A. E. Woods, J. M. O'Bryan, P. T. K. Mui, and R. D. Crowder, Biochemistry **9,** 2334–2338 (1970).
[16] W. E. Bondinell and D. B. Sprinson, Biochem. Biophys. Res. Commun. **40,** 1464–1467 (1970).
[17] J. A. Stubbe and G. L. Kenyon, Biochemistry **10,** 2669–2677 (1971).
[18] A. E. Woods, V. B. Chatman, and R. A. Clark, Biochem. Biophys. Res. Commun. **46,** 1–4 (1972).
[19] M. Adlersberg, J. Dayan, W. E. Bondinell, and D. B. Sprinson, Biochemistry **16,** 4382–4387 (1977).
[20] T. H. Duffy, W. J. Saz, and T. Nowak, Biochemistry **21,** 132–139 (1982).
[21] H. Hoving, T. Nowak, and G. T. Robillard, Biochemistry **22,** 2832–2838 (1983).
[22] R. Silverstein, Biochim. Biophys. Acta **258,** 626–636 (1972).
[23] N. Fujita, K. Izui, T. Nishino, and H. Katsuki, Biochemistry **23,** 1774–1779 (1984).
[24] A. A. Iglesias and C. S. Andreo, Biochim. Biophys. Acta **749,** 9–17 (1983).
[25] A. A. Iglesias and C. S. Andreo, Plant Physiol. **75,** 983–987 (1984).
[26] H. Taussky and E. Shorr, J. Biol. Chem. **202,** 675–685 (1953).
[27] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[28] J. Sedmak and S. Grossberg, Anal. Biochem. **79,** 544–552 (1977).
[29] M. Dixon and E. C. Webb, in Enzymes, 3rd. ed. (M. Dixon and E. C. Webb, eds.), pp. 332–467, Academic Press, New York 1979.
[30] A. A. Iglesias and C. S. Andreo, Photosynth. Res. **5,** 215–226 (1984).
[31] D. H. González, A. A. Iglesias, and C. S. Andreo, Arch. Biochem. Biophys. **245,** 179–186 (1986).
[32] T. H. Duffy and T. Nowak, Biochemistry **23,** 661–670 (1984).