Stereoselectivity of Interaction of Phosphoenolpyruvate Analogues with Various Phosphoenolpyruvate-Utilizing Enzymes†

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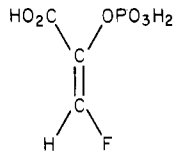
ABSTRACT: The halogenated phosphoenolpyruvate analogues (*Z*)-phosphoenol-3-fluoropyruvate, (*E*)-phosphoenol-3-fluoropyruvate, and (*Z*)-phosphoenol-3-bromopyruvate were synthesized and purified. The analogues were characterized by ^1H and by ^{19}F NMR where applicable. Absolute stereoselectivity of the fluorophosphoenolpyruvate isomers as substrates with the enzymes phosphoenolpyruvate carboxykinase, enolase, and pyruvate phosphate dikinase was observed. The *Z* isomer exhibited substrate activity with these enzymes while no substrate activity was measured with the *E* isomer. Both isomers exhibited substrate activity with the enzyme pyruvate kinase, however, with a substantial decrease in the V_{max}/K_m ratio compared to phosphoenolpyruvate as the substrate. A metal ion dependent stereoselectivity of inhibition was measured for these analogues with the enzymes phosphoenolpyruvate carboxykinase, enolase, and pyruvate kinase. The cation activator appears to affect the specificity and thus the catalytic site of these enzymes. Proton longitudinal relaxation rate titrations demonstrate that the dissociation constants, K_3, of the fluorophosphoenolpyruvate isomers from the enzyme–Mn complex agree, in most cases, with the measured K_I values and analogue binding resembles phosphoenolpyruvate binding. With the enzyme phosphoenolpyruvate carboxykinase, the $K_I \neq K_3$ for (*E*)-fluorophosphoenolpyruvate which suggests that the binding of the *E* isomer is affected by the presence of the other substrates. The halogenated derivatives apparently undergo an enzyme–Mn catalyzed Michael-type addition reaction with the bromo-substituted analogue decomposing much faster than the fluoro analogues.

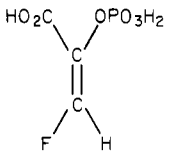
The phosphoenolpyruvate (P-enolpyruvate)[1]-utilizing enzymes P-enolpyruvate carboxykinase, pyruvate kinase, and pyruvate phosphate dikinase belong to a group of enzymes which catalyze homologous reactions. Phosphoryl transfer from P-enolpyruvate to an acceptor molecule occurs concomitant with (although not necessarily concerted with) the addition of a group (i.e., H^+ or CO_2) to the C-3 position of P-enolpyruvate. These enzymes catalyze reactions different from the reaction catalyzed by another P-enolpyruvate-utilizing enzyme, enolase. This enzyme catalyzes a trans hydration of P-enolpyruvate to yield D-2-PGA.

A series of analogues of P-enolpyruvate had previously been synthesized and partially investigated as substrates and as inhibitors of several different enzymes (Stubbe & Kenyon, 1971, 1972). Steric effects seemed to play an important role in ligand interactions. The most useful analogues appeared to be PEB, F-P-enolpyruvate, and Br-P-enolpyruvate, which were synthesized as the *Z* isomers. The *E* and *Z* diastereomers of PEB were synthesized and isolated by Adlersberg et al. (1977) and shown to interact stereospecifically with pyruvate kinase to yield mechanistic information. These diastereomers were shown to interact in a stereoselective manner with other P-enolpyruvate-utilizing enzymes as inhibitory ligands (Duffy et al., 1982). The mechanistic studies with these diastereomers have been extended with pyruvate kinase and with E_I of the sugar transport systems (Hoving et al., 1983).

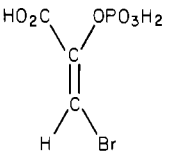
The synthesis of the halogenated analogues has been modified, and the *E* isomers have been prepared for the fluoro and the bromo derivatives.



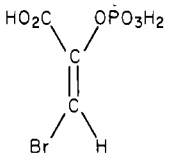
Z-F-P-enolpyruvate



E-F-P-enolpyruvate



Z-Br-P-enolpyruvate



E-Br-P-enolpyruvate

Preliminary experiments with the enzyme P-enolpyruvate carboxykinase showed that *Z*-Br-P-enolpyruvate is a potent competitive inhibitor of the enzyme displaying no substrate activity (Hebda & Nowak, 1982a). This compound, however, apparently acts as a substrate and suicide inhibitor of the related enzyme P-enolpyruvate carboxylase (O'Leary & Diaz, 1982). *Z*-F-P-enolpyruvate acts as a substrate for P-enolpyruvate carboxykinase; results obtained with an *E*–*Z* mixture were interpreted as the *E* isomer eliciting no kinetic effect (Hebda & Nowak, 1982a).

The compounds *Z*-F-P-enolpyruvate and *Z*-Br-P-enolpyruvate were reported to be slowly reacting substrates for pyruvate kinase as was *Z*-PEB (Stubbe & Kenyon, 1971, 1972). The diastereomers of PEB are stereoselective for py-

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§ A Research Career Development Awardee of the National Institutes of Health (AM 00486).

[1] Abbreviations: P-enolpyruvate, phosphoenolpyruvate; PEB, 2-phosphoenolbutyrate; Br-P-enolpyruvate, phosphoenol-3-bromopyruvate; F-P-enolpyruvate, phosphoenol-3-fluoropyruvate; 2-PGA, 2-phosphoglyceric acid; PRR, solvent proton relaxation rate; PP_i, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl.

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ruvate kinase (Adlersberg et al., 1977), and this effect is metal ion dependent (Duffy et al., 1982). Substrate activity with rabbit muscle enolase was observed with *Z*-F-P-enolpyruvate but not with *Z*-Br-P-enolpyruvate or *Z*-PEB (Stubbe & Kenyon, 1972). *Z*-PEB is a competitive inhibitor of enolase (Söling et al., 1971), and the inhibition is stereoselective for the diastereomers; this selectivity is also metal ion dependent (Duffy et al., 1982).

To obtain more detailed information concerning the stereospecificity of ligand interactions to various P-enolpyruvate-utilizing enzymes, the halogenated analogues were studied. Kinetic studies were performed to determine substrate activity and inhibitory effects on chicken liver P-enolpyruvate carboxykinase, rabbit muscle pyruvate kinase, yeast enolase, and pyruvate phosphate dikinase from *Bacteroides symbiosus*. PRR studies with P-enolpyruvate carboxykinase, enolase, and pyruvate kinase were performed to obtain thermodynamic and structural information concerning their interactions.

Experimental Procedures

Materials

P-enolpyruvate carboxykinase was purified by a modification of the method of Hebda & Nowak (1982a). The calcium phosphate adsorption was replaced by chromatography on a DEAE Bio-Gel A column. The extract (4500 mg of protein; 1200 units) was loaded onto the column (2.5 × 40 cm) at pH 8.0 in 5 mM potassium phosphate buffer. The column was washed with 400 mL of this buffer followed by elution with 5 mM potassium phosphate, pH 7.0. The enzyme activity eluted with this buffer with about 60% recovery (specific activity, 1.5–2.1 units/mg). This preparation was then loaded onto a Blue Dextran column as previously described (Hebda & Nowak, 1982a). Yeast enolase was purified by the procedure of Westhead & McLain (1964). Lactate dehydrogenase, malate dehydrogenase, hexokinase, catalase, and pyruvate kinase were purchased from Boehringer Mannheim Corp. Pyruvate phosphate dikinase from *Bacteroides symbiosus*, purified by the method of Milner et al. (1975), was a generous gift of Drs. N. Goss and H. G. Wood, Case Western Reserve University. P-enolpyruvate, IDP, ADP, AMP, pyruvate, α-ketobutyrate, and NADH were purchased from Sigma. Difluorooxalacetate was a gift from Dr. M. Martinez-Carrion. All other reagents were of the highest purity commercially available. All solutions were made by using distilled water which was passed through a mixed-bed deionizing column.

Methods

Kinetic Assays. Initial velocity studies of P-enolpyruvate carboxykinase were performed by measuring the rate of formation of oxalacetate from P-enolpyruvate by a continuous assay coupled with malate dehydrogenase as previously described (Hebda & Nowak, 1982a). The reaction mixture consisted of Tris-HCl, pH 7.4 (65 μmol), KCl (100 μmol), $MnCl_2$ (4 μmol), P-enolpyruvate (2 μmol), IDP (2 μmol), β-mercaptoethanol (143 μmol), $KHCO_3$ (200 μmol), NADH (0.14 μmol), and 22 enzyme units of malate dehydrogenase in a 1-mL volume. The reaction was initiated by the addition of an appropriate amount of P-enolpyruvate carboxykinase. The decrease in absorption of NADH was followed at 340 nm as a function of time by using a Gilford 240 or 250 spectrophotometer. The temperature was controlled at 25 °C.

Initial velocity studies of pyruvate kinase were performed by monitoring the decrease of absorption of NADH at 340 nm by using a coupled pyruvate kinase–lactate dehydrogenase assay. The assay consisted of Tris-HCl, pH 7.4 (50 μmol), KCl (100 μmol), $MnCl_2$ (4 μmol) or $MgCl_2$ (2 μmol), ADP (1 μmol), NADH (0.14 μmol), P-enolpyruvate (2 μmol), and lactate dehydrogenase (20 μg) in a volume of 1 mL. The reaction was initiated by the addition of pyruvate kinase (Mildvan & Leigh, 1964). The temperature was kept at 25 °C.

Initial velocity studies of enolase were performed by monitoring the increase of absorption at 240 nm due to the formation of P-enolpyruvate as a function of time. The assay consisted of Tris-HCl, pH 7.5 (65 μmol), KCl (50 μmol), $MnCl_2$ (0.01 μmol) or $MgCl_2$ (0.5 μmol), and 2-PGA (1 μmol) in a total volume of 1 mL. The reaction was initiated with enzyme. The temperature was controlled at 25 °C.

Initial velocity studies of pyruvate phosphate dikinase were performed by monitoring the decrease of absorption of NADH at 340 nm with a pyruvate phosphate dikinase–lactate dehydrogenase coupled assay. The assay consisted of imidazole chloride, pH 6.8 (50 μmol), NH_4Cl (20 μmol), $MgCl_2$ (20 μmol), PP_i (0.04 μmol), P-enolpyruvate (1 μmol), AMP (0.4 μmol), NADH (0.14 μmol), lactate dehydrogenase (1 enzyme unit), and pyruvate phosphate dikinase in 1 mL (South & Reeves, 1975). The temperature was maintained at 25 °C.

Proton Relaxation Rate (PRR) Studies. Binding studies of ligands were carried out by titrations of the enzyme–Mn^{2+} complex with ligands as previously described (Nowak & Mildvan, 1970; Nowak, 1981). The longitudinal relaxation rates ($1/T_1$) of water protons were measured by using a modified Seimco PS60W pulsed NMR spectrometer. Routine measurements were made at 24.3 MHz. Measurements were taken at room temperature (22 ± 1 °C). Prior to the experiments, all enzymes were passed through a Bio-Rad P-2 column (1.1 × 25 cm) having a 2-cm layer of Chelex-100 on the top. The column was equilibrated with 0.065 M Tris-HCl buffer, pH 7.4. The enzyme was concentrated by using an Amicon Model 8MC ultrafiltration system. The enzyme and Mn^{2+} were combined with the proper buffer and salt, as used in the respective kinetic assays, in a final volume of 0.05 mL. Unless indicated otherwise, the concentration of Mn^{2+} was 40 μM, and the concentration of enzyme sites was in excess of Mn^{2+} concentration (90–150 μM sites). The titrations were performed with an identical sample which also contained the ligand to be titrated. Increments of the second sample were then titrated into the first so that no change in the enzyme or Mn^{2+} concentration occurred, and the PRR was then measured. The paramagnetic contributions to the relaxation rates ($1/T_{1p}$) were calculated by subtracting the relaxation rates observed in the absence of Mn^{2+} ($1/T_{1,0}$) from those observed in its presence ($1/T_{1,obsd}$). Estimates of the observed enhancements, ϵ^*, were calculated by using the equation

$$\epsilon^* = \frac{1/T_{1p}^*}{1/T_{1p}}$$

where $1/T_{1p}^*$ indicates the paramagnetic effect in the presence of enzyme and the denominator gives the paramagnetic effect in the absence of enzyme.

The dissociation constants and enhancements of the ternary complexes, ϵ_T, were determined by a computer fit to the PRR titration data which minimized the percent standard deviation of ϵ_T (Reed et al., 1970). The equilibria and their respective constants which were used are

$$K_1 = \frac{[M][S]}{[MS]} \qquad K_3 = \frac{[E\text{–}M][S]}{[EMS]}$$

$$K_s = \frac{[E][S]}{[ES]} \qquad K_d = \frac{[E][M]}{[EM]}$$

The enhancement value for the binary MS complex is defined as ϵ_a and for the binary EM complex as ϵ_b. These values were independently measured. Values for K_1 and K_d were independently evaluated. The values for K_s and K_3 were varied to obtain the "best fit" to the data. The values reported are the averages obtained from several titrations.

In the treatment of the data, the following constants were used: for the Mn–P-enolpyruvate complex, $K_1 = 1.9 \times 10^{-3}$ M and $\epsilon_a = 1.1$ (Nowak & Lee, 1977); for the P-enolpyruvate carboxykinase–Mn complex, $K_d = 5.0 \times 10^{-5}$ M and $\epsilon_b = 13.5$ (Hebda & Nowak, 1982b). For pyruvate kinase, the K_d of the pyruvate kinase–Mn complex is 5.5×10^{-5} M and ϵ_b is 20 (Mildvan & Cohn, 1966). The constants for enolase are a K_d for enolase–Mn of 5.0×10^{-6} M and an ϵ_b of 15 (Nowak & Mildvan, 1970). The K_1 and ϵ_a for the P-enolpyruvate analogue–Mn complexes were assumed to be identical with the constants for the Mn–P-enolpyruvate complex.

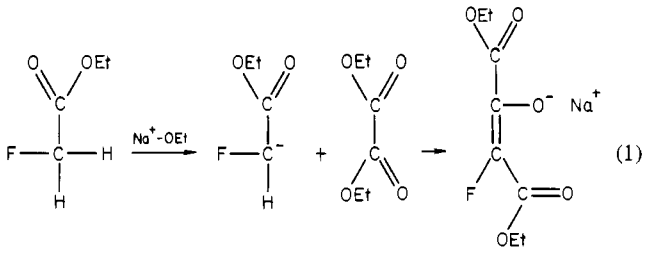
Synthesis of P-enolpyruvate Analogues. Nuclear magnetic resonance spectra were measured at 100 and 94 MHz for ^1H and ^{19}F, respectively, with a Varian XL100 spectrometer equipped with a Nicolet pulse system and 1080 computer, unless otherwise indicated. Chemical shifts were measured in parts per million from an external sample of tetramethylsilane for ^1H and potassium fluoride for ^{19}F NMR. Total phosphate and inorganic phosphate were determined by the method of Ames (1966). These results were compared to those determined by limiting substrate assays with the enzymes pyruvate kinase and lactate dehydrogenase.

Synthesis of (Z)-Cyclohexylammonium Dihydrogen Phosphoenol-3-bromopyruvate. This compound was synthesized by the method of Stubbe & Kenyon (1971). The ^1H nuclear magnetic resonance spectrum (D_2O, pD 2.6) showed a peak at δ 7.75 (1 H, d, $J_{POCCH} = 2.5$ Hz) (Figure 1A).

Synthesis of (E,Z)-Cyclohexylammonium Dihydrogen Phosphoenol-3-bromopyruvate. The Z-Br-P-enolpyruvate was photoisomerized in quartz cuvettes by using a 450-W (medium pressure mercury lamp) UV source (15 min) to give an *E–Z* mixture. The ^1H nuclear magnetic resonance spectrum (D_2O, pD 2.6) showed a peak at δ 7.75 (1 H, d, $J_{POCCH} = 2.5$ Hz) (*Z* isomer) and a peak at δ 7.22 (1 H, d, $J_{POCCH} = 2.2$ Hz) (*E* isomer) (Figure 1B). The mixture was not separated.

Synthesis of (Z)-Tripotassium Phosphoenol-3-fluoropyruvate. Z-F-P-enolpyruvate was synthesized by modifications to the methods of Stubbe & Kenyon (1972) and Bergmann & Shahak (1960).

(*A*) *Bromofluoropyruvic Acid.* To a suspension of sodium ethoxide (0.3 mol) in benzene (150 mL) was added diethyl oxalate (44 g, 0.3 mol) during 10 min with cooling, followed by the addition of ethyl fluoroacetate (31.8 g, 0.3 mol) (reaction 1).



(1)

The enolate (yellow solid) was filtered and washed with anhydrous ether until the washings were colorless. The enolate was suspended in a large volume of benzene and treated dropwise with bromine at such a rate that the temperature did not exceed 50 °C. When the color of bromine remained, the product was washed with water and with a sodium sulfite solution, dried with sodium sulfate, and distilled. The product, diethyl bromofluorooxaloacetate, was distilled at 86 °C (0.7 mmHg) (Bergmann & Shahak, 1960). The diethyl bromofluorooxaloacetate was hydrolyzed by heating at 80 °C for 2 h in concentrated HCl. After removal of excess HCl in vacuo, the product was distilled, and the fraction which boiled from 70 to 100 °C (1.5 mmHg) was collected. (A short-path distillation apparatus was used with no cooling of the condenser.) The fraction was dissolved in a small amount of benzene to remove oxalic acid (oxalic acid is insoluble in benzene) and filtered. The nuclear magnetic resonance spectrum (D_2O) of bromofluoropyruvate showed a peak at δ 6.45 (d, $J_{FCH} = 54$ Hz).

(*B*) *Z-F-P-enolpyruvate.* A 2.5-g portion of bromofluoropyruvic acid in 25 mL of diethyl ether was added to 1.9 g of trimethyl phosphite at 0 °C. The solution was stirred for 5 h while it warmed to room temperature. Solvent was removed at reduced pressure. The product was dissolved in 25 mL of H_2O and stirred for 8 h (Stubbe & Kenyon, 1972). The solution was then diluted to give an $A_{240} \sim 3$ ODU, and the pH was adjusted to 7.8. This solution was loaded on a column (24 × 2.5 cm) of Dowex 1-X8 (1.5 mL/min). The column was washed with 0.02 N HCl. Pure Z-F-P-enolpyruvate was eluted with 0.1 N HCl. The fractions were pooled and neutralized with KOH, and the solvent was removed under reduced pressure. Concentrations were calculated from phosphate tests performed as previously described. An approximate yield of 20% was obtained. The ^1H NMR spectrum (D_2O) showed the vinyl proton peaks centered at δ 8.12 (1 H, dd, $J_{POCCH} = 3.0$ Hz, $J_{FCH} = 77.0$ Hz), pD 7.0. The ^{19}F NMR spectrum (D_2O) showed a peak at δ −5.15 (dd, $J = 4.0$ and 77.0 Hz).

Synthesis of (E)-Tripotassium Phosphoenol-3-fluoropyruvate. The (*Z*)-tripotassium F-P-enolpyruvate was photoisomerized in quartz cuvettes by using a 450-W (medium pressure mercury lamp) UV source to obtain a 21:79 *E:Z* mixture (4.5 h). The ^1H NMR spectra are shown in Figure 2A,B.

The photoisomerized mixture (295 mM) in D_2O (1 mL) which contained Na_2ADP (22 μmol), D-glucose (354 μmol), KCl (2.213 mmol), $MgSO_4$ (266 μmol), and Tris buffer (1.106 mmol) was adjusted to pD 7.5 with DCl (38% solution in D_2O). The solution was warmed to 37 °C, treated with pyruvate kinase (0.443 mg) and hexokinase (0.09 mg), and kept at 37 °C for 34 h. The reaction was monitored for loss of Z-F-P-enolpyruvate by ^1H NMR. The solution was heated to 85 °C for 10 min, cooled, filtered, diluted to 60 mL, adjusted to pH 7.9 with 1.0 N KOH, and loaded on a column (8.5 × 0.75 cm) of Dowex 1-X8 (1.5 mL/min) (Bartlett, 1959). A gradient (300 mL) of 0–0.15 N HCl was used to elute *E*-F-P-enolpyruvate. Pure *E*-F-P-enolpyruvate eluted with approximately 0.07 N HCl (monitored by A_{240}). The fractions were pooled and neutralized with KOH, and the solvent was removed under reduced pressure. Concentrations were calculated from phosphate tests as described previously. An approximate recovery of 75% based on the *E* isomer originally present was obtained. The NMR spectrum (D_2O, pD 7.4) showed the vinyl proton peaks centered at δ 7.74 (1 H, dd, $J_{POCCH} = 2.6$ Hz, $J_{FCH} = 78.0$). The ^{19}F NMR spectrum (D_2O) showed a peak at δ +5.53 (dd, $J = 4.4$ and 78.0 Hz).

Results

Synthesis of Analogues. The pure *Z* isomer of Br-P-enolpyruvate was obtained by the methods of Stubbe & Kenyon (1971). The *Z* isomer was shown to be greater than 95% free of *E* isomer by ^1H NMR (Figure 1A). The exposure to UV

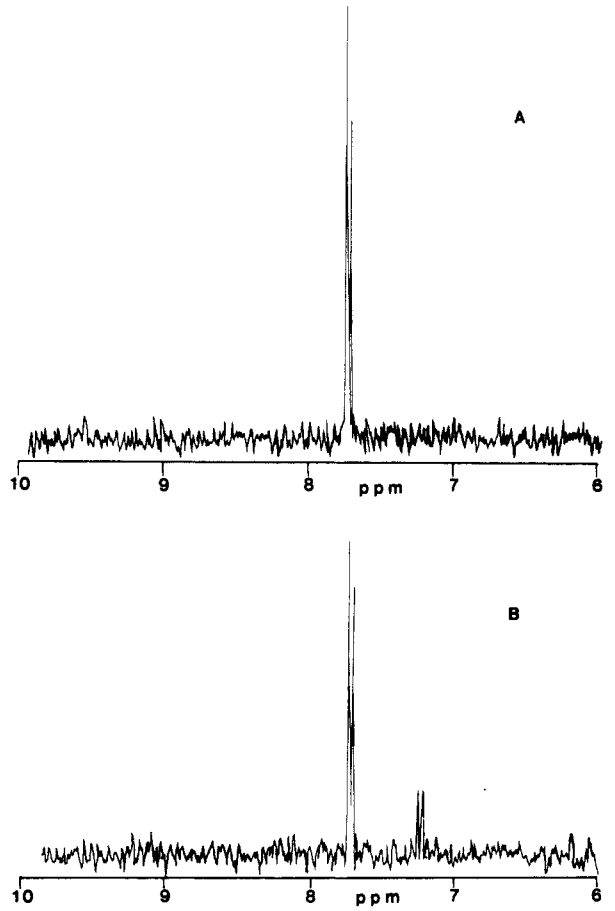


FIGURE 1: Proton nuclear magnetic resonance spectra at 100 MHz (in D_2O) of (A) (*Z*)-cyclohexylammonium phosphoenol-3-bromopyruvate and (B) an *E* and *Z* mixture of cyclohexylammonium phosphoenol-3-bromopyruvate. The pH values for both samples were identical (pD 2.6).

light (15 min) produced a 82:18 *Z*:*E* mixture of Br-P-enolpyruvate with a slight loss of product (Figure 1B). A maximum of a 70:30 *Z*:*E* mixture was obtained after 0.5 h. Additional exposure to UV light provided no increase in the *E* isomer with substantial loss in total product. *Z*- and *E*,*Z*-Br-P-enolpyruvate were found to be unstable in solution. Storage at −78 °C for several days in H_2O showed substantial loss in product. Hence, Br-P-enolpyruvate was stored as the cyclohexylammonium salt in powder form at −78 °C.

The pure *Z* isomer of F-P-enolpyruvate was shown to be greater than 95% free of *E* isomer by ^1H NMR. This isomer was >98% pure as shown by ^{19}F NMR. Exposure of a solution of *Z*-tripotassium F-P-enolpyruvate to UV light for 4.5 h produced a 79:21 *Z*:*E* mixture of F-P-enolpyruvate (Figure 2A,B). Additional exposure provided no increase in the relative formation of the *E* isomer with an overall loss in total product. Pure *E* isomer was obtained by preferential removal of the *Z* isomer from an *E*–*Z* mixture by using the enzymes pyruvate kinase and hexokinase. The *E* isomer was shown to be greater than 95% free of *Z* isomer by ^1H and ^{19}F NMR.

P-enolpyruvate Analogues as Substrates for Various P-enolpyruvate-Utilizing Enzymes. Several analogues of P-enolpyruvate which are halogen substituted at the C-3 position were tested as possible substrates for chicken liver P-enolpyruvate carboxykinase. *Z*-Br-P-enolpyruvate was shown to have no substrate activity; the limits of detection for this experiment were 0.05% of that observed with P-enolpyruvate as the substrate. The *Z* isomer of F-P-enolpyruvate was shown to be a substrate in the P-enolpyruvate carboxykinase reaction. This observation is in contrast to the effects observed with

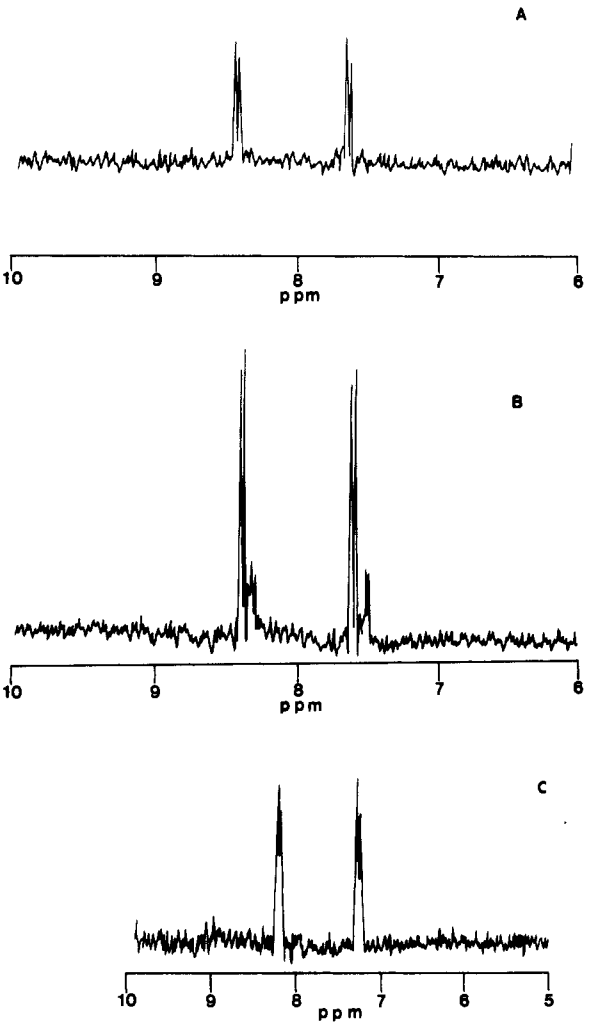


FIGURE 2: Proton nuclear magnetic resonance spectra at 100 MHz (in D_2O) of (A) (*Z*)-tripotassium phosphoenol-3-fluoropyruvate, (B) an *E* and *Z* mixture (25:75) of tripotassium phosphoenol-3-fluoropyruvate, and (C) (*E*)-tripotassium phosphoenol-3-fluoropyruvate. The pH values for each sample were identical (pD 7.0).

E-F-P-enolpyruvate. The *E* isomer shows no substrate activity with P-enolpyruvate carboxykinase with a detection limit of 0.2% activity with P-enolpyruvate. The results are summarized in Table I.

The *Z* isomer of Br-P-enolpyruvate was shown to act as a substrate in the pyruvate kinase catalyzed reaction. The *E* and *Z* isomers of F-P-enolpyruvate were both shown to act as substrates in the pyruvate kinase catalyzed reaction. The cation activator Mg^{2+} enhances the stereoselectivity of pyruvate kinase for these diastereomers. The kinetic parameters for these analogues have been summarized in Table I.

Z-Br-P-enolpyruvate was not a substrate for either the Mn^{2+}- or the Mg^{2+}-activated yeast enolase. The limits of detection for the assay were 0.4% of the activity measured with the substrate 2-PGA.

The *Z* isomer of F-P-enolpyruvate was a substrate for enolase. No substrate activity was observed with *E*-F-P-enolpyruvate. The kinetic parameters have been summarized in Table I. A nonlinear response of absorption with *time* was observed when the reaction with *Z*-F-P-enolpyruvate as substrate was initiated by enzyme. If analogue and metal-free enzyme were preincubated for approximately 5 min and the reaction was initiated by metal, a linear response was then measured. An increase in absorbance at 240 nm which was proportional to analogue concentration was noted during this preincubation period. The kinetics behaved in a normal

Table I: Kinetic Constants for (*Z*)-Phosphoenol-3-bromopyruvate and for (*E*)- and (*Z*)-Phosphoenol-3-fluoropyruvate as Substrates for P-enolpyruvate-Utilizing Enzymes

enzyme	substrate[a]	K_m (μM)	V_{max} (units/mg)	V_m/K_m (mg^{-1} min^{-1})
chicken liver P-enolpyruvate carboxykinase[b]	P-enolpyruvate	167.0	7.0	42.0
	Z-Br-PEP	NR[d]		
	E-F-PEP	NR[d]		
	Z-F-PEP	47.0	1.5	32.0
pyruvate kinase[b]	P-enolpyruvate	19.0	100	5300
	Z-Br-PEP	3.1	0.09	29.0
	E-F-PEP	7.3	0.07	9.6
	Z-F-PEP	5.2	0.11	21.1
pyruvate kinase[c]	P-enolpyruvate	35	200	5700
	Z-Br-PEP	8.3	0.286	34.5
	E-F-PEP	31.0	0.055	1.8
	Z-F-PEP	5.0	0.263	52.6
enolase[b]	P-enolpyruvate	83.0	44	530
	Z-Br-PEP	NR[d]		
	E-F-PEP	NR[d]		
	Z-F-PEP	26.0	0.66	25.4
enolase[c]	P-enolpyruvate	50.0	90	1800
	Z-Br-PEP	NR[d]		
	E-F-PEP	NR[d]		
	Z-F-PEP	29.0	0.15	5.2
pyruvate, phosphate dikinase[c]	P-enolpyruvate	360	1.9	5.3
	Z-Br-PEP	NR[d]		
	E-F-PEP	NR[d]		
	Z-F-PEP	752	0.49	0.65

[a] Abbreviations: *Z*-Br-PEP, (*Z*)-3-bromophosphoenolpyruvate; *E*-F-PEP, (*E*)-3-fluorophosphoenolpyruvate; *Z*-F-PEP, (*Z*)-3-fluorophosphoenolpyruvate. [b] Measured with the Mn^{2+}-activated enzyme. [c] Measured with the Mg^{2+}-activated enzyme. [d] No reaction.

manner after this preincubation. This phenomenon was not investigated further.

Z-Br-P-enolpyruvate showed no substrate activity in the pyruvate phosphate dikinase reaction. The limits of detection for this assay were 0.05% relative to the velocity measured with P-enolpyruvate as substrate.

Z-F-P-enolpyruvate is a substrate for pyruvate phosphate dikinase (Figure 3A). The *E* isomer of F-P-enolpyruvate showed no substrate activity (<0.1%) with this enzyme.

Analogues Z-Br-P-enolpyruvate, Z-F-P-enolpyruvate, and E-F-P-enolpyruvate as Inhibitors. Chicken liver P-enolpyruvate carboxykinase is inhibited by *Z*-Br-P-enolpyruvate. A kinetic study shows linear competitive inhibition with respect

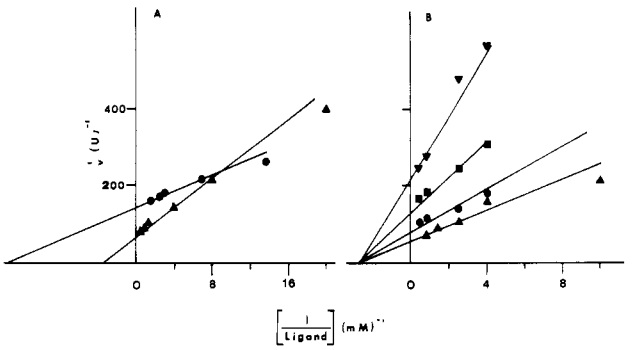


FIGURE 3: Lineweaver–Burk plots of pyruvate phosphate dikinase activity as a function of (*Z*)-phosphoenol-3-fluoropyruvate and phosphoenolpyruvate concentration and inhibition by (*E*)-phosphoenol-3-fluoropyruvate. The reaction mixtures contained, in a final 1.0-mL volume, 65 μmol of Tris-HCl buffer, pH 7.4, 20 μmol of NH_4Cl, 20 μmol of $MgCl_2$, 0.04 μmol of PP_i, 0.4 μmol of AMP, 0.14 μmol of NADH, 1 enzyme unit of lactate dehydrogenase, and pyruvate phosphate dikinase. Initial velocity is defined as micromoles of NADH oxidized per minute. (A) Reciprocal velocity vs. reciprocal concentration of (▲) phosphoenolpyruvate and (●) (*Z*)-phosphoenol-3-fluoropyruvate. (B) Reciprocal velocity vs. reciprocal concentrations of phosphoenolpyruvate at the following fixed concentrations of (*E*)-phosphoenol-3-fluoropyruvate: (▲) 0; (●) 56 μM; (■) 168 μM; (▼) 224 μM.

to P-enolpyruvate. *E-F-P*-enolpyruvate, which is not a substrate for the reaction, is a linear competitive inhibitor with respect to P-enolpyruvate. A summary of the results of the inhibition studies, obtained with the P-enolpyruvate analogues described, is given in Table II.

Z-Br-P-enolpyruvate shows linear competitive inhibition with respect to P-enolpyruvate in the Mn^{2+}-activated and in the Mg^{2+}-activated pyruvate kinase reaction. The *Z–E* mixture showed no significant kinetic difference from the pure *Z* isomer.

Mn^{2+}-activated pyruvate kinase showed little stereoselectivity of inhibition by the two isomers of F-P-enolpyruvate. Both isomers show linear competitive inhibition with respect to P-enolpyruvate. The Mg^{2+}-activated pyruvate kinase demonstrated greater stereoselectivity of inhibition by the two isomers of F-P-enolpyruvate. With Mg^{2+}-activated pyruvate kinase, the *Z* isomer is 10 times as effective an inhibitor as the *E* isomer.

Z-Br-P-enolpyruvate showed no inhibition with respect to 2-PGA in the enolase reaction regardless if the enzyme was

Table II: Summary of Inhibition Constants of (*Z*)-Phosphoenol-3-bromopyruvate and of Phosphoenol-3-fluoropyruvate Isomers for P-enolpyruvate-Utilizing Enzymes

enzyme	analogue	K_I (μM)	K_m/K_I	K_{I_Z}/K_{I_E}
P-enolpyruvate carboxykinase[a]	*Z*-Br-P-enolpyruvate	9.4 ± 1.9	15.6	
	Z-F-P-enolpyruvate			
	E-F-P-enolpyruvate	23.0 ± 3.0	5.65	
pyruvate kinase[a]	*Z*-Br-P-enolpyruvate	0.081 ± 0.015	470	
	Z-F-P-enolpyruvate	0.033 ± 0.009	788	0.647
	E-F-P-enolpyruvate	0.051 ± 0.002	470	
pyruvate kinase[b]	*Z*-Br-P-enolpyruvate	0.16 ± 0.01	150	
	Z-F-P-enolpyruvate	0.057 ± 0.006	544	0.119
	E-F-P-enolpyruvate	0.48 ± 0.004	53	
enolase[a]	*Z*-Br-P-enolpyruvate	ND[c]		
	Z-F-P-enolpyruvate	0.40 ± 0.02	85	1.0
	E-F-P-enolpyruvate	0.40 ± 0.06	40	
enolase[b]	*Z*-Br-P-enolpyruvate	ND[c]		
	Z-F-P-enolpyruvate	0.073 ± 0.006	630	0.261
	E-F-P-enolpyruvate	0.28 ± 0.04	54	
pyruvate, phosphate dikinase[b]	*Z*-F-P-enolpyruvate			
	E-F-P-enolpyruvate[d]	112 ± 32	3.3	

[a] Measured with Mn^{2+}-activated enzyme. [b] Measured with Mg^{2+}-activated enzyme. [c] No inhibition detectable. [d] Noncompetitive inhibitor.

Table III: Summary of PRR Titration Parameters and Comparison of Thermodynamic and Kinetic Constants

enzyme	ligand	K_3 (μM)	ϵ_T	K_I (μM)	K_m (μM)
P-enolpyruvate carboxykinase[a]	P-enolpyruvate	0.25 ± 0.10	8.10 ± 0.20		166
	Z-F-P-enolpyruvate	0.25 ± 0.10	5.92 ± 0.17		47.0
	E-F-P-enolpyruvate	0.15 ± 0.05	6.95 ± 0.05	23.0 ± 3.0	
pyruvate kinase[b]	P-enolpyruvate	2.5 ± 1.0	2.03 ± 0.10		19.0
	Z-F-P-enolpyruvate	0.05 ± 0.01	2.40 ± 0.20	0.033 ± 0.009	5.2
	E-F-P-enolpyruvate	3.0 ± 0.5	2.20 ± 0.05	0.051 ± 0.002	7.3
enolase[c]	P-enolpyruvate	1.0 ± 0.5	6.75 ± 0.50		83.0
	Z-F-P-enolpyruvate	10.0 (50 ± 8)[d]	5.5 ± 0.4 (7.2 ± 0.9)[d]	0.40 ± 0.02	29.0
	E-F-P-enolpyruvate		4.5[e]	0.40 ± 0.06	

[a] ϵ_b = 13.5. [b] ϵ_b = 20.0. [c] ϵ_b = 15.0. [d] A *Z*-F-P-enolpyruvate–3-fluoro-2-PGA mixture. [e] See text.

activated by Mg^{2+} or by Mn^{2+}. If the enzyme was inhibited, the inhibition constant, K_I, was substantially greater than 1 mM. The Mn^{2+}-activated enolase showed no stereoselectivity in inhibition by the two isomers of F-P-enolpyruvate. The Mg^{2+}-activated enolase did show stereoselectivity in inhibition by the two isomers, however. Both isomers showed linear competitive inhibition with respect to P-enolpyruvate. A summary of the results of inhibition studies with *Z*-Br-P-enolpyruvate and the F-P-enolpyruvate isomers is given in Table II.

Z-Br-P-enolpyruvate showed inhibition of the pyruvate formation reaction catalyzed by pyruvate phosphate dikinase which increased as a function of time. This inhibition was observed at various concentrations of P-enolpyruvate, and in each case, the enzyme ultimately lost 100% activity. Increasing the concentration of P-enolpyruvate decreased the rate of inactivation. Preincubation of the enzyme with Br-P-enolpyruvate, PP_i, AMP, and Mg^{2+} also resulted in loss of activity. Under experimental conditions, the loss in activity of pyruvate phosphate dikinase in the presence of Br-P-enolpyruvate appeared irreversible.

Z-F-P-enolpyruvate has a V_{max} comparable to that of P-enolpyruvate for this enzyme which precludes an inhibition study. *E*-F-P-enolpyruvate showed noncompetitive inhibition with a K_I value of 112 ± 32 μM (Figure 3B). Replots of the inhibition data as slope vs. [I] and as intercept vs. [I] gave curves which suggest possible parabolic noncompetitive inhibition. Because of insufficient data, the K_I values were calculated by assuming linear noncompetitive inhibition.

Interactions of P-enolpyruvate and the P-enolpyruvate Analogues with Several Enzyme–Mn^{2+} Complexes As Determined by PRR Studies. The binding of P-enolpyruvate, *Z*-F-P-enolpyruvate, *E*-F-P-enolpyruvate, and *Z*-Br-P-enolpyruvate to the chicken liver P-enolpyruvate carboxykinase–Mn complex was investigated by PRR titration studies.

Complete titrations of P-enolpyruvate carboxykinase–Mn with Br-P-enolpyruvate were not obtainable because of a rapid apparent decomposition of the analogue. An initial observed enhancement (ϵ_T*) of 7.0 ± 0.2, measured in the presence of saturating Br-P-enolpyruvate concentration, was obtained. This value increased as a function of time. The ϵ* value approached the value of ϵ_b*. *E*-F-P-enolpyruvate and *Z*-F-P-enolpyruvate showed similar decomposition but over a much longer time period. The much slower decomposition allowed complete PRR titrations to be obtained with these ligands. The two isomers of F-P-enolpyruvate showed similarities in their binding characteristics (Figure 4). These fits were relatively insensitive to the value of K_s. The results of these titrations are summarized in Table III.

The binding of *Z*-Br-P-enolpyruvate to the pyruvate kinase–Mn complex was investigated by PRR titrations. As with P-enolpyruvate carboxykinase, titration curves were not obtainable because of rapid decomposition of the analogue. Upon apparent completion of the decomposition, the solution was tested enzymatically for the presence of bromopyruvate as a possible decomposition product. No bromopyruvate was found. The limits of the assay for bromopyruvate were <1% with respect to the Br-P-enolpyruvate initially present. The results of the titrations with the isomers of F-P-enolpyruvate indicated that the two isomers interacted with the pyruvate kinase–Mn complex with approximately the same ϵ_T but with very different binding constants. An analysis of the titration curve gives an approximate 1:1 stoichiometry for enzyme–Mn to *E*-F-P-enolpyruvate or *Z*-F-P-enolpyruvate. As noted with P-enolpyruvate, the computer fits expressed by the percent standard deviation were sensitive to values of K_s. The optimal K_s value for both the fluoro isomers was 0.3×10^{-3} M. The results of titrations are summarized in Table III.

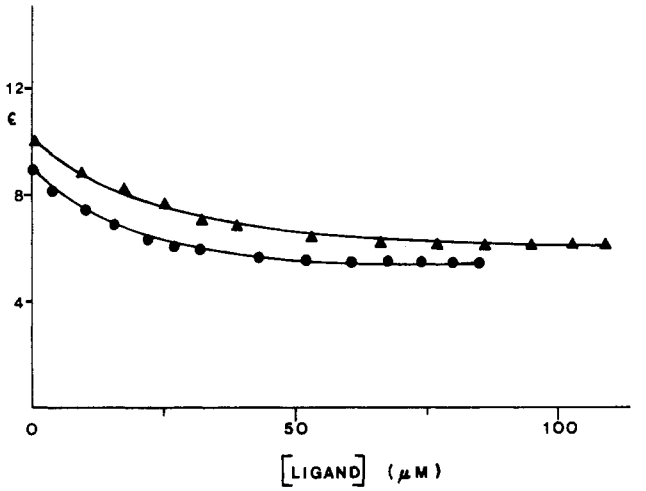


FIGURE 4: Titration of chicken liver P-enolpyruvate carboxykinase–Mn with *Z*-F-PEP and *E*-F-PEP. A plot of observed enhancement (ϵ*) vs. ligand concentration is made. A solution containing 90 μM enzyme in 65 mM Tris-HCl, pH 7.4, containing 0.1 M KCl and 4×10^{-5} M Mn^{2+} in a final volume of 0.05 mL is titrated with a solution containing an identical solution and including either *Z*-F-PEP (●) or *E*-F-PEP (▲). The titrations shown are best fit with K_3 values of 0.25 and 0.15 μM, respectively.

The addition of *Z*-Br-P-enolpyruvate to enolase–Mn also showed a rapid change in ϵ* vs. time. The observed value quickly increased to yield the value of ϵ_b*. *Z*-F-P-enolpyruvate was shown to interact with the enolase–Mn^{2+} complex. A value of 10×10^{-6} M was obtained for K_3, and a value of 5.5 was obtained for ϵ_T* from the experiment shown. Since *Z*-F-P-enolpyruvate has been shown to be a substrate for enolase, although at a low V_{max}, the above values may represent a titration with a mixture of substrate and product. Therefore, the titrations were also repeated after *Z*-F-P-enolpyruvate was allowed to preincubate for 1 h in the presence of enolase and Mn^{2+} to ensure that an equilibrium mixture would be obtained.

Titrations utilizing these mixtures gave both a higher K_3 value and a higher ϵ_T. The large increase in K_3 and the modest increase in ϵ_T could not be entirely accounted for by simple decomposition of *Z*-F-P-enolpyruvate.

Repeated attempts were made to obtain K_3 and ϵ_T values for *E*-F-P-enolpyruvate in the presence of enolase–Mn^{2+}. Rapid decomposition did not allow for valid data to be obtained; however, an initial ϵ_T^* of 4.5 was obtained which increased to the ϵ_b^* of 13. The reaction was assayed for fluoropyruvate as a possible decomposition product by using lactate dehydrogenase. No fluoropyruvate was detected although the limits of the assay for fluoropyruvate were <1% with respect to the *E*-F-P-enolpyruvate initially present. The results of the titration studies are summarized in Table III. A comparison of the thermodynamic constants with the kinetic constants is also given in Table III.

Discussion

Z-Br-P-enolpyruvate was sufficiently stable in solution to be utilized for steady-state kinetic experiments. However, because of its long-term instability in water and its decomposition catalyzed by the enzyme–Mn^{2+} complexes, the analogue was deemed not useful for experiments requiring a longer period of time and higher enzyme concentrations such as proton relaxation rate NMR experiments. Thus, no attempts were made to obtain the pure *E* isomer from an *E*–*Z* mixture of Br-P-enolpyruvate.

Upon photoisomerization of *Z*-F-P-enolpyruvate to form *E*-F-P-enolpyruvate, a contaminant was also formed whose NMR spectrum is partially obscured by the spectrum of *E*-F-P-enolpyruvate (Figure 2A). Pure *E*-F-P-enolpyruvate was obtained by the preferred degradation of the *Z* isomer in the presence of pyruvate kinase. This isomer could also be prepared by specific degradation of *Z*-F-P-enolpyruvate by utilizing P-enolpyruvate carboxykinase. Care was needed not to confuse the contaminant with *Z*-F-P-enolpyruvate, both of which have similar splitting patterns and chemical shifts but can be distinguished on the basis of the coupling constants. This contaminant, which has not been characterized, can be separated from F-P-enolpyruvate by anion-exchange chromatography. The purity of the two isomers was easily determined by high-resolution ^1H and ^{19}F NMR. The *Z* isomer was distinguished from the *E* isomer on the basis of the larger H–P coupling constants normally observed for trans coupling in contrast to cis coupling.

Z-F-P-enolpyruvate, which is a substrate for the P-enolpyruvate carboxykinase catalyzed reaction with a V_{max} of 42% with respect to P-enolpyruvate, gave a binding constant (0.25×10^{-6} M) similar to that for P-enolpyruvate. These results were identical with those reported by Hebda & Nowak (1982a,b). *E*-F-P-enolpyruvate, which is not a substrate but a competitive inhibitor with respect to P-enolpyruvate, gave a binding constant (0.15×10^{-6} M) smaller than its K_I value (23×10^{-6} M). The binding constant is measured in the absence of the other substrates, and the kinetic constant is measured in the presence of the catalytically active complex. The binding of *E*-F-P-enolpyruvate is apparently affected by the presence of the substrates, causing $K_I \neq K_3$. The K_3 values for P-enolpyruvate and for *Z*-F-P-enolpyruvate with this enzyme are also much lower than their K_m values (Table III).

The effect of fluorine substitution in the substrate was tested in the reverse reaction. 3-Fluorooxalacetate, obtained from an intermediate in the synthesis of F-P-enolpyruvate, was 88–90% pure with a 8–12% contaminant of fluoropyruvate. This compound was tested as a substrate in the decarboxylation direction by using ITP as the second substrate. No activity was observed with this compound. This analogue serves as a linear competitive inhibitor, however. Under conditions where the K_m for oxalacetate is 69 μM, fluorooxalacetate yields a $K_I = 65 \pm 4$ μM. Neither pyruvate nor fluoropyruvate have an effect on this reaction. The substitution of a fluorine for a hydrogen atom at C-3 of oxalacetate does not affect the steady-state affinity of the enzyme for this ligand, but this substitution does preclude catalysis. Substrate activity and inhibition by 3,3-difluorooxalacetate toward P-enolpyruvate carboxykinase were also tested. Neither substrate activity nor inhibition was observed. This lack of a kinetic effect is likely due to the much higher distribution of the hydrated form of difluorooxalacetate and of the slow rate of dehydration of the hemiketal form. These results suggest that the enzyme P-enolpyruvate carboxykinase utilizes the keto form of oxalacetate as opposed to the hydrate as the substrate.

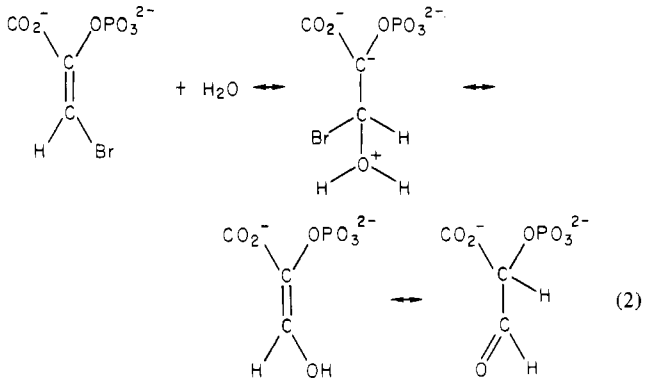
Binding studies of P-enolpyruvate carboxykinase–Mn using PRR methods give titration curves with P-enolpyruvate, *Z*-F-P-enolpyruvate, and *E*-F-P-enolpyruvate nearly parallel and yield similar ϵ_T values. These data suggest similar environmental changes occur about the bound Mn^{2+} in the formation of the respective ternary complexes. Effects at the site of carboxylation of the substrate, which is more remote from the bound Mn^{2+}, may be responsible for the differences in specificity for these ligands. The technique of PRR appears not to be sensitive to such differences with this enzyme.

The P-enolpyruvate analogues *Z*-PEB, *E*-PEB (Duffy et al., 1982), *Z*-Br-P-enolpyruvate, and *E*-F-P-enolpyruvate all show competitive inhibition of P-enolpyruvate carboxykinase with respect to P-enolpyruvate. These data suggest that these analogues bind at the P-enolpyruvate site on the enzyme. Steric factors appear to play a role in the strength of interaction of the ligand with the enzyme when the hydrogen on the C-3 carbon, either cis or trans to the phosphoryl group of P-enolpyruvate, is replaced by another group. The van der Waals radii for H, F, Br, and CH_3 are 120, 150–160, 180–200, and 200 pm (Bondi, 1964), respectively. The binding constants for P-enolpyruvate carboxykinase–Mn complex binding to P-enolpyruvate analogues, with substitution of the cis C-3 proton ($H > F > Br > CH_3$), roughly parallel the van der Waals radii of these substituents. The methyl and bromo groups are similar in size; however, the bromo group is more polarizable, and hence, its interaction is possibly less sterically hindered. When the hydrogen on the C-3 carbon trans to the phosphoryl group of P-enolpyruvate is replaced by a halogen, steric factors do not explain the strength of the interaction. The methyl group of *E*-PEB (200 pm) and the fluoro group of *E*-F-P-enolpyruvate (150–160 pm) are quite different in size although *E*-PEB (Duffy et al., 1982) and *E*-F-P-enolpyruvate have comparable K_I values. Possibly, electronic factors are also important when comparing strengths of interaction with the enzyme.

Of the P-enolpyruvate analogues utilized, only *Z*-F-P-enolpyruvate showed substrate activity in the carboxylation reaction of P-enolpyruvate carboxykinase. Several explanations are possible for this specificity. Steric hindrance by groups substituted at C-3 could be such that the substrate CO_2 could not bind at the catalytic site, or the CO_2 bound at the catalytic site is misalligned, preventing electron overlap and thus catalysis. Ligand (P-enolpyruvate analogue) binding could also be altered such that the nucleotide is not bound properly to allow the phosphoryl transfer to occur. The binding of P-enolpyruvate to enzyme–Mn does differ from the binding of *E*-PEB and *Z*-PEB (T. H. Duffy and T. Nowak, unpublished results). These effects could occur from a conformational

change in the enzyme upon ligand binding. Another possible explanation is that the CO_2 is activated as an electrophile by a protonated group on the enzyme. An electronegative group placed trans to the phosphoryl group at the C-3 position of P-enolpyruvate can interfere with the CO_2 activation. This electronegative group may interact with the conjugate acid and prevent the bound CO_2 from activation. This latter possibility is currently under investigation.

Because of incomplete titrations with *Z*-Br-P-enolpyruvate, similar comparisons of K_I and K_3 could not be made. *Z*-Br-P-enolpyruvate is unstable in aqueous solutions, and its decomposition also appears to be enzyme catalyzed. A simple hydrolysis (dephosphorylation) reaction was ruled out, since no bromopyruvate had been detected by the lactate dehydrogenase assay. A likely possibility for this decomposition is that *Z*-Br-P-enolpyruvate undergoes an enzyme-catalyzed Michael-type addition (reaction 2). Kinetic studies suggest



Z-Br-P-enolpyruvate binds to the P-enolpyruvate site, which is in proximity to the divalent metal activator. Upon binding to the enzyme–metal complex, the *Z*-Br-P-enolpyruvate becomes activated for a Michael-type addition.

Muscle pyruvate kinase was inhibited by the P-enolpyruvate analogues *Z*-Br-P-enolpyruvate, *Z*-F-P-enolpyruvate, and *E*-F-P-enolpyruvate. All the analogues were also found to be slowly reacting substrates for the pyruvate kinase reaction with V_{max} values less than 1% compared to P-enolpyruvate. *Z*-Br-P-enolpyruvate and *Z*-F-P-enolpyruvate were previously shown to be substrates for pyruvate kinase (Stubbe & Kenyon, 1971, 1972). PRR titrations of these ligands to pyruvate kinase–Mn^{2+} were performed. The K_3 value for *Z*-F-P-enolpyruvate was in agreement with the kinetically determined K_I value and less than the K_m value. The K_3 value for *E*-F-P-enolpyruvate was not in agreement with the K_I value. The binding of *E*-F-P-enolpyruvate may be affected by the presence of the substrate ADP. The K_3 value was the same as the K_m value (Table III). These results suggest that the kinetic mechanism of pyruvate kinase differs, depending upon the substrate. A change in the kinetic mechanism for pyruvate kinase has been demonstrated with the two diastereomers *E*- and *Z*-PEB.[2] Incomplete titrations due to ligand decomposition preclude similar comparisons with *Z*-Br-P-enolpyruvate. This decomposition seems to be catalyzed by the pyruvate kinase–Mn complex. We speculate that this decomposition is similar to that seen in the presence of P-enolpyruvate carboxykinase–Mn.

Structural differences were noted in the binding of these ligands to the pyruvate kinase–Mn complex. The ligands P-enolpyruvate, *Z*-F-P-enolpyruvate, and *E*-F-P-enolpyruvate all show similar ϵ_T values. The low ϵ_T value for P-enolpyruvate has been ascribed to a conformational change around the Mn binding site upon P-enolpyruvate binding to the pyruvate kinase–Mn complex (James et al., 1973). The low ϵ_T values for *Z*-F-P-enolpyruvate and *E*-F-P-enolpyruvate also suggest a similar conformational change around the Mn binding site. However, these substrates have a much lower V_{max} than does P-enolpyruvate. The stereochemical results obtained with *E*-PEB and with *Z*-PEB suggest that the rate-determining step with these substrates is the protonation of the enolate (Adlersberg et al., 1977; Hoving et al., 1983) as opposed to product departure when P-enolpyruvate is the substrate (Robinson & Rose, 1972). The rate-determining step with *Z*-F-P-enolpyruvate, *E*-F-P-enolpyruvate, and *Z*-Br-P-enolpyruvate may be the same as that with *Z*-PEB and *E*-PEB. Effects on the enzyme, at the site of protonation of the enolate, which presumably is more remote from the bound Mn^{2+}, may be responsible for differences in substrate specificity. The technique of PRR may not be sensitive to such differences for this enzyme.

The difference in K_m values between the two F-P-enolpyruvate isomers with the Mg^{2+}-activated enzyme allows for the preferential degradation of the *Z* isomer utilized in the preparation of the pure *E* isomer. The difference in K_m values of the two F-P-enolpyruvate isomers is small, and therefore, more care than required in the preparation of *E*-PEB (Duffy et al., 1982) is needed in utilizing these kinetic differences in a preparatory manner.

The measured K_I values for pyruvate kinase roughly parallel the van der Waals radii of the substituents, F > Br > CH_3, which are at the C-3 position of P-enolpyruvate. This correlation is seen for both Mg^{2+}- and Mn^{2+}-activated pyruvate kinase. If it is postulated that the large decrease in V_m/K_m, compared to P-enolpyruvate, for these analogues is because that are misaligned with respect to a specific base on the enzyme, it appears steric hindrance is not solely responsible for this misalignment. Also, catalysis appears not to be simply a function of the "strength" of binding of the substrate. This is consistent with the hypothesis that the conformation of the substrate is also important in catalysis. The nature of the activating divalent cation appears to affect the specificity of the ligand binding.

Z-F-P-enolpyruvate binds to the enolase–Mn complex with a K_3 of 10×10^{-6} M, which is nearly 1 order of magnitude larger than the kinetically determined K_I value. *Z*-F-P-enolpyruvate is also a substrate, and the K_3 value probably represents a mixture of substrate and product. The product appears to have a higher binding constant than the F-P-enolpyruvate. When the mixture is allowed to come to equilibrium and a PRR titration is then performed, a 5-fold increase in K_3 is obtained, which supports the premise of a higher binding constant for the product 3-fluoro-2-PGA than for F-P-enolpyruvate. Because of incomplete titrations with *E*-F-P-enolpyruvate, comparisons of K_I and K_3 cannot be adequately made for this ligand.

It appears that *E*-F-P-enolpyruvate undergoes an enzyme-catalyzed degradation reaction with enolase similar to that observed with Br-P-enolpyruvate. To check this possibility, the decomposition of *E*-F-P-enolpyruvate by Mg^{2+}–enolase was followed by ^{19}F and ^1H NMR. Fluoride ion was shown to be a product of the decomposition, and the ^1H NMR spectrum showed a doublet at δ 7.5 (J = 3.0 Hz). No fluoropyruvate or pyruvate was detected by using the lactate dehydrogenase assay upon completion of the *E*-F-P-enolpyruvate decomposition. Control experiments with metal in the absence of enolase showed no decomposition of *E*-F-P-enolpyruvate over

[2] T. Nowak, H. Hoving, and G. Robillard, unpublished results.

the 3-h time course of the experiment. The product of the Mg–enolase-catalyzed decomposition of Br-P-enolpyruvate yields a ^1H spectrum with a doublet at δ 7.5 (J = 3.0 Hz) identical with that obtained from *E*-F-P-enolpyruvate decomposition. The chemical shift is characteristic of an aldehydic proton. These results are consistent with our hypothesis of a Michael addition (reaction 2).

The analogue *Z*-F-P-enolpyruvate gave a similar ϵ_T value with enolase–Mn as did P-enolpyruvate, which suggests that both ligands bind in a structurally similar fashion to the enolase–Mn complex. Nowak et al. (1973) showed that the addition of P-enolpyruvate to the enolase–Mn complex reduced the number of rapidly exchanging water molecules from 2 to 0.3–1.0. PRR studies suggested that the reduction in the number of rapidly exchanging water molecules was not due to the displacement of water on Mn^{2+} by the ligands but due to an increase in the residence time (τ_m) of coordinated water ligands. The data for P-enolpyruvate and *Z*-F-P-enolpyruvate are also consistent with this model. A role for the divalent metal activator to activate the "immobilized" water which attacks the vinylic substrate rather than to activate the substrate was suggested (Nowak et al., 1973). The data for *Z*-F-P-enolpyruvate, which is also a substrate, are consistent with this role for the divalent metal activator.

In contrast to the correlation found with P-enolpyruvate carboxykinase and pyruvate kinase, the strength of the interaction of these analogues with enolase does not parallel their van der Waals radii. These results also show that catalysis is not simply a function of the strength of substrate interaction.

Z-Br-P-enolpyruvate showed 100% inhibition of pyruvate phosphate dikinase as a function of time. Yoshida & Wood (1978) showed that relatively low concentrations of bromopyruvate irreversibly inactivate the enzyme. It appears that *Z*-Br-P-enolpyruvate is a substrate for the enzyme which turns over to form bromopyruvate. The bromopyruvate formed then inactivates the enzyme. Thus, Br-P-enolpyruvate apparently behaves as a suicide substrate with this enzyme. Since no detectable bromopyruvate is measured, the inactivation appears to be selective and rapid. An analogous inactivation of yeast pyruvate kinase by Br-P-enolpyruvate was reported (Blumberg & Stubbe, 1975) although we did not observe this reaction with pyruvate kinase from muscle. An irreversible inhibition of P-enolpyruvate carboxylase by Br-P-enolpyruvate (O'Leary & Diaz, 1982) occurs, indicating that this analogue is a suicide substrate for that enzyme. We looked for such an effect with the homologous enzyme P-enolpyruvate carboxykinase but failed to show any irreversible inhibition. Potent competitive inhibition was confirmed (Hebda & Nowak, 1982a), however.

The two diastereomers of F-P-enolpyruvate showed contrasting effects with pyruvate phosphate dikinase. *Z*-F-P-enolpyruvate is a substrate with a V_m/K_m value of 0.65 mg^{-1} min^{-1} (V_m/K_m for P-enolpyruvate = 5.3 mg^{-1} min^{-1}). *E*-F-P-enolpyruvate showed noncompetitive inhibition with a K_I value of approximately 110 μM. One possible explanation for these data is that *E*-F-P-enolpyruvate is an irreversible inhibitor. Thus, enzyme was preincubated with *E*-F-P-enolpyruvate in the standard assay mix, and after 20 min, no loss in activity was noted, indicating *E*-F-P-enolpyruvate does not act as an irreversible inhibitor. Replots of the steady-state inhibition data as slope vs. [I] and as intercept vs. [I] gave curves suggesting parabolic noncompetitive inhibition. Various possibilities exist which could explain these data. Isotope exchange data by Milner & Wood (1976) were used to suggest the enzyme has three functionally distinct and independent substrate sites (P-enolpyruvate, pyruvate; AMP, ATP; PP_i, P_i). If *E*-F-P-enolpyruvate binds such that it overlaps the nucleotide and/or PP_i, P_i site or if more than one *E*-F-P-enolpyruvate molecule bound per active site, then replots of slopes vs. [I] and of intercept vs. [I] would result in inhibition proportional to $[I]^2$. The nature of this inhibition is still not understood.

The three enzymes P-enolpyruvate carboxykinase, pyruvate kinase, and enolase all appear to form enzyme–Mn–ligand-type bridge complexes with the P-enolpyruvate analogues *Z*-Br-P-enolpyruvate, *E*-F-P-enolpyruvate, and *Z*-F-P-enolpyruvate. These complexes are analogous to those found with the physiological substrate P-enolpyruvate. It appears that the effects upon catalysis which these ligands elicit upon binding take place remote from the metal site (i.e., at the carboxylation site). That portion of the catalytic site is sensitive to steric and electronic effects.

Registry No. PEP, 138-08-9; *Z*-Br-PEP, 88253-15-0; *E*-Br-PEP, 88253-19-4; *Z*-F-PEP, 88253-16-1; *E*-F-PEP, 88253-17-2; FCH_2COOEt, 459-72-3; EtOCOCOOEt, 95-92-1; EtOCOCOCFBrCOOEt, 2707-82-6; bromofluoropyruvic acid, 684-95-7; PEP carboxykinase (GTP), 9013-08-5; pyruvate kinase, 9001-59-6; enolase, 9014-08-8; pyruvate phosphate dikinase, 9027-40-1; Mg, 7439-95-4; Mn, 7439-96-5.

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Selective Inhibition of Two Soluble Adenosine Cyclic 3′,5′-Phosphate Phosphodiesterases Partially Purified from Calf Liver†

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ABSTRACT: "Low K_m" cAMP phosphodiesterase and cGMP-stimulated cyclic nucleotide phosphodiesterase activities were partially purified from calf liver supernatant by chromatography on DEAE-cellulose and DEAE-Sepharose and ammonium sulfate precipitation. The low K_m phosphodiesterase was not retained on N^6-$H_2N(CH_2)_2$-cAMP-agarose and could be separated from the cGMP-stimulated phosphodiesterase which was absorbed by this matrix. From the proteins that did not bind, two distinct low K_m cAMP phosphodiesterases were separated on Ultrogel AcA 34. One form (fraction C) hydrolyzed cAMP with an apparent K_m of $\sim$0.5 μM and was very sensitive to inhibition by cGMP. Lineweaver-Burk plots of cAMP hydrolysis by a second form (fraction B) were nonlinear, with an apparent low K_m component of $\sim$2 μM. This form was rather insensitive to inhibition by cGMP. With both fractions, hydrolysis of cAMP relative to cGMP was much greater at low ($\sim$1 μM) than at high ($\sim$100 μM) substrate concentrations. Maximal velocities for cAMP and cGMP were similar. From sedimentation equilibrium, the apparent weight-average molecular weight of fraction B was estimated as 174 000, and that of fraction C was 85 000. Another fraction (A) of cAMP phosphodiesterase eluted at the void volume of the AcA 34 column. On the basis of the relative affinities for cAMP and cGMP and inhibition by cGMP, fraction A is most likely an aggregated form of fraction B. No apparent interconversion of fractions A, B, or C was observed on high-performance liquid chromatography. Fractions B and C differed in their sensitivity to phosphodiesterase inhibitors as well as in other characteristics. The order of potency for inhibition of fraction B was RO 20-1724 (IC_{50}, 2.2 μM) > papaverine > isobutylmethylxanthine (IBMX) > cilostamide > theophylline > cGMP. The order for fraction C was cilostamide (IC_{50}, 0.03 μM) > cGMP (IC_{50}, 0.75 μM) > papaverine > IBMX > theophylline > RO 20-1724. The use of specific inhibitors may facilitate understanding the role of specific phosphodiesterases in the regulation of intracellular cAMP content.

Multiple forms of cyclic nucleotide phosphodiesteraes (EC 3.4.1.17) with distinct physcial, catalytic, and regulatory properties have been described in mammalian tissues [for a review, see Wells & Hardman (1977), Strada & Thompson (1978), and Appleman et al. (1973)]. One form, referred to as "low K_m" cAMP phosphodiesterase, has been purified from dog kidney (Thompson et al., 1979) and from bovine lung (Moore & Schroedter, 1982). Particulate fractions of several mammalian tissues also contain low K_m cAMP phosphodiesterases, one of which is known as insulin-stimulated cAMP phosphodiesterase (Weber & Appleman, 1982; Marchmont & Houslay, 1980; Houslay & Marchmont, 1981; Elks et al., 1983).

The different forms of cyclic nucleotide phosphodiesterases can be more or less selectively inhibited by certain inhibitors (Hidaka et al., 1979; Kramer et al., 1977; Chasin & Harris, 1976; Weiss & Hait, 1977; Kuo et al., 1978; Helfman & Kuo, 1982; Hidaka & Endo, 1983), as might be expected from their distinct properties. The calmodulin-activated phosphodiesterase is inhibited by trifluoperazine (Weiss & Hait, 1977) or W7 (Hidaka & Endo, 1983), drugs that presumably bind to calmodulin. It has recently been reported that this enzyme is directly and selectively inhibited by compound TCV-3B (Hidaka & Endo, 1983) and the platelet calmodulin-insensitive cGMP phosphodiesterase by MY 5445 (Hidaka & Endo, 1983). A low K_m cAMP phosphodiesterase was selectively inhibited by cilostamide (Hidaka et al., 1979) and a cCMP phosphodiesterase by Sch 15280 (Kuo et al., 1978).

We describe here characteristics of two forms of low K_m cAMP phosphodiesterase, partially purified from calf liver, with distinct hydrodynamic properties and different sensitivities to phosphodiesterase inhibitors. One form, with an apparent weight-average molecular weight (M_w^{app}) of $\sim$85 000 and an apparent K_m for cAMP of $\sim$0.5 μM, was selectively inhibited by cilostamide and was also very sensitive to inhibition by cGMP. In this regard, this enzyme was analogous to a recently

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