Mechanistic Studies of Phosphoenolpyruvate Carboxylase from *Zea mays* Utilizing Formate as an Alternate Substrate for Bicarbonate†

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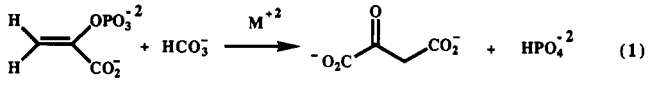
ABSTRACT: Formate is an alternate substrate for bicarbonate in the reaction with PEP catalyzed by phosphoenolpyruvate carboxylase from *Zea mays*, producing formyl phosphate and pyruvate. The K_m for formate is 25 ± 2 mM, and the maximum velocity is 1% of that for bicarbonate at pH 8.0. Use of [^{18}O]formate produces inorganic phosphate containing 1 equiv of ^{18}O, but no label is incorporated into residual phosphoenolpyruvate. PEP carboxylase catalyzes the hydrolysis of phosphoglycolate or L-phospholactate 2000 times more slowly and D-phospholactate 4000 times more slowly than the reaction between bicarbonate and PEP.

Phosphoenolpyruvate carboxylase [orthophosphate:oxaloacetate carboxy-lyase, phosphorylating (EC 4.1.1.31)] catalyzes the initial step of carbon fixation in C_4 and crassulacean acid metabolism plants (eq 1). PEP[1] carboxylase requires



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a divalent cation for activity, with the most effective activators being Mg^{2+} and Mn^{2+} (O'Leary, 1982). Bicarbonate is the substrate rather than CO_2 (Cooper & Wood, 1971; O'Leary & Hermes, 1987), and this appears to be the only non-biotin-dependent carboxylase to utilize bicarbonate rather than CO_2 (Knowles, 1989). In the reaction catalyzed by PEP carboxylase, one atom of oxygen is transferred from bicarbonate to phosphate (Maruyama & Lane, 1962; Maruyama et al., 1966). Isotope effect (O'Leary et al., 1981) and stereochemical studies (Hansen & Knowles, 1982) are consistent with a stepwise mechanism in which carboxy phosphate and the enolate of pyruvate are intermediates. Recent work of Fujita et al. (1984) and O'Laughlin (1988) with methyl-PEP and work of ours with fluoro-PEP (Janc et al., 1992b) have shown that CO_2 is formed reversibly at the active site as the result of decarboxylation of carboxy phosphate. These results suggest that CO_2, rather than carboxy phosphate, is the electrophile that is attacked by the enolate of pyruvate.

Numerous analogues of PEP have been tested as alternate substrates for PEP carboxylase (Gonzalez & Andreo, 1989). However, to date no analogues for bicarbonate have been found. We report here the substrate activity of the bicarbonate analogue, formate, in the reaction catalyzed by PEP carboxylase from *Zea mays*. This is the first time formate has been found to be active as a surrogate for bicarbonate. Additionally, a bicarbonate-independent phosphatase activity of PEP carboxylase has been identified and characterized.

Materials and Methods

Materials. HEPES (free acid), BICINE (free acid), EPPS (free acid), NADH (disodium salt), phosphoglycolate (tricyclohexylammonium salt), bromopyruvate (free acid), ATP (disodium salt), and L-lactic acid were from Sigma Chemical Co. Acetyl phosphate (lithium/potassium salt) and formic acid were from Aldrich Chemical Co. Sodium formate was from Fisher Scientific Co. D-Lactic acid (lithium salt) was from Calbiochem. $H_2{}^{18}O$ (95% enriched) was from EG and G Mound Applied Technologies, and [^{14}C]formic acid (sodium salt) was from ICN Radiochemicals. PEP (monocyclohexylammonium salt) was synthesized by Peter Henke as described by Hirshbein et al. (1982). All other chemicals were of reagent grade and were used without further purification. Alkaline phosphatase (bovine intestinal mucosa), carbonic anhydrase (bovine erythrocytes), and bovine serum albumin were from Sigma Chemical Co. Lactate dehydrogenase (LDH) (porcine muscle), malate dehydrogenase (MDH) (porcine heart), and pyruvate kinase (PK) (rabbit muscle) were from Boehringer Mannheim Biochemicals. PEP carboxylase was purified from *Z. mays* as previously described by O'Leary et al. (1981) and Diaz (1986) to a specific activity of 20 units/mg. Analysis of the purified enzyme by SDS–polyacrylamide gel electrophoresis revealed that the enzyme was essentially homogeneous (>95% pure).

Methods. Kinetic and NMR methods were as described by Janc et al. (1992b).

Formate Substrate Activity and Requirements. To examine the substrate activity of formate with PEP carboxylase, reactions were carried out in the absence of bicarbonate. Assays

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[1] Abbreviations: PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; BICINE, *N*,*N*-bis-(2-hydroxyethyl)glycine; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PK, pyruvate kinase.

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contained 100 mM EPPS, 5.0 mM $MgCl_2$, 10 mM PEP, 0.3 mM NADH, 24 units/mL MDH, 24 units/mL LDH, and 1 unit/mL PEP carboxylase, pH 8.0, at 27 °C. Any exogenous bicarbonate present in the assay mixtures was converted to malate by the action of PEP carboxylase and MDH prior to initiation of the reaction with formate. Solutions used in the kinetic experiments were sparged with CO_2-free N_2 prior to use in order to minimize the level of contaminating bicarbonate that had to be consumed prior to initiation of the reaction. The concentration of residual bicarbonate in the assays was typically 20–40 μM, as determined by end-point analysis with PEP carboxylase. Reactions were performed in cuvettes which were purged of CO_2 with N_2 prior to use and sealed with rubber septa. Solutions were delivered to the cuvette using Hamilton syringes.

Inactivation of PEP Carboxylase by Bromopyruvate. Inactivation was initiated by diluting 100 μL of 20 mM bromopyruvate into 900 μL of a PEP carboxylase stock solution (18 units/mL). A control reaction was run in parallel in which 100 μL of water was added to 900 μL of the stock solution of PEP carboxylase. Both solutions were incubated at 25.0 °C in a thermostated water bath throughout the experiment. At various times, aliquots were withdrawn and the residual activity of PEP carboxylase was measured. The assay of PEP carboxylase activity in which bicarbonate was supplied as the substrate contained 100 mM HEPES (pH 8.0), 10.0 mM $MgCl_2$, 5.0 mM bicarbonate, 20.0 mM PEP, 0.2 mM NADH, and 24 units/mL MDH. The assay of PEP carboxylase activity in which formate was supplied as substrate contained 100 mM formate in place of bicarbonate.

Phosphoglycolate Inhibitor Studies of PEP Carboxylase. Reactions contained 100 mM EPPS, 5.0 mM $MgCl_2$, and 0.25 mM NADH, pH 7.8, at 25 °C. Phosphoglycolate was varied from 0 to 18.0 mM, and PEP was varied from 0.45 to 6.0 mM. Reactions were initiated with formate (final concentration, 100 mM) after the residual bicarbonate present in the assay was converted to malate. A similar experiment was performed in which the inhibition of PEP carboxylase by phosphoglycolate was examined with 1 mM bicarbonate in place of formate as the nonvariable substrate.

Synthesis of ^{18}O-Labeled Formate. Forty microliters of formic acid, 950 μL of $H_2{}^{18}O$ (95% enriched), and 10 μL of 6 M HCl were incubated at 70 °C for 80 h. The extent of ^{18}O incorporation in formate was assayed by ^{13}C NMR. Three formate resonances were observed, corresponding to formate labeled with zero, one, and two ^{18}O atoms, with an isotope-induced chemical shift (upfield) of 0.031 ppm for each atom of ^{18}O incorporated into formate (Risley & Van Etten, 1980). The extent of ^{18}O incorporation in formate was estimated to be 75% on the basis of peak height. The labeled formate was neutralized with 250 μL of 6 M NaOH, frozen, and lyophilized. The resulting solid was dissolved in 1 mL of water and adjusted to pH 8.0. Five milligrams of carbonic anhydrase (10 000 units) was added, and the solution was placed under vacuum for 30 min to remove any contaminating bicarbonate.

^{18}O Transfer Experiment. The enzymatic reaction with ^{18}O-labeled formate contained 100 mM BICINE (pH 8.5), 10.0 mM PEP, and 4.0 mM $MgCl_2$. Dithiothreitol (1.0 mM) and bovine serum albumin (0.1% w/v) were included in the reaction to stabilize PEP carboxylase. The concentration of ^{18}O-labeled formate was 50 mM, and the total volume of the assay was 5 mL. Reactions were initiated with 10 units of PEP carboxylase. A control reaction was run in which PEP carboxylase was omitted. Solutions used for the experiment, except the stock solution of PEP carboxylase and the stock solution of labeled formate, were sparged with N_2 for 16 h prior to the start of the reaction. Reactions were incubated at 23 °C for 18 h, after which time the solutions were loaded onto separate Chelex-100 columns (Bio-Rad; Na^+ form; 1 × 6 cm) to remove Mg^{2+}. The samples were eluted with 10 mL of H_2O and then lyophilized. The resulting solid was dissolved in 1 mL of 50% H_2O/D_2O containing 1 mM EDTA. The pH of the samples was adjusted to 10.0 with NaOH, and the ^{31}P NMR spectra were obtained.

Assay for Formyl Phosphate. Solutions contained 100 mM EPPS (pH 8.0), 10.0 mM PEP, 10 mM $MgCl_2$, 0.2 mM NADH, 1.0 mM dithiothreitol, and 50 mM formate in 4-mL volume at 25 °C. Aliquots (0.8 mL) were withdrawn at 5-min intervals and added to 0.5 mL of 1.8 M hydroxylamine. After 10 min, 0.5 mL of 0.735 M trichloroacetic acid was added to precipitate the protein. The solution was vortexed, and 1 mL of 0.22 M $FeCl_3$ in 1 M HCl was added. The solution was centrifuged, and the absorbance of the supernatant was measured at 505 nm. Succinohydroxamate (2.0 mM) synthesized from succinic anhydride and hydroxylamine (Pechere & Capony, 1968) and 4 mM acetyl phosphate were used to calibrate the assay. In parallel reaction mixtures the rate of pyruvate production was measured spectrophotometrically by coupling the reaction with LDH.

[^{14}C]Formate Studies. The PEP carboxylase catalyzed reaction was performed with [^{14}C]formate as a substrate. Reactions contained 100 mM EPPS (pH 8.0), 5.0 mM PEP, 10.0 mM NADH, 2.5 mM $MgCl_2$, 5.0 mM unlabeled formate, 100 units of LDH, 0.5 unit of PEP carboxylase, and 5.0 μCi of [^{14}C]formate (specific activity = 57.5 mCi/mmol). Reactions were incubated for 21 h at 23 °C. After 21 h the pH was adjusted to 2.0 with 0.1 N HCl containing 5.0 mM formic acid. Reaction solutions were transferred to a water bath at 37 °C and evaporated with a stream of N_2. When the solutions had been completely evaporated (about 4 h), 2.0 mL of the HCl/formic acid mixture was added to each sample and the evaporation procedure repeated. A total of five evaporations were performed in order to remove the last traces of [^{14}C]formate. The amount of radioactivity remaining in each sample was determined by liquid scintillation counting.

Characterization of the PEP Carboxylase Catalyzed Hydrolysis of Phosphate Monoesters. Experiments were performed by incubating the particular monoester with PEP carboxylase for a fixed length of time and then assaying for inorganic phosphate with the colormetric assay of Heinonen and Lahti (1981). Reactions contained 100 mM EPPS (pH 8.0), 5.0 mM $MgCl_2$, and 10 mM phosphoglycolate and were initiated with 2.5 units of PEP carboxylase and incubated for 47 h at 23 °C. Bicarbonate was supplied at 0, 1.0, 5.0, 10.0, and 50 mM. To examine the phosphatase activity of PEP carboxylase in the absence of bicarbonate, 0.2 mM PEP, 0.2 mM NADH, and 25 units of MDH were included in the basic assay mixture to ensure that any residual bicarbonate present would be converted to malate to create an assay that was bicarbonate free.

Inactivation of PEP Carboxylase with Bromopyruvate. To test whether the phosphatase activity of PEP carboxylase decreased at the same rate as the carboxylase activity upon treatment with bromopyruvate, PEP carboxylase (20 units/mL) was incubated in 0, 2.0, or 10.0 mM bromopyruvate, pH 7.3, at 30 °C in a total volume of 400 μL. The solution of PEP carboxylase containing 10.0 mM bromopyruvate was incubated for 100 min ($t_{1/2}$ for inactivation was 20 min). The solution of PEP carboxylase incubated in 2.0 mM bromopyruvate was inactivated for 1 half-life (40 min). The control,

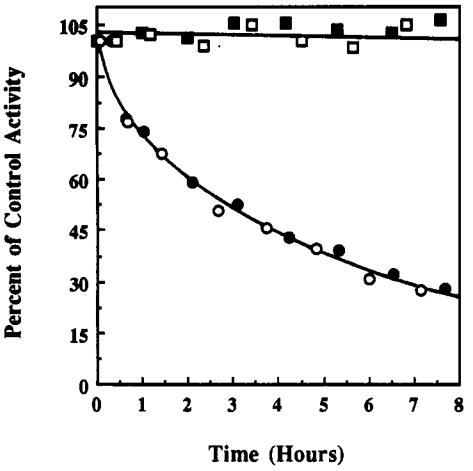

FIGURE 1: Inactivation of PEP carboxylase by bromopyruvate. PEP carboxylase was incubated with (○, ●) or without (□, ■) 2 mM bromopyruvate. Aliquots of enzyme were removed and assayed with 5 mM bicarbonate (●, ■) or with 100 mM formate (○, □) present as substrate.

which was not treated with bromopyruvate, was diluted to the same volume as the samples of PEP carboxylase that were partially inactivated with bromopyruvate. Each of the three samples of PEP carboxylase was transferred to a separate Centricon ultrafiltration apparatus (30 000 MW cutoff; Amicon) and centrifuged at 3000*g* for 20 min at 4 °C. After the centrifugation step, 1.5 mL of 25 mM HEPES (pH 7.3) containing 5.0% glycerol was added, and each sample was centrifuged again to remove any remaining bromopyruvate. The rinsing procedure was repeated three additional times, and then the activities of the three solutions of PEP carboxylase were measured.

Data Processing. Kinetic data were fitted to the appropriate equation with the FORTRAN programs of Cleland (1979).

$$v = VA/(K + A) \quad (2)$$

$$y = m(x) + b \quad (3)$$

$$v = VA/[K(1 + I/K_{is}) + A] \quad (4)$$

Linear double reciprocal plots were fitted to eq 2. Data fitting a straight line were fitted to eq 3. Competitive inhibition data were fitted to eq 4.

RESULTS

Formate Substrate Activity. When formate was added to a bicarbonate-free reaction mixture containing PEP, Mg^{2+}, NADH, LDH, and PEP carboxylase, a linear decrease in absorbance at 340 nm was observed. The rate increased linearly with PEP carboxylase concentration. No rate was observed if PEP, Mg^{2+}, or PEP carboxylase was omitted, nor was any reaction observed when LDH was replaced by MDH. This latter control was performed to ensure that the rate observed was not due to contaminating bicarbonate in the reaction mixture. The reaction followed saturation kinetics with respect to formate, and the K_m value for formate at pH 8.0 was 25 ± 2 mM, 250 times the K_m for bicarbonate. The V_{max} with formate supplied was 100 times lower than that measured with bicarbonate.

Bromopyruvate Inactivation of the Formate Substrate Activity. PEP carboxylase was inactivated with 2.0 mM bromopyruvate at pH 7.3. The activity of PEP carboxylase was monitored throughout the course of the inactivation with either formate or bicarbonate supplied as a substrate. The rate of inactivation of PEP carboxylase was identical with both

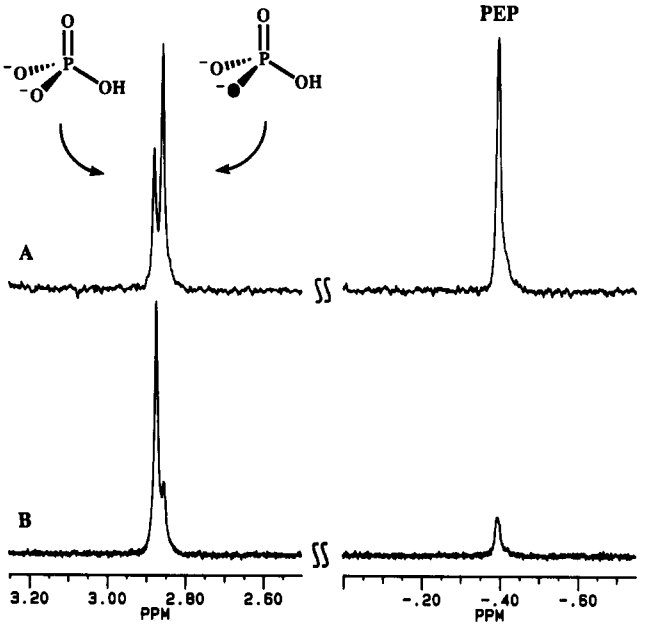

FIGURE 2: (A) Proton-decoupled ^{31}P NMR spectrum of the ^{18}O transfer experiment which contained PEP carboxylase. (B) Identical to (A) but after the addition of 10 mg of Na_2HPO_4.

methods for determining residual PEP carboxylase activity (Figure 1). The $t_{1/2}$ for inactivation of PEP carboxylase by bromopyruvate obtained by fitting the natural logarithm of the percent of control activity remaining versus time to eq 3 was 223 ± 31 min with bicarbonate and 202 ± 32 min with formate as the substrate.

Inhibition of PEP Carboxylase with Phosphoglycolate. Phosphoglycolate was examined as an inhibitor versus PEP, with formate or bicarbonate supplied as the second substrate. Linear competitive inhibition of PEP carboxylase by phosphoglycolate versus PEP was observed in both cases, and the data were fitted to eq 4. The K_I for phosphoglycolate was 2.9 ± 0.2 mM when bicarbonate was the nonvariable substrate and 6.2 ± 1.0 mM when formate was the nonvariable substrate.

^{18}O Transfer Experiment. An ^{18}O-labeling study was performed in which the PEP carboxylase catalyzed reaction was run with formate containing 75% ^{18}O. The transfer of ^{18}O from formate to phosphate was monitored by ^{31}P NMR. Spectra were externally referenced to 200 mM D_3PO_4 and were proton decoupled. Figure 2A shows the proton-decoupled ^{31}P NMR spectrum of the reaction which contained PEP carboxylase. The singlet at −0.41 ppm is due to PEP, and the two resonances at 2.8 ppm correspond to inorganic phosphate containing zero (downfield resonance) and one (upfield resonance) atoms of ^{18}O. The separation between the two resonances was 0.021 ppm, similar to the results of Cohn and Hu (1978). Figure 2B shows that the resonance corresponding to unlabeled phosphate increased in intensity relative to the other resonances upon addition of 10 mg of Na_2HPO_4. In a control in which PEP carboxylase was omitted, two singlet resonances were observed in the ^{31}P NMR spectrum: one at −0.47 ppm due to PEP and one at 2.8 ppm due to unlabeled inorganic phosphate resulting from the hydrolysis of PEP. When 10 mg of Na_2HPO_4 was added to the control sample, the resonance corresponding to unlabeled phosphate increased in intensity.

Assay for the Production of Formyl Phosphate. A hydroxamate assay was performed to test for the production of formyl phosphate when formate was the substrate. The hydroxamate assay is specific for acyl phosphates and has been

used to detect the presence of formyl phosphate in other enzymatic reactions (Sly & Stadtman, 1963; Mejillano et al., 1989). A linear increase in absorbance at 505 nm was observed with time, corresponding to the production of formyl phosphate with a rate of 48 ± 8 μM/min. The assay was calibrated with acetyl phosphate and succinohydroxamate standards. In parallel reactions the rate of the reaction when coupled to LDH was 34 ± 5 μM/min. Controls were performed in which either formate or PEP carboxylase was excluded from the reaction. No increase in absorbance at 505 nm was observed in either of the controls over the same time period used to measure the rate of formyl phosphate production in the complete reaction.

[^{14}C]Formate Studies. An experiment was performed using [^{14}C]formate as the substrate. The control reactions in which either PEP carboxylase or PEP was omitted contained 1600 and 2300 cpm (respectively) after the reaction had been completed and the unreacted [^{14}C]formate removed by evaporation. The complete reaction contained 23 000 cpm (10× the amount measured in either of the controls). However, the residual radioactivity detected in the complete reactions only corresponded to 1 out of every 140 turnover events on the basis of the rate of pyruvate production when the reaction was coupled to LDH.

Initial Characterization of the Phosphatase Activity of PEP Carboxylase. When phosphoglycolate was incubated with Mg^{2+}, bicarbonate, and PEP carboxylase at pH 8.0, inorganic phosphate was liberated. The amount of phosphate produced was independent of the concentration of bicarbonate (up to 50 mM) present in the reaction mixture. No phosphate was detected in the reaction in which Mg^{2+} was omitted, indicating that the apparent phosphatase activity of PEP carboxylase requires Mg^{2+}. The phosphatase activity measured with phosphoglycolate was 2000 times less than the carboxylase activity under identical conditions. PEP carboxylase activity was assayed throughout the experiment, and no decrease in activity was detected.

An experiment was performed in which the contaminating bicarbonate present in the assay components was converted to malate to create an assay that was bicarbonate free. This was accomplished by including sufficient PEP and NADH together with MDH in the basic assay to consume the exogenous bicarbonate. The amount of phosphate liberated in the bicarbonate-free assay was identical to that in the reaction containing 10 mM bicarbonate, indicating that the phosphatase activity of PEP carboxylase does not require bicarbonate.

Phosphatase Activity of PEP Carboxylase with D- *and* L-*Phospholactate Supplied as Substrates.* D- and L-phospholactate were synthesized enzymatically from the corresponding lactate isomers and MgATP in the presence of pyruvate kinase (Ash et al., 1984). L-Phospholactate was hydrolyzed by PEP carboxylase at approximately the same rate as phosphoglycolate, while D-phospholactate was hydrolyzed at half the rate observed with L-phospholactate or phosphoglycolate.

Inactivation of the Phosphatase Activity of PEP Carboxylase with Bromopyruvate. PEP carboxylase was partially inactivated with bromopyruvate to determine whether loss of carboxylation activity parallels the loss of phosphatase activity. As shown in Table I, the loss in phosphatase activity of PEP carboxylase directly correlates with the loss of carboxylase activity when PEP carboxylase is inactivated with the affinity label, bromopyruvate. At the highest degree of inactivation, the phosphatase activity does appear somewhat higher than the carboxylase activity; this is due either to the difficulty of measuring small amounts of phosphate accurately or to a small contamination with a phosphatase.

Table I: Inactivation of the Phosphatase Activity of PEP Carboxylase by Bromopyruvate[a]

reaction	% carboxylase act. remaining	phosphate liberated (mM)	av phosphate liberated as % of control
1A	100	2.30	100
1B	100	2.38	
2A	32	0.60	26
2B	32	0.62	
3A	0.4	0.16	6
3B	0.4	0.15	

[a] Reactions contained 100 mM HEPES (pH 7.7), 5 mM $MgCl_2$, 2 mM $NaHCO_3$, and 15 mM phosphoglycolate. Reactions were incubated at 23 °C for 72.5 h, and all reactions were performed in duplicate (A and B). Reaction 2 was run with enzyme preincubated for 40 min with 2 mM bromopyruvate and reaction 3 with enzyme preincubated for 100 min with 10 mM bromopyruvate. Reaction 1 was run with uninhibited enzyme. See Materials and Methods for handling of preincubated enzyme samples.

Table II: Inactivation of the Phosphatase Activity of PEP Carboxylase by Heat[a]

reaction	% carboxylase act. remaining	phosphate liberated (mM)	av phosphate liberated as % of control
1A	100	3.64	100
1B	100	3.09	
2A	35	1.50	41
2B	35	1.35	
3A	2.2	0.24	7
3B	2.2	0.25	

[a] Reactions contained 100 mM EPPS (pH 8.0), 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM $NaHCO_3$, and 15 mM phosphoglycolate. Reactions were incubated at 23 °C for 67 h. Reactions were performed in duplicate (A and B). Enzyme used for reactions 1–3 was preincubated for 3 h at 4 °C (1), 25 min at 50 °C (2), and 3 h at 50 °C (3).

Inactivation of the Phosphatase Activity of PEP Carboxylase by Heat. An experiment similar to that described above in which PEP carboxylase was thermally inactivated was carried out in order to determine whether the loss in carboxylation activity of PEP carboxylase is paralleled by a loss in phosphatase activity. The data in Table II show that heat inactivation of the carboxylase activity of PEP carboxylase produces a corresponding decrease in phosphatase activity, although again there is a slight excess of phosphatase over carboxylase activity at high levels of inactivation.

Discussion

Earlier studies of PEP carboxylase have suggested that carboxy phosphate and the enolate of pyruvate are intermediates in the reaction (O'Leary et al., 1981; Hansen & Knowles, 1982). Our recent work (Janc et al., 1992b) indicates that carboxy phosphate is not attacked directly by the enolate of pyruvate but decomposes at the active site to form CO_2, which is the actual carboxylating species. Formate has been examined as an alternate substrate replacing bicarbonate in an effort to obtain independent evidence concerning the molecule reacting with the enolate of pyruvate. The first step in the reaction catalyzed by PEP carboxylase with formate would involve the formation of formyl phosphate and the enolate of pyruvate. Since formyl phosphate cannot undergo decarboxylation to form CO_2, as can carboxy phosphate, the next step would necessarily be attack of the enolate on formyl phosphate to form 2,4-dioxobutyrate. Therefore, if formylation were occurring, it would be expected to occur via attack of

the enolate on formyl phosphate.

We have demonstrated that PEP carboxylase will utilize formate as an alternate substrate replacing bicarbonate. The reaction requires Mg^{2+} and follows saturation kinetics with respect to formate. In order to demonstrate that the reaction was catalyzed by PEP carboxylase and not a contaminating protein, two types of experiments were performed. The first involved the affinity label bromopyruvate. Previous studies have shown that PEP carboxylase is alkylated at the active site by bromopyruvate (Gonzalez et al., 1986; O'Laughlin, 1988). The demonstration that the rate of inactivation of PEP carboxylase by bromopyruvate was the same regardless of whether assays were conducted with formate or bicarbonate strongly suggests that the reaction observed with formate was catalyzed by PEP carboxylase. The inhibition of PEP carboxylase by phosphoglycolate was examined as a second means of demonstrating that the reaction observed with formate is catalyzed by PEP carboxylase. In the presence of phosphoglycolate, linear competitive inhibition of PEP carboxylase versus PEP was observed with either formate or bicarbonate present as the nonvariable substrate. The apparent K_I for phosphoglycolate was 2.9 ± 0.2 mM in the experiment with bicarbonate and 6.2 ± 1.0 mM in the experiment with formate. The reason for the different K_I values is that bicarbonate and formate bind to the E–phosphoglycolate complex to decrease the apparent K_I value, and the level of bicarbonate used was higher relative to its dissociation constant than was the case with formate.

The PEP carboxylase catalyzed reaction in which formate is the substrate is predicted to form formyl phosphate in a reaction analogous to the formation of carboxy phosphate. There is precedent for the formation of formyl phosphate in other enzyme-catalyzed reactions. For example, formyl phosphate has been shown to be a chemically competent intermediate in the reaction catalyzed by formyltetrahydrofolate synthetase (Smithers et al., 1987; Mejillano et al., 1989). A chemical characterization of formyl phosphate has recently been reported by Jahansouz et al. (1989). At neutral pH, the hydrolysis of formyl phosphate occurs with 50% C–O bond cleavage, while at extremes of pH the hydrolysis of formyl phosphate occurs with C–O bond cleavage exclusively. In the presence of nucleophilic buffers such as Tris and glycine, the hydrolysis of formyl phosphate occurs with 100% C–O bond cleavage accompanied by the formation of formyl-Tris and *N*-formylglycine. The half-life of formyl phosphate in 100 mM HEPES at 20 °C, pH 7.0, is 48 min (Smithers et al., 1987).

In order to detect the formation of formyl phosphate in the reaction catalyzed by PEP carboxylase, an isotope transfer experiment was performed. When ^{18}O-labeled formate was used as a substrate, 1 equiv of ^{18}O was transferred to inorganic phosphate during the reaction. This result is consistent with the formation of formyl phosphate, with its subsequent hydrolysis occurring with C–O bond cleavage. Additionally, a hydroxamate assay was used to monitor the production of formyl phosphate directly. It was determined that the rate of formyl phosphate production was identical to the rate of pyruvate production on the basis of the LDH coupled assay, indicating that hydrolysis of formyl phosphate occurs in solution rather than at the active site. When the reaction was performed with [^{14}C]formate, a small amount of residual nonvolatile radioactivity was detected in the complete assay corresponding to less than 1% of the reaction measured with LDH. The residual radioactivity is probably the result of nonspecific formylation of reaction components by formyl phosphate rather than the formation of 2,4-dioxobutyrate; however, the latter possibility cannot be ruled out.

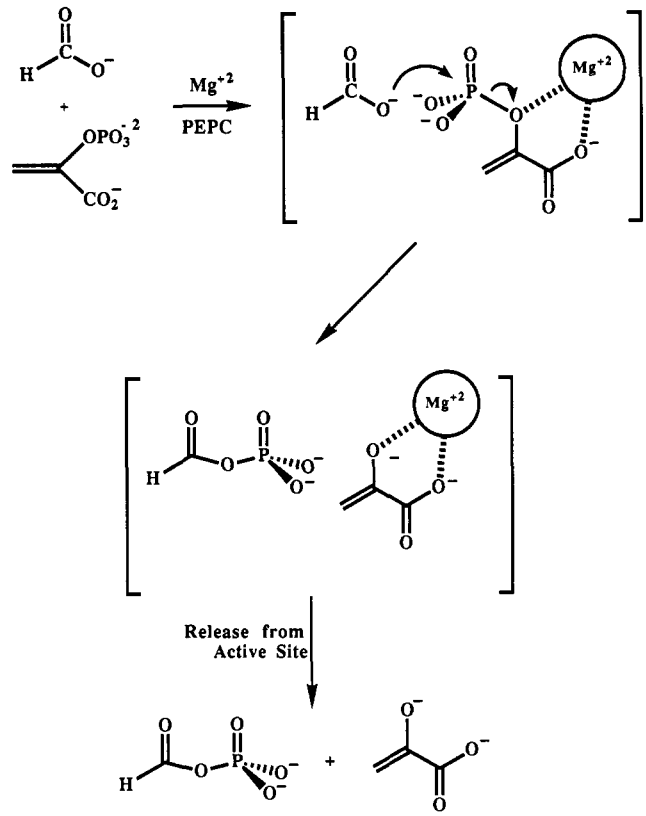

FIGURE 3: Reaction catalyzed by PEP carboxylase with formate as a substrate.

In summary, the reaction catalyzed by PEP carboxylase with the bicarbonate analogue, formate, produces the enolate of pyruvate and formyl phosphate, which is subsequently released from the active site. It is not known whether the enolate of pyruvate is released directly or whether it ketonizes to form pyruvate prior to being released. Studies of the PEP carboxylase catalyzed hydrolysis of methyl-PEP have demonstrated that the enolate of α-ketobutyrate is released into solution (Gonzalez & Andreo, 1988; O'Laughlin, 1988). These results would suggest that the enolate of pyruvate is released from the active site rather than pyruvate, but the reaction is too slow to detect the presence of enolpyruvate in solution, as was done with methyl-PEP (Gonzalez & Andreo, 1988) (Figure 3). The failure of formyl phosphate to be attacked by the enolate of pyruvate to produce 2,4-dioxobutyrate, in spite of the fact that formyl phosphate would be expected to be more electrophilic than carboxy phosphate, is consistent with the decarboxylation of carboxy phosphate to form CO_2 as an obligatory step prior to attack by the enolate of pyruvate in the normal carboxylation.

PEP carboxylase has been found to catalyze the hydrolysis of phosphoglycolate and L-phospholactate approximately 2000 times more slowly than the carboxylase activity. D-Phospholactate was hydrolyzed 4000 times more slowly than the carboxylase activity. The phosphatase activity requires Mg^{2+} but does not require bicarbonate. It is not surprising that the phosphatase activity does not require bicarbonate because the formation of carboxy phosphate from a monoester would be a very unfavorable reaction thermodynamically. However, phosphoryl transfer from an alcohol (the monoester) to H_2O is a thermodynamically favorable reaction. The general base that has been implicated to catalyze the decarboxylation of carboxy phosphate may play a role in catalyzing the attack of water on the monoester (Janc et al., 1992a).

To ensure that the hydrolysis activity observed was due to PEP carboxylase rather than a nonspecific phosphatase, PEP carboxylase was inactivated with the affinity label bromopyruvate. The loss in carboxylase activity as a result of inactivation by bromopyruvate directly parallels the loss in phosphatase activity. Thermal inactivation of the carboxylation activity of PEP carboxylase was also paralleled with a corresponding loss in phosphatase activity. The fact that both bromopyruvate and heat are able to inactivate the phosphatase activity at the same rate as the carboxylase activity strongly suggests that PEP carboxylase is responsible for the phosphatase activity observed rather than a contaminating phosphatase.

Conclusion. It has long been assumed that the mechanism of action of PEP carboxylase involves as a first step the transfer of phosphate from PEP to bicarbonate, forming carboxy phosphate and the enolate of pyruvate. Isotope-labeling studies (Maruyama et al., 1966) and early studies of carbon isotope effects (O'Leary et al., 1981) are consistent with this mechanism. It was originally assumed that carboxy phosphate served as a direct carboxylating agent, forming, in a single step, OAA and phosphate. Mechanistically, the difficulty with such a mechanism is that reactions of nucleophiles with carboxylate anions are unusual or unknown in the organic chemical literature, and carboxy phosphate would not be expected to be reactive toward nucleophiles.

The studies in this paper and the three preceding papers provide a convincing alternative mechanism that overcomes this dilemma. Instead of reacting directly with the enolate, carboxy phosphate decomposes to CO_2 and phosphate in the active site, and CO_2 is the actual carboxylating species.

Several lines of evidence support this point of view and mitigate against the simpler mechanism. First, transfer of ^{18}O from $HC^{18}O_3^-$ to the nonbridging oxygens of the phosphate group of the substrate accompanies reaction of several PEP analogues (O'Laughlin, 1988; Fujita et al., 1984; Janc et al., 1992b); this reaction is most easily interpreted as being due to reaction of CO_2 with phosphate to form carboxy phosphate and, ultimately, return of this material to the starting state. This same mechanism explains why certain substrates produce phosphate containing more than a single atom of ^{18}O (O'Laughlin, 1988; Janc et al., 1992b) and why certain substrates apparently produce carboxy phosphate but not carboxylated product (Ausenhus & O'Leary, 1992). This same mechanism provides a ready explanation for the fact that formate can replace bicarbonate in the early stages of the reaction, producing formyl phosphate, but no formylation product is produced. Carbon isotope effects with the two isomers of fluoro-PEP are quantitatively consistent with this mechanism but not with the simpler one (Janc et al., 1992b).

Thus these studies, together with the recent analysis of Knowles (1989), suggest that enzymes which use bicarbonate as a substrate invariably dehydrate it to form CO_2 at the active site before reaction; these enzymes presumably have evolved to use bicarbonate because of the potential for greater affinity of this substrate when compared to CO_2, thus providing a selective advantage for survival in an environment of low CO_2 concentration.

Registry No. PEP carboxylase, 9067-77-0; formic acid, 64-18-6; phosphoglycolic acid, 13147-57-4; phosphatase, 9013-05-2; D-phospholactate, 28238-06-4; L-phospholactate, 28238-07-5.

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