# Hydrolysis of Phosphoenolpyruvate Catalyzed by Phosphoenolpyruvate Carboxylase from *Zea mays*†

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ABSTRACT: In addition to the normal carboxylation reaction, phosphoenolpyruvate carboxylase from *Zea mays* catalyzes a $HCO_3^-$-dependent hydrolysis of phosphoenolpyruvate to pyruvate and $P_i$. Two independent methods were used to establish this reaction. First, the formation of pyruvate was coupled to lactate dehydrogenase in assay solutions containing high concentrations of L-glutamate and aspartate aminotransferase. Under these conditions, oxalacetic acid produced in the carboxylation reaction was efficiently transaminated, and decarboxylation to form spurious pyruvate was negligible. Second, sequential reduction of oxalacetate and pyruvate was achieved by initially running the reaction in the presence of malate dehydrogenase with NADH in excess over phosphoenolpyruvate. After the reaction was complete, lactate dehydrogenase was added, thus giving a measure of pyruvate concentration. At pH 8.0 in the presence of $Mg^{2+}$, the rate of phosphoenolpyruvate hydrolysis was 3–7% of the total reaction rate. The hydrolysis reaction catalyzed by phosphoenolpyruvate carboxylase was strongly metal dependent, with rates decreasing in the order $Ni^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$. These results suggest that the active site metal ion binds to the enolate oxygen, thus stabilizing the proposed enolate intermediate. The more stable the enolate, the less reactive it is toward carboxylation and the greater the opportunity for hydrolysis.

Phosphoenolpyruvate (PEP)[1] carboxylase (EC 4.1.1.31) catalyzes the reaction of PEP with bicarbonate to form OAA and $P_i$ (Utter & Kolenbrander, 1972; O'Leary, 1982, 1983; Andreo et al., 1987). The enzyme has an absolute requirement for a divalent metal ion. Highest activity is observed with $Mg^{2+}$ or $Mn^{2+}$, but $Cd^{2+}$, $Co^{2+}$, and $Fe^{2+}$ also activate the enzyme to a lesser extent (O'Leary et al., 1981a; Nguyen et al., 1988).

The observation that one atom of $^{18}O$ is transferred from $HC^{18}O_3^-$ to $P_i$ led to the suggestion of a concerted cyclic mechanism for the reaction (Maruyama & Lane, 1962; Maruyama et al., 1966). However, the small $^{13}C$ isotope effect determined for the bicarbonate carbon (O'Leary et al., 1981a) and the occurrence of inversion of configuration at phosphorus (Hansen & Knowles, 1982) argue against such a mechanism. Instead, the mechanism appears to be stepwise, involving rate-determining formation of a carboxy phosphate intermediate, followed by carboxylation of the resulting enolate.

Numerous analogues of PEP substituted at carbon 3 have been tested as substrates or inactivators of PEP carboxylase (González & Andreo, 1989). Unexpectedly, these compounds are principally hydrolyzed to give pyruvate derivatives rather than being carboxylated to yield OAA analogues (Table I), although in some cases both hydrolysis and carboxylation are observed. The PEP analogues with a halogen substituted at carbon 3 are particularly interesting: Whereas 3-bromo-PEP is exclusively hydrolyzed (Diaz et al., 1988), the corresponding fluoro (Diaz et al., 1988; González & Andreo, 1988a; Janc et al., 1992) and chloro compounds (Liu et al., 1990) partition between the hydrolysis and carboxylation reactions.

The predominance of hydrolysis with analogues of PEP raises the important question of whether PEP itself might partition to a limited extent from the primary carboxylation reaction toward hydrolysis to give pyruvate and $P_i$. Experimentally, this is a difficult question to answer in a fully convincing manner. It is essential to demonstrate that any pyruvate formed arises from direct hydrolysis of PEP catalyzed by PEP carboxylase, rather than from the decarboxylation of OAA, a reaction which is particularly facile in the presence of metal ions (Leussing, 1982). Additionally, it is necessary to demonstrate the absence of any contaminating phosphatase activity. We report here on the ability of PEP carboxylase to catalyze the hydrolysis of PEP and factors which govern the partitioning between hydrolysis and carboxylation.

Table I: Hydrolysis and Carboxylation of Analogues of PEP by Maize PEP Carboxylase

| compd | % carboxylation | % hydrolysis | ref |
|---|---|---|---|
| alleno-PEP | 0 | 100 | Wirshing & O'Leary, 1988 |
| thio-PEP | 0 | 100 | Sikkema & O'Leary, 1988 |
| (*Z*)-methyl-PEP | 0 | 100 | Gonzalez & Andreo, 1988b; O'Laughlin, 1988 |
| dimethyl-PEP | 0 | 100 | Gonzalez & Andreo, 1986 |
| (*Z*)-bromo-PEP | 0 | 100 | Diaz et al., 1988 |
| (*Z*)-chloro-PEP | 25 | 75 | Liu et al., 1990 |
| (*Z*)-fluoro-PEP | 3 | 97 | Janc et al., 1992 |
| (*E*)-fluoro-PEP | 86 | 4 | Janc et al., 1992 |

## Materials and Methods

*Materials.* Monosodium pyruvate, monosodium L-glutamate, L-aspartic acid, monosodium α-ketoglutarate, tricyclohexylammonium phosphoglycolate, trisodium D-2-

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[1] Abbreviations: PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; OAA, oxalacetic acid.

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phosphoglycerate, disodium D-3-phosphoglycerate, disodium NADH, aspartate aminotransferase (porcine heart, type I), L-lactate dehydrogenase (rabbit muscle, type XI), and malate dehydrogenase (bovine heart) were purchased from Sigma Chemical Co. 1-Hydroxycyclopropanecarboxylic acid phosphate was available from previous work (O'Leary et al., 1981b). Monocyclohexylammonium PEP was synthesized by Peter Henke according to the procedure of Hirshbein et al. (1982). L-Phospholactate was synthesized by William DeGooyer by the procedure of Friedkin and Lehninger (1947). OAA and $ZnCl_2$ were purchased from Aldrich. $CaCl_2$ and $MnCl_2$ were from Fischer; $MgCl_2$ and $CoCl_2$ were purchased from Mallinckrodt; $CdCl_2$, $NiCl_2$, and ammonium molybdate were from J. T. Baker. All buffers were from Sigma except Tris (Boehringer Mannheim) and HEPES (Research Organics). Water was purified by a Millipore Super-Q filtration system.

PEP carboxylase was purified by a modification of the procedure of O'Leary et al. (1981a) in which chromatography on Blue Sepharose CL-6B (Pharmacia) was substituted for the final Sephacryl S-200 gel filtration and hydroxylapatite chromatography steps. PEP carboxylase was eluted from the Blue Sepharose column (2.5 × 50 cm) with 100 mM HEPES, 10% glycerol, 25 mM NaCl, and 1 mM dithiothreitol, pH 7.50, after first dialyzing the enzyme against the same solution. The Blue Sepharose column binds contaminating proteins including malate dehydrogenase, but PEP carboxylase elutes near the void volume. In some instances, it was necessary to remove remaining trace contaminants by Sephacryl S-400 chromatography (2.5 × 100 cm) eluted with 100 mM Tris, pH 7.5, containing 10% glycerol, 50 mM KCl, and 1 mM dithiothreitol. Purified enzyme had a specific activity of 24–25 units/mg and was pure by SDS gel electrophoresis.

Recent studies suggest that PEP carboxylase can be altered by proteolysis during purification. McNaughton et al. (1989) reported that a proteolyzed form of maize PEP carboxylase had lost not only a 4-kDa peptide fragment from its N- or C-terminus but also its malate sensitivity and the ability to be phosphorylated in vitro. Jiao and Chollet (1991) suggest that the way in which the enzyme is purified influences the enzymatic and dissociation properties of the enzyme in vitro. They report that PEP carboxylase from both $C_4$ and CAM plants loses its sensitivity to malate when the protein is proteolyzed, and conventional purification procedures are likely to lead to such proteolysis. Amino acid sequencing from the N-terminus reveals that the protein used in these studies is >99% homogeneous but is missing the first 22 amino acids and cannot be phosphorylated by the usual procedure (J.-A. Jiao and R. Chollet, unpublished results).

*Methods.* Rate measurements were made in 1.0 mL of solution using a Cary 118 spectrophotometer equipped with a thermostated cell compartment at 25 °C.

*PEP Carboxylase Assay.* Solutions contained 5 mM PEP, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.2 mM NADH, 100 mM HEPES, pH 8.0, and 4 units of malate dehydrogenase. Reactions were initiated by the injection of an aliquot of PEP carboxylase, and the carboxylation rate was monitored by following the decrease in absorbance at 340 nm.

*PEP Hydrolysis: Transaminase Method.* Assay solutions contained 5 mM PEP, 5 mM $NaHCO_3$, 0.2 mM NADH, 50 mM L-glutamate, 100 mM buffer, 6 units of lactate dehydrogenase, and 3.5 units of aspartate aminotransferase. Buffers were either PIPES (pH 6.5 and 7.0), HEPES (pH 7.5 and 8.0), or TAPS (pH 8.5 and 9.0). Metal ion dependence studies were conducted at pH 8.0 in solutions containing 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, or $NiCl_2$. Studies at other pH values used 5 mM $Mg^{2+}$. Reaction was initiated by injection of PEP carboxylase. Initial rates were determined by observing the decrease in NADH absorbance at 340 nm resulting from the lactate dehydrogenase catalyzed reduction of pyruvate.

To determine the efficacy of aspartate aminotransferase in eliminating decarboxylation of OAA, 1.0 mM OAA was substituted for PEP in the above assay mixture, and the rate of pyruvate formation was determined from the absorbance decrease at 340 nm. Under all conditions used, rates of pyruvate formation so obtained were less than 5% of the rates observed with PEP. When carboxylase and hydrolysis activities were compared, account was taken of the fact that glutamic acid inhibits PEP carboxylase.

*PEP Hydrolysis: Sequential Dehydrogenase Method.* Assay solutions contained 0.2 mM PEP, 5 mM $NaHCO_3$, 0.27 mM NADH, 1 mM metal, 100 mM HEPES, pH 8.0, and 20 units of malate dehydrogenase. Experiments with $MgCl_2$ were conducted with 1.7, 17, or 200 units of malate dehydrogenase in order to assure that the concentration of this enzyme was not limiting.

Prior to addition of PEP carboxylase, an initial baseline absorbance was established at 340 nm. The reaction was then initiated by the addition of PEP carboxylase (1.2 units). Total OAA formed was determined by observing the decrease in NADH absorbance at 340 nm. When reaction stopped (all PEP consumed), 3.8 units of lactate dehydrogenase was added. The ensuing absorbance change reflected the pyruvate concentration in the solution. All absorbance changes were corrected for dilution.

*Bicarbonate Requirement for PEP Hydrolysis.* A solution containing 5 mM PEP, 5 mM $MgCl_2$, 0.2 mM NADH, 50 mM L-glutamate, and 100 mM HEPES, pH 7.5, was sparged with $N_2$ for approximately 24 h. An aliquot of this solution was transferred by syringe to a sealed, $N_2$-flushed, 1-mL cuvette. The absorbance at 340 nm was recorded, and then aspartate aminotransferase (10 units) and lactate dehydrogenase (20 units) were added by syringe. PEP carboxylase (0.10 unit) was then injected to react with any traces of $HCO_3^-$. After the absorbance at 340 nm stabilized, an aliquot of $NaHCO_3$ was added to the reaction mixture to give a final concentration of 5 mM. The rate of $HCO_3^-$-dependent PEP hydrolysis was measured by following the change in absorbance at 340 nm.

*Heat Inactivation of PEP Carboxylase.* PEP carboxylase in 100 mM HEPES, pH 7.5, containing 10% glycerol was heated at 47 °C for 0–20 min, after which time the enzyme solution was centrifuged for 3 min and then assayed for carboxylation and hydrolysis activity using the transaminase method.

*Inhibition of PEP Carboxylase by 1-Hydroxycyclopropanecarboxylic Acid Phosphate.* Inhibition of the carboxylation and hydrolysis activities of PEP carboxylase by 1-hydroxycyclopropanecarboxylic acid phosphate was determined by the transaminase method using assay solutions containing 2 mM PEP, 5 mM $NaHCO_3$, 15 mM $MgCl_2$, 0.2 mM NADH, 50 mM L-glutamate, 0–1.0 mM inhibitor, and 100 mM HEPES, pH 8.0.

*Decarboxylation of OAA.* Solutions were identical to those used for the transaminase assay and contained 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, or $NiCl_2$, pH 8.0. A fresh OAA solution, pH 1, was used as a source of OAA. Spontaneous decarboxylation velocities were calculated from the absorbance change at 340 nm produced by the lactate dehydrogenase catalyzed reduction of pyruvate. Rates in each case were

determined both with and without the addition of aspartate aminotransferase (3.5 units). Up to 1.7 units of PEP carboxylase was used per 1-mL assay to check for enzyme-catalyzed decarboxylation. The reaction rate in every case was indistinguishable from that observed in the absence of PEP carboxylase.

*Hydrolysis of Phosphate Monoesters.* Rates of hydrolysis of various phosphate monoesters catalyzed by PEP carboxylase were measured by the phosphate assay method of Heinonen and Lahti (1981). Reaction mixtures contained 5 mM substrate, 10 mM $NaHCO_3$, 0.25 mM metal chloride, and 23 mM HEPES, pH 7.8. Phosphate monoesters tested included phosphoglycolate, 2-phosphoglycerate, 3-phosphoglycerate, and L-phospholactate. Metals used were $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$, $Co^{2+}$, and $Ni^{2+}$. Reactions were initiated by addition of 8.3 units of PEP carboxylase to give a total reaction volume of 0.125 mL. Blank reactions in which enzyme was omitted were run in parallel for each trial. Reactions were run at 23 °C and quenched after 14.5 h by adding 0.125 mL of 15% $HClO_4$. Color development was achieved by the addition of 2 mL of a 2:1:1 mixture of acetone, 5 N $H_2SO_4$, and 10 mM $(NH_4)_6Mo_7O_{24}$ to the quenched reaction solution. After 30 s, 0.2 mL of 1 M citric acid was added. Absorbances at 355 nm were measured, and concentrations were derived from a standard curve prepared from $KH_2PO_4$ standards. In all cases, the rate in the presence of PEP carboxylase did not significantly exceed the rate in the absence of enzyme. Hydrolysis of all these PEP analogues was at least 1500–2000 times slower than the total rate of $P_i$ release from PEP. PEP carboxylase showed no loss of activity over the time course of the reaction.

## Results

*PEP Hydrolysis: Transaminase Method.* The PEP carboxylase catalyzed hydrolysis of PEP was measured by coupling pyruvate formation to lactate dehydrogenase. Formation of pyruvate by decarboxylation of OAA was prevented by the inclusion of aspartate aminotransferase and high concentrations of L-glutamate (50 mM) in the assay mixture. Under these conditions, a decrease in absorbance at 340 nm was observed, indicating the PEP carboxylase catalyzed formation of pyruvate.[2] When the same measurement was made with malate dehydrogenase substituted for aspartate aminotransferase and lactate dehydrogenase, it was determined that the hydrolysis rate was 3.2 ± 0.3% of the carboxylation rate.

*Metal Dependence of Carboxylation and Hydrolysis.* When this same experiment was conducted in the absence of a divalent metal, the rate of pyruvate formation was 60 times slower than the rate in the presence of 5 mM $MgCl_2$. However, upon the addition of $MgCl_2$ to a final concentration of 5 mM, the rate of pyruvate formation was the same as that typically obtained with a complete assay solution containing 5 mM $MgCl_2$.

The ability of a variety of divalent metals to activate PEP carboxylase for both carboxylation and hydrolysis reactions was measured using both the transaminase assay and the sequential dehydrogenase assay at pH 8.0 and 25 °C (Table II). When expressed as absolute rates, the carboxylase activity of PEP carboxylase showed a larger metal ion dependence than the hydrolysis rate. However, when expressed relative to the total activity, metal activation toward the hydrolysis reaction varied significantly and decreased in the order $Ni^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$ (Table II).

Table II: Metal Dependence of Hydrolysis and Carboxylation Activities of Maize PEP Carboxylase at pH 8.0 and 25 °C

| | metal ion | | | | |
|---|---|---|---|---|---|
| | $Ca^{2+}$ | $Mg^{2+}$ | $Mn^{2+}$ | $Co^{2+}$ | $Ni^{2+}$ |
| transaminase method | | | | | |
| hydrolysis rate[a] (units/mg) | NR[b] | 0.70 | 0.66 | 0.73 | 0.46 |
| carboxylation rate[a] (units/mg) | 0.84 | 21.4 | 4.80 | 1.95 | 0.17 |
| % hydrolysis | 0 | 3.2 | 12 | 27 | 73 |
| sequential dehydrogenase method | | | | | |
| % hydrolysis | ND[c] | 6.8 | 16 | 32 | 67 |

[a] Solutions contained 5 mM PEP, 5 mM $NaHCO_3$, 0.2 mM NADH, 50 mM L-glutamate, 100 mM HEPES, and 5 mM indicated metal chloride, pH 8.0, at 25 °C. Hydrolysis reactions contained 6 units of lactate dehydrogenase and 3.5 units of aspartate aminotransferase. Carboxylation reactions contained 2.8 units of malate dehydrogenase. [b] No hydrolysis reaction observed. [c] Not determined.

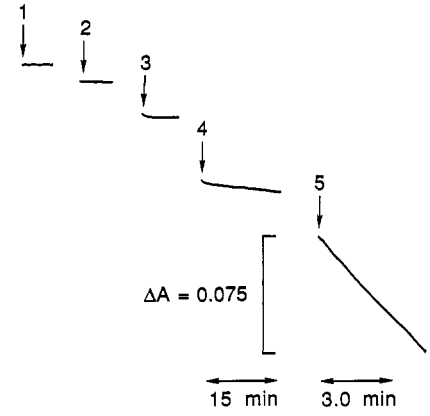


FIGURE 1: $HCO_3^-$ dependence of PEP hydrolysis rate. The change in NADH absorbance at 340 nm with time is shown: (1) initial bicarbonate-free solution containing 5 mM PEP, 5 mM $MgCl_2$, 0.2 mM NADH, 50 mM L-glutamate, and 100 mM HEPES, pH 7.5, at 25 °C; (2) addition of 10 units of aspartate aminotransferase; (3) addition of 20 units of lactate dehydrogenase; (4) addition of 0.10 unit of PEP carboxylase; (5) addition of $NaHCO_3$ (final concentration equals 5 mM).

*$CO_2$ Requirement.* When this same experiment was conducted in the absence of added $HCO_3^-$ using a solution that had been purged of bicarbonate by sparging with $N_2$, the observed rate of NADH disappearance was approximately 30 times slower than that observed in solutions containing 5 mM $NaHCO_3$. The subsequent addition of an aliquot of $NaHCO_3$ to the bicarbonate-free solution to give a final concentration of 5 mM resulted in a rate of NADH utilization comparable to complete assay solutions already containing 5 mM $NaHCO_3$ (Figure 1).

*pH Dependence.* The transaminase assay method was also used to determine the pH dependence of the proportion of hydrolysis in the presence of 5 mM $Mg^{2+}$ at 25 °C. When expressed as a fraction of the total hydrolysis and carboxylation rates, partitioning toward hydrolysis was 2.6 ± 0.2% at pH 9.0 and increased regularly to 3.7 ± 0.4% at pH 7.0.

*PEP Hydrolysis: Sequential Dehydrogenase Method.* In order to be doubly sure that the pyruvate being produced in the above experiments was arising from direct PEP carboxylase action, a second assay method was devised in which a sample of PEP was carboxylated by PEP carboxylase in the presence of malate dehydrogenase and a slight excess of NADH. After the reaction was complete and the absorbance at 340 nm stabilized, the presence of pyruvate could be determined directly by addition of lactate dehydrogenase (Figure 2). The

[2] The initial rate of pyruvate formation determined in the presence of $Mg^{2+}$ at pH 8.0 was the same with or without aspartate aminotransferase, because under these conditions the concentration of OAA is essentially zero. Without aspartate aminotransferase, the observed rate of pyruvate formation increased at longer times, presumably reflecting the accumulation of OAA and its subsequent decarboxylation.

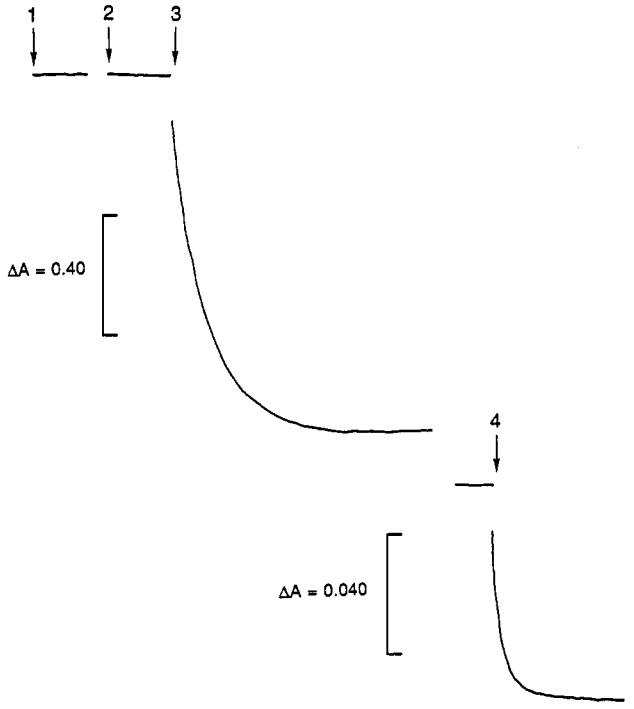


FIGURE 2: Time course of the double dehydrogenase experiment conducted at pH 8.0 with $Mg^{2+}$. The reaction mixture contained (1) 0.2 mM PEP, 5 mM $NaHCO_3$, 0.27 mM NADH, and 100 mM HEPES, at 25 °C with (2) 20 units of malate dehydrogenase added, (3) 1.2 units of PEP carboxylase added, and (4) 3.8 units of lactate dehydrogenase added.

ratio of the second absorbance change to the first gave a measure of the proportions of hydrolysis and carboxylation. Data obtained by this method are consistent with those obtained by the first method (Table II) and indicate that, in the presence of $Mg^{2+}$, about 5% of PEP is converted directly to pyruvate at pH 8. The same results were obtained whether 1.7, 17, or 200 units of malate dehydrogenase were used.

*Heat Inactivation of PEP Carboxylase Activities.* If the pyruvate formation reported above is in fact due to PEP carboxylase, then partial heat inactivation of the enzyme should result in parallel decreases in both carboxylase and hydrolysis activities. To test this, PEP carboxylase was denatured to varying extents by heating aliquots of the enzyme at 47 °C. When the enzyme was assayed for both carboxylation and hydrolysis activity (transaminase method), both activities exhibited nearly identical susceptibilities to heat inactivation (data not shown).

*Inhibition of PEP Carboxylase Activities.* Similarly, an inhibitor of PEP carboxylase would be expected to have parallel effects on both activities. Inhibition of PEP carboxylase carboxylation activity and hydrolysis activity (transaminase method) was observed in solutions containing 2 mM PEP and 0.1–1.0 mM 1-hydroxycyclopropanecarboxylic acid phosphate. The inhibitor had similar effects on both activities (data not shown).

*Additional Control Experiments.* A number of additional checks were performed to demonstrate that pyruvate formation was an authentic activity associated with PEP carboxylase:

(a) PEP carboxylase and all coupling enzymes were demonstrated to be free of malate dehydrogenase, and results were independent of levels of coupling enzymes.

(b) The PEP used was demonstrated to be free of pyruvate.

(c) L-Glutamate, L-aspartate, and α-ketoglutarate were demonstrated to show no activity toward lactate dehydrogenase.

(d) PEP carboxylase was found not to catalyze the decarboxylation of OAA at pH 7.0–9.0 in the presence of either $Mg^{2+}$ or $Ni^{2+}$, even in the presence of up to 1.7 units of enzyme per 1-mL assay.

(e) No hydrolysis of other phosphate monoesters, including L-phospholactate, phosphoglycolate, 2-phosphoglycerate, and 3-phosphoglycerate, was found in this work, establishing that these reactions were at least 2000-fold slower than the normal carboxylation. In fact, Janc et al. (1992) have detected a very slow hydrolysis of a number of phosphate esters catalyzed by PEP carboxylase.

(f) Consistent hydrolysis results were obtained even with PEP carboxylase purified by different procedures and by different investigators.

## Discussion

The principal purpose of this work was to determine whether the carboxylation of PEP by PEP carboxylase is accompanied by significant hydrolysis to form pyruvate and $P_i$, analogous to the reactions seen with a variety of PEP analogues (Table I). Two independent methods have been used to show that this reaction does in fact occur and constitutes about 5% of the total reaction at pH 8 in the presence of $Mg^{2+}$. The extent of hydrolysis depends on pH and metal ion.

Throughout this investigation our concern was to validate the apparent hydrolysis of PEP as an authentic activity of PEP carboxylase. Reagents and enzymes were carefully checked for contaminants that might lead to spurious results. In addition, a large number of control experiments were used to eliminate a variety of possible problems:

(1) The OAA formed during the normal carboxylation decarboxylates readily to form pyruvate, especially in the presence of divalent metal ions (Leussing, 1982). Concentrations of OAA were kept at very low levels by use of aspartate aminotransferase, and control experiments with added OAA demonstrated that this procedure reduced pyruvate formation via OAA to insignificant levels.

(2) A small amount of a contaminating phosphatase might result in direct hydrolysis of PEP. However, phosphatase activity against a number of other phosphate esters was very low. Further, pyruvate formation required the presence of both metal ion and $HCO_3^-$, as is required for normal carboxylase activity. Heat denaturation of PEP carboxylase caused parallel decreases in both carboxylase and hydrolysis activity, as did addition of a competitive inhibitor of PEP carboxylase.

*Mechanism.* The parallel between carboxylase and hydrolysis activities, in particular the $HCO_3^-$ dependence of the velocity, suggests that the two reactions occur by a common mechanism. In the case of other substrates which are hydrolyzed by PEP carboxylase [e.g., bromo-PEP (Diaz et al., 1989), methyl-PEP (Fujita et al., 1984), and fluoro-PEP (Janc et al., 1992)], hydrolysis is accompanied by $^{18}O$ transfer from $HCO_3^-$ to $P_i$. For this reason, it is likely that the hydrolysis mechanism diverges from the carboxylation mechanism at the point of or after the formation of carboxy phosphate.

Several mechanisms for the hydrolysis can be envisioned:

(a) The enolate might be protonated on the enzyme to give pyruvate. However, the enzyme-bound intermediates are likely to be shielded from the solvent, and it is more likely that this protonation occurs after the enolate dissociates from the enzyme, rather than before.

(b) The enolate might dissociate from the enzyme and then be protonated. In the case of methyl-PEP, the protonation of the corresponding enolate to give α-ketobutyrate occurs in a stereo-random fashion, and the free enol has been observed (Gonzales & Andreo, 1988b), thus giving credence to this possibility.

(c) Carboxy phosphate might dissociate from the enzyme and then be hydrolyzed in solution.

(d) Carboxy phosphate might decompose on the enzyme to form $CO_2$ and $P_i$. In fact, several lines of evidence suggest that the carboxylation step in the normal reaction involves $CO_2$, rather than carboxy phosphate, as the actual carboxylating species (Janc et al., 1992). Dissociation of either $CO_2$ or $P_i$ from the enzyme might then initiate the sequence of events leading to formation of pyruvate. Any of these possible scenarios might also involve an enzyme conformation change, perhaps connected with the release of the first product from the enzyme.

Pyruvate kinase is a useful analogue of PEP carboxylase, in the sense that both enzymes form the enolate of pyruvate by phosphate transfer from PEP. Lodato and Reed (1987) demonstrated that oxalate (an isoelectronic analogue of the enolate of pyruvate) is stabilized by bidentate coordination to one of the required metals. The analogy would suggest that the enolate might also be stabilized by bidentate coordination in the active site of PEP carboxylase.[3] The poor inhibition of PEP carboxylase by the phosphonate analogue of PEP (O'Leary, 1983) provides indirect evidence for metal coordination at the bridging oxygen of PEP.

The partitioning of the PEP carboxylase reaction between carboxylation and hydrolysis is strongly metal ion dependent, with hydrolysis decreasing in the order $Ni^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$. Irving and Williams (1953) found that the stability constant of oxalate with the above metals decreased in the same order. To examine more closely the relationship between partitioning toward hydrolysis and complex stability, the logarithm of the relative hydrolysis rate was plotted against the logarithm of the corresponding metal–oxalate stability constant (Willen & Martell, 1964). When the dehydrogenase method data are used, this plot is linear, with a slope of 0.34 and a correlation coefficient of 0.99. The more stable the complex, the greater the extent of hydrolysis.

This suggests that the activity ratio may be governed, at least in part, by the degree to which the metal stabilizes the enolate. In particular, when the enolate is strongly stabilized (e.g., by $Ni^{2+}$), the decrease in the reactivity of the enolate may be sufficient to allow time for either carboxy phosphate or $CO_2$ to dissociate from the enzyme.

Interestingly, mutagenesis of a conserved histidine of the related PEP carboxylase from *Escherichia coli* produces a protein having no carboxylase activity but a significant $HCO_3^-$-dependent PEP phosphatase activity (Terada & Izui, 1991). This histidine may be responsible for both the pH dependence of the hydrolysis reaction observed here and the pH dependence of the normal carboxylation reaction observed previously (O'Leary et al., 1981a).

*Conclusion.* These results provide closure to the survey with numerous analogues of PEP which undergo almost exclusively the hydrolysis reaction (Table I). PEP itself clearly undergoes hydrolysis, though generally in a much smaller proportion than seen with PEP analogues. Under optimum conditions used to assay PEP carboxylase (pH 8.0 with $Mg^{2+}$), about 5% of the total reaction flux is diverted to hydrolysis, rather than carboxylation. Diversion occurs at the stage of the enolate, and the proportion of hydrolysis can be increased by using metals that stabilize the enolate, thereby presumably reducing the reactivity of carbon 3. Interestingly, in the case of $Ni^{2+}$, hydrolysis accounts for more than half of the total reaction flux.

[3] This possibility is currently being investigated in collaboration with Dr. George Reed.

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