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Reaction of Phosphoenolpyruvate Carboxylase with (*Z*)-3-Bromophosphoenolpyruvate and (*Z*)-3-Fluorophosphoenolpyruvate†

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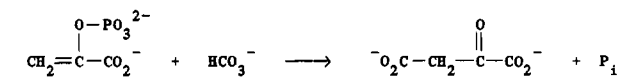
ABSTRACT: (*Z*)-3-Bromophosphoenolpyruvate inactivates phosphoenolpyruvate carboxylase from maize in the presence of HCO_3^- and either Mg^{2+} or Mn^{2+}. The inactivation rate follows saturation kinetics. Inactivation is slower in the presence of phospholactate or epoxymaleate, both of which are inhibitors of the enzyme, or dithiothreitol. Inactivation is completely prevented by the presence of lactate dehydrogenase and NADH, and 3-bromolactate is formed during this treatment. If the reaction is conducted by using $HC^{18}O_3^-$, the inorganic phosphate produced contains ^{18}O. This and other evidence indicate that phosphoenolpyruvate carboxylase catalyzes conversion of bromophosphoenolpyruvate into bromopyruvate by way of the usual carboxyphosphate–enolate intermediate, and bromopyruvate is the species responsible for enzyme inactivation. (*Z*)-3-Fluorophosphoenolpyruvate is transformed by the enzyme into a 6:1 mixture of 3-fluoropyruvate and 3-fluorooxalacetate, presumably by the same mechanism. The enzyme is not inactivated during this treatment.

Phosphoenolpyruvate (PEP)[1] carboxylase (EC 4.1.1.31) catalyzes the reaction



The enzyme is widely distributed in plants but is apparently absent from animal tissues (Vennesland & Mazelis, 1958;

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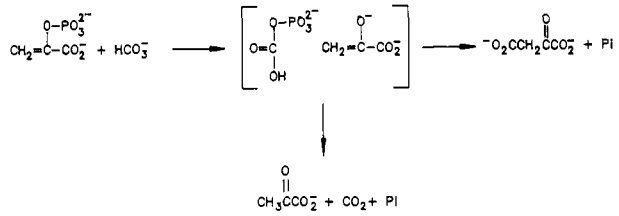
Scheme I



Utter & Kolenbrander, 1972; Hatch & Slack, 1966; O'Leary, 1982, 1983; Andreo et al., 1987). The role of PEP carboxylase in C_4 plants is particularly important: incorporation of atmospheric CO_2 into oxalacetate is the first step in the C_4 pathway of photosynthesis. Because of the number of C_4 plants that are noxious weeds, there is particular interest in the development of specific inhibitors of PEP carboxylase and other enzymes of the C_4 pathway.

PEP carboxylase uses HCO_3^- rather than CO_2 as substrate (Cooper & Wood, 1971; Maruyama et al., 1966; Reibach & Benedict, 1977; O'Leary & Hermes, 1987). pH studies indicate that the enzyme binds the trianion form of PEP (O'Leary et al., 1981) and that bicarbonate, rather than carbonate, is the substrate (R. Gill and M. H. O'Leary, unpublished data). The enzyme requires a divalent metal. Highest activities are observed with Mg^{2+} and Mn^{2+}, but several other metals have limited activity (O'Leary et al., 1981). The product of the reaction is the keto form of oxalacetate (Chen et al., 1955), and pyruvate is neither a substrate nor an intermediate (Maruyama & Lane, 1962a,b).

When $HC^{18}O_3^-$ is used as a substrate for the carboxylation, the P_i produced contains ^{18}O (Maruyama et al., 1966; O'Leary & Hermes, 1987). The concerted cyclic mechanism that was originally proposed to account for this observation (Maruyama et al., 1966) is not consistent with current evidence. O'Leary et al. (1981) reported that only a small ^{13}C isotope effect is observed for the carbon atom arising from HCO_3^-, whereas a large isotope effect would be expected for the concerted mechanism. Hansen and Knowles (1982) reported that when the phosphorus of PEP is made chiral by using ^{16}O, ^{17}O, ^{18}O, and S, chiral thiophosphate is formed in the reaction with inversion of configuration at phosphorus. The cyclic concerted mechanism should proceed with adjacent attack at phosphorus, which would give retention of configuration.

The alternative to the concerted cyclic mechanism is a stepwise mechanism (Scheme I) in which the first step is phosphate transfer to form carboxyphosphate and the enolate of pyruvate. Such a scheme provides a reasonable mechanism for activation of HCO_3^- toward carbon–carbon bond formation and at the same time provides a method for activating carbon-3 of PEP. In the second step, the enolate carbon attacks the carbon atom of carboxyphosphate, forming oxalacetate and P_i. The stepwise mechanism is consistent with the isotope effect results and requires that formation of the intermediate is rate-determining (O'Leary et al., 1981). The stereochemical results require that the intermediate undergo a conformational change prior to carboxylation (Hansen & Knowles, 1982). This mechanism is also consistent with synergistic inhibition exhibited by oxalate and carbamyl phosphate, which is an analogue of the proposed carboxyphosphate intermediate (M. H. O'Leary and J. E. Rife, unpublished results).

PEP carboxylase from *Escherichia coli* catalyzes hydrolysis of phosphoenol-α-ketobutyrate, and this reaction is accompanied by ^{18}O transfer from HCO_3^- to P_i (Fujita et al., 1984). The maize enzyme catalyzes a similar hydrolysis (Gonzalez & Andreo, 1986), also with incorporation of ^{18}O into P_i (J. O'Laughlin and M. H. O'Leary, unpublished results). The isotope transfer suggests that the hydrolysis also occurs via the carboxyphosphate–enolate intermediate (cf. Scheme I), but instead of the enolate being carboxylate, it is hydrolyzed.

In this paper and the following three we report synthesis and study of a variety of analogues of PEP that are intended to serve as mechanism-based inhibitors of PEP carboxylase and other enzymes that metabolize PEP. Surprisingly, for all compounds tested, we find that hydrolysis, rather than carboxylation, is the principal reaction.

We previously suggested that (*Z*)-phosphoenol-3-bromopyruvate (Br-PEP) inactivates PEP carboxylase by way of bromooxalacetate, which alkylates the enzyme (Díaz & O'Leary, 1982). Results reported in this paper indicate that this mechanism is not correct. Instead, Br-PEP is converted by the enzyme into bromopyruvate in a HCO_3^--dependent process involving the same type of intermediate as is believed to occur in the normal carboxylation reaction. Details of the inactivation are described in this paper, as are analogous studies with F-PEP.

Experimental Procedures

Materials. Water was purified in a Millipore Super-Q water filtration system. PEP carboxylase was purified from maize as described by O'Leary et al. (1981), except that the final chromatography was carried out with Sephacryl S-300. The final specific activity of the enzyme was about 20 units/mg. Other enzymes were purchased from Sigma Chemical Co. PEP, NADH, bromopyruvate, and all buffers used were purchased from Sigma. Dithiothreitol was obtained from Sigma or Chemalog. Ion-exchange resins were obtained from Bio-Rad. NaB^3H_4 was obtained from ICN Radiochemicals. Aquasol was obtained from New England Nuclear. Pyruvic acid (Aldrich Chemical Co.) was distilled at reduced pressure before use. 1-Butanol (Aldrich) and CH_2Cl_2 were purified as described in Perrin and Perrin (1966). Aldrich Gold Label trifluoroacetic anhydride was used without purification. Other compounds were of reagent grade and were used without further purification.

Cyclohexylammonium Br-PEP [*Z*-isomer; see Duffy and Nowak (1984)] was prepared as described by Stubbe and Kenyon (1972). Epoxymaleic acid was prepared by the method of Payne and Williams (1959). Bromomalic acid was prepared from epoxymalic acid (Kuhn & Ebel, 1925). Bromolactic acid was prepared by reduction of bromopyruvic acid with sodium cyanoborohydride (Borch et al., 1971). The product was recrystallized from chloroform–pentane: mp 88–89 °C [lit. (Jones, 1978) 92–94 °C]; mass spectrum, *m/e* 171 [MH^+ (^{81}Br)], 169 [MH^+ (^{79}Br)], 125 [$M^+ - CO_2$ (^{81}Br)], 123 [$M^+ - CO_2$ (^{79}Br)].

S-Carboxyhydroxyethylcysteine was synthesized by the procedure of Meloche (1970). F-PEP (90% *Z*, 10% *E* isomer by NMR) was synthesized by Dr. Douglas Fluornoy by the method of Stubbe and Kenyon (1972).

Methods. Rates were measured on a Gilford Model 222 spectrophotometer or a Cary 118 spectrophotometer with a cell compartment thermostated at 25 °C. Counting of radioactive samples was done on a Beckmann Model LS-8000 scintillation counter, using glass scintillation vials and Aquasol scintillation solution. Amicon ultrafiltration cells Models 12 and 202 were used with PM-10 and PM-30 ultrafiltration

[1] Abbreviations: PEP, phosphoenolpyruvate; Br-PEP, (*Z*)-3-bromophosphoenolpyruvate; F-PEP, (*Z*)-3-fluorophosphoenolpyruvate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

membranes for the filtration of protein solutions. Nuclear magnetic resonance spectra were taken on either a JEOL MH-100 spectrometer (^1H), an IBM-WP-200 spectrometer (^1H), a JEOL FX-60 spectrometer (^{13}C), a JEOL FX-200 spectrometer (^{13}C), or a Bruker AM-500 spectrometer (^{31}P). Mass spectra were taken on a Kratos MS-80 spectrometer.

(*a*) *PEP Carboxylase Assay.* Enzyme activity was measured by coupling the carboxylation of PEP to the reduction of oxalacetate catalyzed by malate dehydrogenase and monitoring the disappearance of NADH at 340 nm. A typical assay solution contained 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.2 mM NADH, 5 mM PEP, 100 mM Tris, pH 8.0, and 6.7 units of malate dehydrogenase in 1 mL of solution.

Studies with Br-PEP and F-PEP were conducted in the same way, with malate dehydrogenase or lactate dehydrogenase being used as noted.

(*b*) *Inactivation of PEP Carboxylase by Br-PEP.* PEP carboxylase was incubated with 0.4 mM $MnCl_2$ or 10 mM $MgCl_2$, 100 mM Tris, pH 7.5 or 8.2, 0.1 mM EDTA, 7.5 mM $NaHCO_3$, 12.5% glycerol, and various concentrations of Br-PEP at 25 °C. Aliquots were withdrawn at various times and assayed for PEP carboxylase activity. Blank solutions without Br-PEP were treated in the same way. Protection experiments were done in the same way in the presence of phospholactate, epoxymaleic acid, dithiothreitol, or 0.5 mM NADH and 20 units of lactate dehydrogenase.

(*c*) *Radioactive Labeling of PEP Carboxylase.* PEP carboxylase was incubated with 0.4 mM $MnCl_2$, 100 mM Tris, pH 8.2, 0.1 mM EDTA, 12.5% glycerol, 500 mM Br-PEP, and 5 mM $NaHCO_3$ for 2 h. The inactivated enzyme solution (10 mL) was mixed with 0.1 mL of NaB^3H_4 (5 mCi, 1.4 mCi/mmol) in 14 N NaOH. The pH was adjusted to 8 with 6 N HCl, and the solution was left at room temperature for 1 h and then dialyzed against four changes of H_2O. The water was removed by lyophilization, and the solid enzyme was hydrolyzed in 6 N HCl under vacuum at 110 °C for 20 h. The HCl was removed from the hydrolyzed protein under high vacuum, and 1 mL of 1 N acetic acid was added to the hydrolysate. This solution was added to an AG 1-X8 column (acetate form, 100–200 mesh, 1.2 × 40 cm) and eluted with 1 N acetic acid (Hirs, et al., 1954). Four fractions containing radioactivity were obtained. The acetate was removed from all fractions under high vacuum at 60 °C.

(*d*) *Derivatization.* Hydrolysis products were esterified and acetylated by the procedure described by Kaiser et al. (1974). To the lyophilized fractions was added 1 N butanol–HCl, and the mixture was refluxed under N_2 for 1 h. The butanol was removed under high vacuum at 60 °C. For acetylation, 10 mL of dichloromethane and 5 mL of trifluoroacetic anhydride were added to the dry samples in vials. The vials were covered with aluminum foil, capped, and left at room temperature in a jar containing $CaSO_4$ until they were analyzed.

(*e*) *Thin-Layer Chromatography of Derivatized Fractions.* Each fraction was applied together with the standard onto TLC plates (silica gel) and eluted with the following solvent systems: cyclohexane–ethyl acetate, 2:1; cyclohexane–ethyl acetate, 1:1; and ethyl acetate–methanol, 5:1. The plates were immersed in a solution containing 0.05% $PdCl_2$ in 0.5% HCl [test positive for sulfur (Zweig & Sherma, 1972)]. Sections of the plates were scraped off and counted.

(*f*) *Br-PEP as Alternate Substrate.* Solutions containing 5 mM $NaHCO_3$, 10 mM $MgCl_2$ or 0.4 mM $MnCl_2$, 50 units/mL lactate dehydrogenase, 0.2 mM NADH, 100 mM Tris, pH 8.2, and 0.0745–0.745 mM Br-PEP were equilibrated to 25 °C. PEP carboxylase was added, and the change in absorbance at 340 nm was measured as before. For studies at low HCO_3^- concentration, the buffer was purged with CO_2-free N_2 for 1.5 days, and the assays were carried out in stoppered cuvettes.

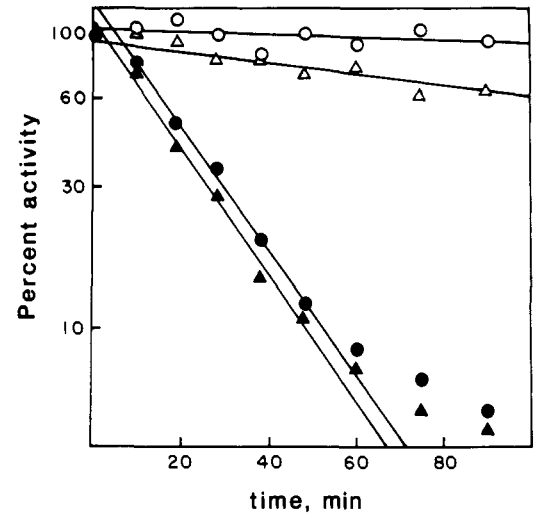


FIGURE 1: Inactivation of PEP carboxylase by Br-PEP at pH 8.2, 25 °C, in the presence of 0.4 mM $MnCl_2$ and 6 mM $NaHCO_3$ with (○) no Br-PEP, with (▲) 590 μM Br-PEP, with (●) 590 μM Br-PEP and 20 units of lactate dehydrogenase, and with (△) 590 μM Br-PEP, 20 units of lactate dehydrogenase, and 500 μM NADH.

(*g*) *Phosphate Determination.* Phosphate concentrations were determined as described by Bencini et al. (1983). All glassware was throughly rinsed with 1 N HCl and water before use.

(*h*) ^{18}O *Transfer from Bicarbonate to Phosphate.* A 3 M $K_2C^{18}O_3$/1 M $KHC^{18}O_3$ solution was prepared by adding the appropriate amounts of K_2CO_3 and $KHCO_3$ to $H_2{}^{18}O$ and leaving it at room temperature for 4 days. ^{13}C NMR of the solution showed four peaks in the bicarbonate region, indicating that exchange into carbonate had taken place. Five milliliters of 50 mM Tris, pH 8.2, containing 10 mM $MgCl_2$ was added to a 10-mL flask. The flask was covered with a rubber septum, and the solution was degassed for 5 h by bubbling it with CO_2-free N_2. Then 100 μL of 105 mM Br-PEP, 40 units of PEP carboxylase (in 100 mM PIPES, pH 6.5), and 50 μL of 4 M [^{18}O]bicarbonate solution were successively added to the flask. The reaction was stopped after 2 min by addition of 5 drops of concentrated H_2SO_4. A second flask containing the same solution plus 1.5 mg of carbonic anhydrase was processed in the same way. Both solutions were ultrafiltered, and the pH of both solutions was adjusted to 6 with 4 N NaOH. Each solution was passed through a Chelex-100 column (Na^+ form, 50–100 mesh, 0.6 cm × 5 cm), after which the water was removed by lyophilization. The dried samples were dissolved in 0.5 mL of D_2O. EDTA (0.05 mL, 100 mM) was added, the pH was adjusted to 12–13, and ^{31}P NMR spectra were taken.

RESULTS

Inactivation of PEP Carboxylase by Br-PEP. When PEP carboxylase was incubated with Br-PEP in the presence of either Mg^{2+} or Mn^{2+} and HCO_3^-, the enzyme was gradually inactivated (Figure 1). In a given experiment, the inactivation followed first-order kinetics, and the activity approached zero at long times, as reported previously (Díaz & O'Leary, 1982). When the Br-PEP concentration was varied, the rate showed saturation kinetics. A double-reciprocal plot of rate constant vs Br-PEP concentration gave a maximum rate constant for inactivation of 0.0736 min^{-1} and an apparent Michaelis constant of 62.9 μM when Mn^{2+} was present and a rate constant

of 0.0490 min^{-1} and a Michaelis constant of 261 μM when Mg^{2+} was present. Similar results were obtained at pH 7.5. No PEP carboxylase activity could be recovered after extensive dialysis of the inactivated enzyme. When DTT was present, less inactivation occurred.

A decrease in the rate of PEP carboxylase inactivation at high Br-PEP concentrations was observed with both metals. The presence of Br-PEP at the active site of the enzyme seems to prevent the binding of the inactivating species, which suggests that this species is released into solution but may bind again at the active site.

Epoxymaleate, a competitive inhibitor vs PEP of PEP carboxylase, protects the enzyme against inactivation by Br-PEP. In the presence of 12.5% glycerol, 100 mM Tris, 0.751 mM Br-PEP, 7.5 mM $NaHCO_3$, and 0.4 mM $MnCl_2$ at pH 8.2, 1.98 mM epoxymaleate completely inhibited the inactivation of PEP carboxylase.

Effect of Lactate Dehydrogenase. The presence of lactate dehydrogenase and NADH also protects PEP carboxylase from inactivation by Br-PEP (Figure 1). In the presence of 10% glycerol, 0.05 mM EDTA, 0.590 mM Br-PEP, 6 mM $NaHCO_3$, 50 mM Tris at pH 8.2, 0.33 mM $MnCl_2$, and 0.5 mM NADH, 20 units of lactate dehydrogenase reduced the rate of inactivation of the enzyme by a factor of 8.5. When lactate dehydrogenase was replaced by malate dehydrogenase, inactivation occurred at the same rate as in the absence of dehydrogenase.

When PEP carboxylase was incubated with Br-PEP at pH 8.2, 25 °C, in the presence of 5 mM $NaHCO_3$, 0.2 mM NADH, 50 units/mL lactate dehydrogenase, and either 10 mM $MgCl_2$ or 0.4 mM $MnCl_2$, a decrease in NADH concentration with time was observed. A double-reciprocal plot of the rate of NADH disappearance vs Br-PEP concentration was linear and gave a Michaelis constant of 457 ± 25 μM and a maximum rate constant that is 25% of that observed with PEP and malate dehydrogenase under the same conditions. Similar results were observed with Mn^{2+}.

The rate of change in NADH concentration decreased at low bicarbonate concentration. When 2.3 mM Br-PEP was incubated with 0.2 unit of PEP carboxylase, 5 mM $MgCl_2$, 0.3 mM NADH, 50 units of lactate dehydrogenase, 100 mM Tris, and 40 μM HCO_3^- at pH 8.2, a rate of of 15 μM/min was observed. When 4.2 mM bicarbonate was added, the rate was 27 μM/min.

Phosphate Release during Incubation of PEP Carboxylase with Br-PEP. Phosphate release was detected during incubation of PEP carboxylase with Br-PEP through its complexation to $(NH_4)_6Mo_7O_{24}$. When 0.565 mM Br-PEP was incubated with 5 mM $NaHCO_3$, 10 mM $MgCl_2$, 100 mM Tris, pH 8.2, and 0.2 unit of PEP carboxylase at 25 °C, a rate of phosphate release of 69.1 ± 0.6 μM/min was obtained. When the same system was coupled to 50 units of lactate dehydrogenase and 0.2 mM NADH in two separate experiments, a rate of NADH disappearance of 73.2 ± 7.6 μM/min was obtained.

^{18}O Transfer during Incubation of PEP Carboxylase with Br-PEP. PEP carboxylase was incubated with Br-PEP in the presence of $HC^{18}O_3^-$ and Mg^{2+} at pH 8.2 and room temperature. A ^{31}P NMR spectrum of the reaction mixture after inactivation of PEP carboxylase showed that the signal for P_i contained two resonances, separated by 0.02 ppm, corresponding to P_i containing zero and one ^{18}O (Figure 2) (O'Leary & Hermes, 1987). When carbonic anhydrase was added to the incubation mixture, only one phosphate resonance was observed.

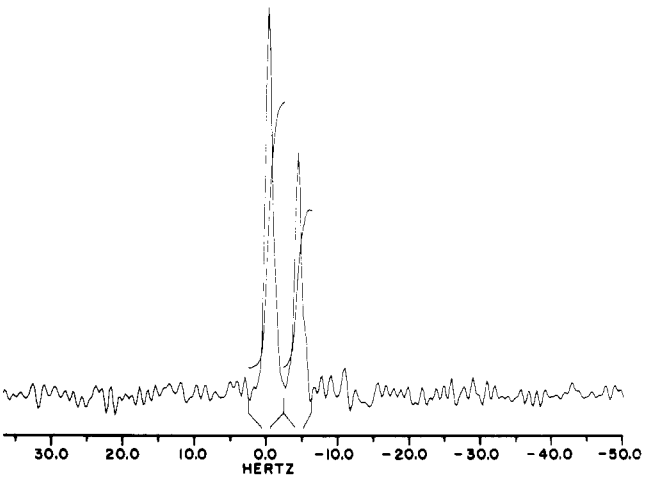


FIGURE 2: ^{31}P NMR spectra of P_i formed by the PEP carboxylase catalyzed reaction of Br-PEP with $HC^{18}O_3^-$ at pH 8.2. Chemical shifts are given relative to P_i.

Product Identification. PEP carboxylase was inactivated with Br-PEP and reduced with $NaBH_4$ as previously described (O'Leary & Díaz, 1982). The mixture was chromatographed on an Aminex HPX-87H HPLC column. Only bromolactic acid, glyceric acid, and Br-PEP were detected; bromomalate, tartrate, glycerate, and Br-PEP were absent. The system was capable of detecting as little as 2.4% of product, and at least 87% of the initial Br-PEP could be accounted for.

When 1.00 mM Br-PEP was incubated with 5 mM $MgCl_2$, 5 mM $NaHCO_3$, 100 mM Tris, pH 8.2, 1 mM NADH, 0.6 unit of PEP carboxylase, and 50 units of lactate dehydrogenase, a total change in NADH concentration of 0.53 mM was obtained. HPLC analysis of the reaction mixture gave a final bromolactate concentration of 0.56 mM.

Labeling Experiments. Previous experiments demonstrated that radioactive label was retained in PEP carboxylase when the enzyme was incubated with Br-PEP in the presence of $NaH^{14}CO_3$ and then treated with 0.5 N $NaBH_4$ immediately after inactivation (Díaz & O'Leary, 1982). However, more recent results have shown that a similar amount of label is incorporated when the same experiment is conducted in the absence of Br-PEP. Label incorporation apparently results from reaction of HCO_3^- with the enzyme rather than carboxylation of Br-PEP.

Incubation of PEP carboxylase with 500 μM Br-PEP, 0.4 mM $MnCl_2$, 100 mM Tris, pH 8.2, 0.050 mM EDTA, 12% glycerol, and 5 mM $NaHCO_3$ for 2 h resulted in 95% inactivation of the enzyme. Reduction of this material with NaB^3H_4 followed by extensive dialysis gave enzyme that retained 0.2 equiv of ^3H per enzyme subunit. The resulting protein was hydrolyzed and chromatographed on Amberlite AG 1-X8. The radioactive fractions were esterified with 1-butanol and acetylated with trifluoroacetic anhydride and analyzed by thin-layer chromatography. Ninety percent of the total radioactivity cochromatographed with authentic *S*-carboxyhydroxyethylcysteine, the product expected if bromopyruvate reacts with one or more cysteine residues. *S*-Carboxyhydroxyethylcysteine is stable under the hydrolysis conditions used (Meloche, 1970).

Reaction of F-PEP with PEP Carboxylase. In normal assays, F-PEP is a competitive inhibitor (vs PEP) of PEP carboxylase. The inhibition constant at pH 8.0 is 85 μM in the presence of 5 mM Mg^{2+} and 35 μM in the presence of 0.8 mM Mn^{2+}.

When F-PEP was incubated with PEP carboxylase at pH 8.0 in the presence of HCO_3^-, malate dehydrogenase, NADH,

and Mg^{2+}, a decrease in NADH absorbance was observed. The reaction follows saturation kinetics, with a K_m value of 1150 μM and a maximum velocity that is 8% of that observed with PEP under the same conditions. When the same experiment was conducted with lactate dehydrogenase, rather than malate dehydrogenase, a similar change in NADH absorbance was observed. The rate observed in this experiment is 40% of the rate observed with PEP and malate dehydrogenase.

The enzyme-catalyzed reaction of F-PEP can also be observed by measuring the rate of P_i production during the reaction. In the presence of Mn^{2+}, the rate obtained by this method equals the sum of the rates observed by measuring NADH oxidation with lactate dehydrogenase and malate dehydrogenase. A comparison of rates of P_i release with PEP and with F-PEP under similar conditions gives relative reactivities of approximately 2:1 for the two substrates.

No inactivation of PEP carboxylase was observed during reaction of F-PEP with the enzyme.

Discussion

We have previously shown that in short-term measurements of enzyme activity Br-PEP is a competitive inhibitor of PEP carboxylase (Díaz & O'Leary, 1982). Inhibition constants are similar for Mg^{2+} (6.7 μM at pH 8.0) and Mn^{2+} (26 μM at pH 8.0) and are similar to those observed for other PEP analogues.

In longer term measurements, PEP carboxylase is inactivated by Br-PEP. In the following paragraphs we summarize the evidence which leads to the conclusion that Br-PEP is transformed into bromopyruvate, which inactivates the enzyme.

Inactivation of PEP carboxylase by Br-PEP is accompanied by enzymatic transformation of Br-PEP and release of P_i. The inhibition is slower at low HCO_3^- concentration, although the precise concentration dependence has not been determined. The inhibition is slower in the presence of phospholactate or epoxymaleate, both of which are competitive inhibitors of the enzyme. Qualitatively, it appears that high concentrations of Br-PEP also protect against inactivation. Inactivation is completely prevented by the presence of lactate dehydrogenase and NADH. Inactivation is also slowed or prevented by DTT (Br-PEP itself does not react with DTT). Contrary to earlier reports (O'Leary & Díaz, 1982), no ^{14}C is incorporated into the enzyme when the inactivation is conducted in the presence of $H^{14}CO_3^-$.

Bromolactate is formed when the inactivation is conducted in the presence of lactate dehydrogenase and NADH. Bromolactate is also formed if the inactivation of PEP carboxylase is carried out in the absence of lactate dehydrogenase and NADH, but then $NaBH_4$ is added after the inactivation is complete.

When the inactive enzyme is reduced with radioactive $NaBH_4$ and then hydrolyzed, the product that is found is the product expected from reaction of bromopyruvate with cysteine. Thus, it is likely that PEP carboxylase is acting on Br-PEP to form bromopyruvate, which in turn inactivates the enzyme. The fact that bromopyruvate reacts with one or more cysteine residues in PEP carboxylase has also been observed in inactivation studies with bromopyruvate itself (Gonzalez et al., 1986; J. O'Laughlin and M. H. O'Leary, unpublished results). High reactivity of bromopyruvate toward sulfhydryl groups is consistent with the observation that the inactivation of PEP carboxylase is largely prevented by the presence of dithiothreitol.

An independent approach to the chemistry of Br-PEP is obtained by using lactate dehydrogenase to study the kinetics of PEP carboxylase catalyzed formation of bromopyruvate from Br-PEP. The reaction shows saturation kinetics, with K_m values similar to those observed in the inactivation. The rate of formation of bromopyruvate is essentially the same as the rate of formation of of P_i. Comparison of the rate of oxidation of NADH with the rate of inactivation of the enzyme reveals that less than 1 time in 10^3 does formation of bromopyruvate lead to inactivation of the enzyme. There is no evidence that Br-PEP is ever converted to bromooxalacetate. The latter is not a known compound, but if formed, its reduction product, 3-bromomalate, should have been detected chromatographically following reduction of reaction mixtures with $NaBH_4$. In addition, the stoichiometric equivalence of P_i formation and NADH oxidation in the experiments with lactate dehydrogenase suggests that all of the substrate is being converted to bromopyruvate. The lack of an induction period in experiments with lactate dehydrogenase is also consistent with the direct formation of bromopyruvate. To our knowledge, bromooxalacetate is not a known compound; thus, we cannot be sure of the rate at which any bromooxalacetate formed would decarboxylate. On the basis of the fact that fluorooxalacetate decarboxylates about fivefold more slowly than oxalacetate itself (Dummel et al., 1971), the rate of decarboxylation of bromooxalacetate should be similar to that of oxalacetate. Experiments with malate dehydrogenase and NADH are inconclusive because it is not known whether bromooxalacetate is a substrate for malate dehydrogenase; experiments that we conducted with bromomalate suggest that it is not.

The ability of lactate dehydrogenase and NADH to protect the enzyme against inactivation by Br-PEP indicates that bromopyruvate is released into solution before inactivation occurs. Previous studies (Gonzalez et al., 1986) indicate that reaction of PEP carboxylase with bromopyruvate follows saturation kinetics and occurs at the active site. This is consistent with our observation that inactivation of PEP carboxylase by Br-PEP is slower in the presence of competitive inhibitors of the enzyme.

The reaction of PEP carboxylase with F-PEP leads to a mixture of products. The majority of the substrate (about 85%) is converted to fluoropyruvate, which has been detected by reducing it to fluorolactate with lactate dehydrogenase. However, a small fraction of the substrate (about 15%) is carboxylated, leading to fluorooxalacetate, which has been detected by reducing it to fluoromalate. F-PEP is an excellent substrate for PEP carboxylase, being converted at about half the rate for PEP itself and approximately twice the rate for Br-PEP.

The mechanism of PEP carboxylase catalyzed formation of bromopyruvate from Br-PEP and of fluoropyruvate from F-PEP is of particular interest. The possibility that PEP carboxylase simply has a nonspecific phosphatase activity (or is simply contaminated by a phosphatase) is eliminated by the observation that ^{18}O is transferred from HCO_3^- to P_i during the action of PEP carboxylase on Br-PEP. A similar isotope transfer accompanies the carboxylation of PEP itself (Maruyama & Lane, 1962a,b; O'Leary & Hermes, 1987). This and other evidence (O'Leary, 1982; O'Leary et al., 1981) indicate that the reaction occurs by way of a carboxyphosphate–enolate intermediate (Scheme I, X = H). In the case of Br-PEP the isotopic evidence suggests that the reaction proceeds to the normal carboxyphosphate–enolate intermediate, but rather than being carboxylated, the enolate is protonated, leading to bromopyruvate and eventually to CO_2 and P_i (Scheme I, X

= Br). A similar hydrolysis appears to occur with 3-methyl-PEP, and this reaction also shows ^{18}O transfer to P_i (Fujita et al., 1984; Gonzalez & Andreo, 1986; J. O'Laughlin and M. H. O'Leary, unpublished results). In the case of F-PEP, both carboxylation and hydrolysis products are formed, presumably because protonation of the enolate intermediate competes with carboxylation. This is the only case to date for which both carboxylation and hydrolysis have been demonstrated for a single substrate. The maize enzyme also catalyzes the hydrolysis of phosphoenolthiopyruvate (Sikkema & O'Leary, 1988), alleno-PEP (Wirsching & O'Leary, 1988), and (*Z*)-3-chloro-PEP (J. Liu and M. H. O'Leary, unpublished results). A small amount of hydrolysis (3–4%) is observed with PEP itself (S. Ausenhus and M. H. O'Leary, unpublished results).

The factors that control this diversion of the intermediate to the hydrolysis pathway are not currently known, but it is interesting that all substrates examined to date except PEP itself undergo principally hydrolysis rather than carboxylation. Given the variety of substrates for which this abnormal reaction occurs, it is likely that both steric and electronic effects are important in diverting the reaction to the hydrolysis pathway.

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