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Phosphoenol Acetylphosphonates: Substrate Analogues as Inhibitors of Phosphoenolpyruvate Enzymes

STEPHEN L. BEARNE AND RONALD KLUGER[1]

The Lash Miller Chemical Laboratories, Department of Chemistry, University of Toronto, Toronto, Canada M5S 1A1

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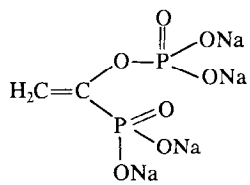
Phosphoenol acetylphosphonate **(1)** and its bis methyl ester **(2)** are structurally and electrostatically defined analogues of phosphoenolpyruvate (PEP) in which the COO^- moiety of PEP is replaced by a $P(O)O^-(OR)$ group. It is expected that such materials will bind to electrostatic receptors but the stereoelectronic properties of the phosphonate group will align the molecule at an angle that differs from that of the carboxylate substrate.



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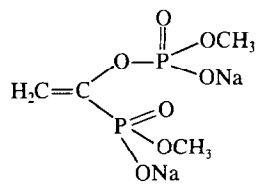

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The ability of these analogues to bind in place of PEP was evaluated with three enzymes that utilize PEP as a substrate in distinct reactions. Both **1** and **2** inhibit enolase and PEP carboxylase but they do not bind to pyruvate kinase. The inhibition of enolase at pH 6.8 is competitive with respect to 2-phosphoglycerate (the substrate in the reverse of the reaction that utilizes PEP) with K_i = 2.2 ± 0.4 mM for **1** and 27 ± 4 mM for **2**. The inhibition of enolase by **1** follows a titration curve consistent with the trianionic form of **1** being the active inhibitory form of the analogue. The specificity for the trianionic form shows that the compound is not an analogue of the *aci*-carboxylate reaction intermediate, which is a tetraanion. Neither **1** nor **2** are substrates for any of the enzymes. Inhibition of PEP carboxylase is pH dependent: at pH 7.8, **1** is a competitive inhibitor of the enzyme with respect to PEP (K_i = 2.2 ± 0.6 mM) while at pH 6.8 it is an activator; **2** has no effect on this enzyme. The diversity of the effects of the phosphonate analogues is consistent with the existence of distinct classes of binding sites for PEP. The variation in binding patterns suggests that the relationship of the points of contact of the substrate with the enzyme and with other substrates differs markedly in each of the enzymes studied.

INTRODUCTION

We have undertaken the systematic design and testing of phosphonates as inhibitors of enzymes which catalyze reactions of carboxylates. The choice of phosphonates is based on the recognition that ionic interactions are an important

[1] To whom correspondence should be addressed.

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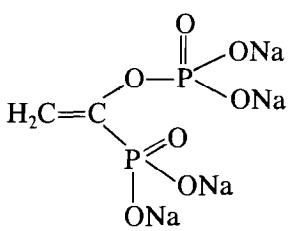
component of enzymic specificity (*1, 2*) and thus a major factor in the design of inhibitors. A cationic group of the enzyme (such as the guanidinium group of an arginine side chain) should be complementary to the carboxylate and thus analogues of the carboxylate should also contain delocalized charge. Although phosphonates provide an analogy in this regard, they are stereoelectronically distinct since the functional group is tetrahedral: the phosphonyl substituent in the monoanionic form will orient itself at a different angle than will a carboxylate (*3, 4*). The electrostatic similarity combined with the stereochemical dissimilarity makes it likely that the phosphonate will be an inhibitor rather than a substrate where reaction occurs at a site remote from the anionic group. Examples of such phenomena include the inhibition of pyruvate enzymes by methyl acetyl phosphonate (*5, 6*) and inhibition of acetoacetate enzymes by methyl acetonyl phosphonate (*3*).

In designing the PEP analogues for this study, we reasoned that since the enol phosphate group of PEP is uncommon, specific inhibition can be achieved if the analogue is itself an enol phosphate. There are no reported studies of analogues in which the C_1 position of PEP has been modified to an anionic function while all other structural components of the PEP molecule are conserved. Previously reported analogues of PEP which have been successful enzyme inhibitors have involved alteration of the enol phosphate group in some manner: structural modification at C_3 (*7–11*), conversion of the phosphate group to a related group (*12–16*), and saturation of the carbon–carbon double bond (*17–19*).

We have prepared the PEP analogue phosphoenol acetylphosphonate **(1)** and its bis(methyl) derivative **(2)** in which the carboxylate moiety is replaced by a phosphonate group. The phosphonate, in a monoanionic form, is electrostatically similar to the carboxylate but structurally different. The analogues retain the enol phosphate group, including hybridization and substituents on the carbon–carbon double bond. We have tested these as inhibitors toward three PEP-specific enzymes in which the normal reaction occurs at different sites on the substrate molecule: enolase, phosphoenolpyruvate carboxylase, and pyruvate kinase.



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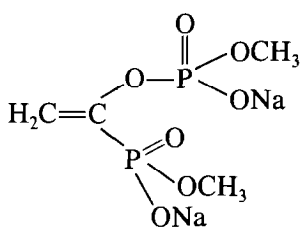

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EXPERIMENTAL

Materials

Enolase (rabbit muscle), pyruvate kinase (beef heart), phosphoenolpyruvate carboxylase (*Escherichia coli*), buffers, and reagents were purchased from the

Sigma Chemical Co. Phosphoenol pyruvate carboxylase (wheat) was from Boehringer Mannheim.

Enzyme assays were performed by monitoring absorbance changes at appropriate wavelengths with a Perkin–Elmer Coleman Hitachi 124 spectrophotometer whose cell holder was maintained at a constant temperature with a Heto circulating bath. Initial velocity was used to measure the catalytic rates under steady-state conditions. Data were analyzed using the program *GraFit* (Erithacus Software) on a microcomputer (MS-DOS, Windows 3.0).

Proton NMR spectra were recorded on a Varian T-60 (60 MHz) or a Varian Gemini (200 MHz) spectrometer. Phosphorus spectra were recorded on a Varian XL-200 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tennessee.

Synthesis

Phosphoenolacetylphosphonate **(1)** as the tetracyclohexylammonium salt was prepared from the tetramethyl ester by the procedure of Sekine *et al.* (*20*). Properties: mp 171–174°C (decomp.) (lit. mp 174°C (decomp.)); ^{1}H NMR (D_2O/DSS) δ 0.8–2.10 (m, 40H, $(CH_2)_5$), δ 3.10 (m, 4H, NCH), δ 4.87–5.50 (m, 2H, $=CH_2$); ^{31}P NMR (33% D_2O, 0.1 M Tris–HCl buffer, pH 7.8), ^{1}H decoupled, δ −0.41 (d, OPO_3^{-2}, $J_{P—P}$ = 22.9 Hz), δ 5.77 (d, PO_3^{-2}, $J_{P—P}$ = 22.9 Hz), ^{1}H coupled, δ −0.41 (d, $J_{P—P}$ = 22.9 Hz), δ 5.77 (ddd, $J_{P—P}$ = 22.9 Hz, $J_{P—H(trans)}$ = 30.6 Hz, $J_{P—H(cis)}$ = 9.9 Hz).

The bis(methyl) derivative of phosphoenolacetonylphosphonate **(2)** was prepared by the reaction of tetramethyl phosphoenolacetonylphosphonate with sodium iodide (*21*). A solution of 6.0 g (23 mmol) of the tetramethyl ester and 10.8 g (72 mmol) sodium iodide in 150 ml acetone was refluxed for 1 h. After the solution cooled, the resulting yellow precipitate was collected by suction filtration, washed with cold acetone, and air dried. The product was recrystallized from hot 95% ethanol. The hot solution was filtered and acetone was then added until the solution became cloudy. The solution was then boiled and stirred vigorously for 5 min before cooling. The precipitate was collected by suction filtration and washed with cold acetone. This procedure was repeated twice to afford white crystals of **2** which were dried *in vacuo* (74% yield): mp 228–232 (decomp.); ^{1}H NMR (D_2O/DSS) δ 3.56 (d, 3H, $J_{P—H}$ = 10.8 Hz), 3.61 (d, 3H, $J_{P—H}$ = 10.8 Hz), 5.07–5.77 (m, 2H, $=CH_2$); ^{31}P NMR (33% D_2O, 0.1 M Tris–HCl buffer, pH 7.8), ^{1}H-decoupled, δ −2.95 (d, OPO_3^{2-}, $J_{P—P}$ = 29.3 Hz), 8.16 (d, PO_3^{2-}, $J_{P—P}$ = 29.3 Hz). *Anal.* Calcd for $C_4H_8O_7P_2Na_2$: C, 17.39; H, 2.92; P, 22.45. Found: C, 17.50; H, 3.02; P, 22.21.

Enolase Assay

Enolase was assayed using either 2-phosphoglycerate (2-PGA) (0.1–0.6 mM) or PEP (0.15–0.30 mM) as the substrate, monitoring the change in absorbance at 240 nm due to phosphoenol pyruvate. Routine assays were done at 25°C in 0.10 M imidazole buffer, pH 6.8, which contained 0.4 M KCl, 0.01 mM EDTA, and 1.0 mM $MgSO_4$. The 2-PGA or PEP was dissolved in the buffer solution. The enzyme

preparation (50 μl containing 0.22 units) was then added to 3.0 ml of the substrate solution to initiate the reaction. Imidazole buffer (0.10 M) was also used for assays at pH 7.3 and 7.8. Compounds tested as inhibitors were dissolved in the buffer together with the substrate. The enzyme preparation was then added to give 3.0 ml of the solution as described above.

Pyruvate Kinase Assay

Pyruvate kinase was assayed using the method of Bücher and Pfleiderer (*22*). Reactions were maintained at 37°C. Assays were done at pH 7.6 in 0.05 M imidazole buffer which contained 0.12 M KCl and 0.06 M $MgSO_4$. Solutions of NADH (5 mg/ml H_2O), ADP (20 mg/ml H_2O), and phosphoenolpyruvate (5 mg/ml H_2O) were prepared. Reaction volumes were 3 ml. This included 2.6 ml of buffer, and 0.1 ml of each of the ADP, NADH, and phosphoenolpyruvate solutions. To this mixture, 10 μl of lactate dehydrogenase was added. The reaction was initiated by the addition of 100 μl pyruvate kinase solution and the decrease in absorbance at 340 nm was monitored. Approximately 0.4 units of lactate dehydrogenase and 1 μg of pyruvate kinase were used per 3 ml assay solution.

Phosphoenolpyruvate Carboxylase Assay

Phosphoenolpyruvate carboxylase (PEPC, Ec 4.1.1.31) was assayed at 25°C using the coupled assay of Lane *et al.* (*23*), which employs malate dehydrogenase to reduce the product. Reaction mixtures contained 2.6 ml of 0.10 M Tris–HCl buffer, pH 7.8 (which was also 0.01 M $KHCO_3$ and 2.0 mM $MgCl_2$), 50 μl NADH solution, 50 μl PEP solution, and 50 μl malate dehydrogenase solution. The final concentrations of NADH and PEP in the assay were 1.5 mM and 0.1–2.0 mM, respectively. Approximately 6 units of malate dehydrogenase was used for each assay. The reaction was initiated by the addition of 25 μl of PEPC solution (2 units/ml in a 12.5% glycerol solution of buffer) and the increase in absorbance at 340 nm was monitored. For inhibition studies the concentrations of **1** and **2** were 0.0 to 4.6 and 20.0 mM, respectively. Studies at pH 6.8 and pH 8.8 utilized 0.1 M Mops–HCl and 0.1 M Tris–HCl buffers, respectively.

*Tests of **1** as a Substrate*

Analysis of reaction solutions with ^{31}P NMR was used to determine whether **1** is a substrate for the enzymes enolase and PEP carboxylase. Solutions of **1** (20 mM) were prepared in the assay buffers (pH 6.8 for enolase and pH 7.8 for PEP carboxylase) with all reaction components present as described above, except that the substrate PEP was absent. The solutions also contained 20% added deuterium oxide. The reaction was initiated by the addition of approximately five times the amount of enzyme used in the regular assays and monitored for 15 h at 25°C by NMR. For PEP carboxylase the *E. coli* preparation was used (with a concentration of acetyl-CoA of 10 mM) since the wheat preparation was found to possess a small amount of phosphatase activity.

TABLE 1

Inhibition of Enolase by **1**

pH	Pattern	K_m (mM)	K_i (mM)	K_{ii} (mM)
6.8	Competitive	0.14 ± 0.04	2.2 ± 0.4	
7.3	Linear mixed	0.12 ± 0.01	2.4 ± 0.3	17 ± 3
7.8	Linear mixed	0.12 ± 0.005	4.5 ± 0.3	29 ± 8

RESULTS

Inhibition of Enolase

Both **1** and **2** are competitive inhibitors of enolase with respect to 2-PGA. K_i for **1** with enolase was determined at pH 6.8, 7.3, and 7.8 (Table 1). Above pH 6.8, linear mixed-type inhibition (*24*) was observed. K_m for the enzyme stayed relatively constant over this pH range. However, the K_i to K_m ratio increased approximately two-fold. The enzyme was also assayed in the reverse direction at pH 6.8 using PEP as the substrate. Again, competitive inhibition was observed. K_i for **2** toward enolase at pH 6.8 is 27 ± 4 mM, which is an order of magnitude larger than that for **1**.

Inhibition by 1 of PEP Carboxylase

1 is a potent inhibitor of phosphoenolpyruvate carboxylase in solutions more basic than pH 7.8. The inhibition is competitive at pH 7.8 (K_i = 2.2 ± 0.6 mM) and linear mixed-type at pH 8.8 (K_i = 0.3 mM and K_{ii} = 2.6 ± 0.2 mM). At pH 6.8, **1** activated the enzyme (Fig. 1). The K_m values for PEP as a function of pH

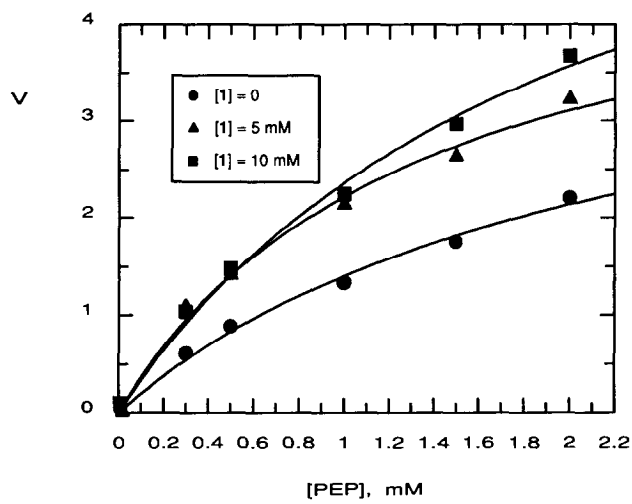


FIG. 1. Activation of PEP carboxylase by **1**.

are: 2.2 ± 0.6 mM, pH 6.8; 0.24 ± 0.03 mM, pH 7.8; 0.19 ± 0.01 mM, pH 8.8. The bis methyl ester, **2**, had no effect on the activity of this enzyme at a concentration of 20 mM.

Tests of Inhibition of Pyruvate Kinase

Neither **1** nor **2** inhibited pyruvate kinase at concentrations of 20 mM. ADP was present in the assay solution and only inhibition against PEP was tested.

*Tests of **1** as a Substrate*

No change in the ^{31}P NMR spectrum of **1** was noted over a 15-h period during which the compound was incubated under conditions described for assays of enolase and phosphoenolpyruvate carboxylase. Therefore **1** is not a substrate for these enzymes.

DISCUSSION

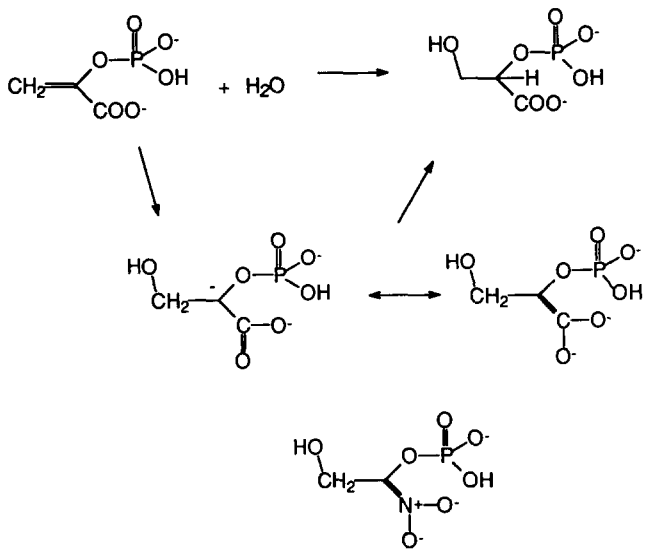
1 as a PEP Analogue

With the exception of the phosphonate function, all structural features of **1** are identical to PEP. As a result, variation in the interaction of **1** with PEP-dependent enzymes is likely to be due to a change in the electrostatic interaction between the phosphonate and the corresponding region of the enzyme normally responsible for the recognition of the carboxyl moiety of PEP. In this study we show that **1** and **2** interact differently with the enzymes pyruvate kinase, enolase, and PEP carboxylase.

Enolase

Enolase (EC 4.2.1.11) catalyzes the reversible dehydration of 2-phospho-D-glycerate (2-PGA) to PEP. Wold (*25*) compared the "fit" of various PEP and 2-PGA analogues as measured by the competitive inhibition constant, K_i, to the active site of enolases (mainly the yeast and rabbit enzymes) to describe the active site. There is a fairly rigidly defined spatial relationship between the phosphate and the primary hydroxyl group involved in the dehydration of 2-PGA. Both the carboxylate and the phosphate groups appear to be required for binding with the geometrical restrictions on the carboxylate being less stringent than those imposed on the other groups.

In our study we have shown that **1** and **2** bind to enolase although the carboxyl group has been replaced. The phosphonate and methyl phosphonate groups, therefore, are successful mimics of the carboxyl function and this may be due to the less stringent geometrical restrictions in the enzyme's carboxylate binding pocket. **2**, on the other hand, is a poor inhibitor of enolase probably because of the increased steric requirements of the methyl phosphate moiety in the rigidly defined phosphate binding site.



SCHEME 1. Enolase mechanism and nitronate analogue of the intermediate.

1 as an Intermediate Analogue in Enolase Catalysis

Enolase catalysis has been demonstrated to proceed via the formation of a carbanion intermediate (*26, 27*). This mechanism, which involves the formation of a carbanion adjacent to a carboxyl group, has also been demonstrated to occur in the elimination reactions of aspartase, fumarase (*28*), and aconitase (*29*) and in the condensation reaction of isocitrate lyase (*30*). The enzyme induces carbanion formation in these reactions by stabilizing the *aci*-carboxylate anion (Scheme 1), a form of the carbanion which has the negative charge delocalized into the carboxyl function.

The nitronate group may act as a mimic of the *aci*-carboxylate as shown in Scheme 1. Cleland and co-workers (*31*) have reported a number of nitronate analogues of the *aci* form of the normal carbanion intermediate which are potent inhibitors of enolase. The nitronate function is isosteric and isoelectronic with the *aci* form of the carbanion intermediate generated in these enzymatic reactions; the only major difference being an extra positive charge introduced by the nitrogen substitution. All the 2-PGA analogues in those studies were phosphonates since 2-nitrophosphates are unstable.

The dianionic phosphonate function may also be viewed as a mimic of the *aci*-carboxylate as indicated in Scheme 1. The fourth pK_a of **1** is 7.46 and corresponds to the second ionization of the phosphonate group (*32*). Therefore at pH 6.8 only about 18% of **1** is in the form of the tetraanion, while at pH 7.8 this is increased to about 69%. For phosphoenolpyruvate the pK_a values are (1), 3.5, and 6.8, the trianion being the substrate (*32*). K_i for **1** increases from pH 6.8 to 7.8 while the K_m for PEP is nearly constant. Thus, over this narrow pH range, increased ionization of the phosphate group does not significantly affect binding. The decreased

binding of **1** may be attributed to the slightly higher concentration of the tetraanionic species. Thus, the tetraanionic form of **1** does not act as an *aci*-carboxylate analogue since the phosphonate group in its dianionic ionization state binds the enzyme *less* favorably than in its monoanionic state. Therefore, the form of **1** which inhibits enolase is the trianion, an analogue of the substrate. This is similar to observations with other phosphonate analogues of carboxylates in enzyme inhibition studies (*3, 33, 34*). The competitive inhibition of enolase by **2** is a further indication that the enzyme can bind a monoanionic function in the C_1 position of PEP.

Linear Mixed-Type Inhibition of Enolase

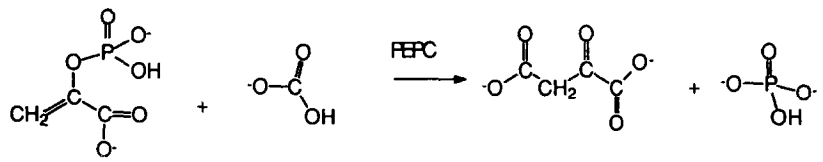
Above pH 6.8, **1** produces linear mixed-type inhibition kinetics. This kinetic pattern of inhibition normally arises when the inhibitor can bind to the enzyme–substrate complex (*36*). This means that **1** not only can compete with PEP for the active site but also can bind to enolase after the enzyme has bound a molecule of substrate. Studies using substrates and substrate analogues have demonstrated that rabbit muscle enolase has two binding sites per dimer, both of which are probably catalytically active (*35–37*). The binding of **1** to one of these sites after the initial binding of the substrate, PEP, can account for the observed kinetics. At pH 6.8 the two active sites behave as two independent active sites, each being inhibited by **1**. However, above pH 6.8 the two sites may not behave independently. When the substrate has bound to the enzyme, the binding of **1** is altered.

Substrate Studies with Enolase

The dehydration of 2-PGA catalyzed by enolase involves the abstraction of a proton at C_2 of the substrate, which is believed to be stabilized by the formation of an *aci*-carboxylate. By analogy, if **1** was a substrate for enolase the reaction intermediate would have a negative charge on the carbon atom attached to the phosphonate phosphorus. The Hammett σ_{para} values for the $-COO^-$ and $-PO_3H^-$ groups are 0.00 and 0.26, respectively (*38*). The phosphonate group, therefore, is more electron-withdrawing and might be better able to stabilize the adjacent carbanion. However, delocalization of electrons into the phosphonate group of **1** from the adjacent C_2 is stereoelectronically prohibited. The localized structure would not resemble the *aci*-carboxylate.

X-ray crystallographic studies of a yeast enolase–Mg^{2+}–PEP complex at 2.2 Å resolution (*39*) have shown that the carboxylate group of the substrate interacts with histidine and lysine side chains while the phosphate function is hydrogen-bonded to the guanidinium group of an arginine residue. Both of these interactions partially neutralize the anionic charges on the substrate and consequently reduce electron density at C_2. The electron withdrawal stabilizes the anionic reaction intermediate.

The histidine and lysine residues on the enzyme which are responsible for electrostatic interaction with the carboxylate moiety of PEP should also interact with the anionic phosphonate function of **1**. If each of the carboxylate oxygens of



SCHEME 2. PEPC reaction.

PEP interact with positive charges at the active site, the alignment of the acyl substituent is established. In the phosphonate analogue, the interaction of the two phosphonate oxygens with the cationic site can be approximately the same as the interaction of the carboxylate group with the site. However, the resulting position of the remainder of the molecule will be very different due to the tetrahedral geometry of the function group (*3, 4*).

Thus, although enolase is capable of binding the analogue, the perturbation in orientation generated by introduction of the phosphonate function causes a misalignment with respect to catalytic groups, preventing catalysis. The importance of properly aligning the substrate in the active site has recently been demonstrated in crystallographic studies of the complex of enolase and Ca^{2+}–2-PGA (*40*). This inhibitory complex is bound in the active site in what is referred to as an "orthonormal position." The enzyme, having the substrate bound perpendicular to the plane of the carboxyl groups of the proposed catalytic glutamic acid residues, is catalytically inert.

Phosphoenolpyruvate Carboxylase

Phosphoenolpyruvate carboxylase (PEPC) catalyzes the irreversible addition of bicarbonate to phosphoenolpyruvate to produce oxaloacetate and inorganic phosphate in the presence of a divalent metal ion (Scheme 2). This enzyme is widespread in higher plants (*41*) where it is involved in CO_2 fixation by the C_4-dicarboxylic acid pathway of photosynthesis (*42*). Many C_4 plants are weeds and, as a consequence, the topography of the active site of PEPC has been studied in the design of herbicides (*43*).

O'Leary examined the interaction of a large number of substrate analogues with PEPC (*44, 45*). Inorganic phosphate and simple phosphate esters are inhibitors of the enzyme. However, compounds which contain both a phosphate and a carboxylate group are much better inhibitors. Many of the substrate analogue studies have involved variations at C_3 of phosphoenolpyruvate while retaining the phosphate and carboxylate moieties. As a result, very little information is available about the geometrical requirements of the enzyme with respect to these groups.

1 is a competitive inhibitor of PEPC against PEP at pH 7.8. However **2** does not interact with the enzyme. As is the case with enolase, it appears that although the steric requirements of the carboxylate binding pocket are not stringent enough to prevent the binding of **1**, they exclude **2** from the binding pocket. **1** is the first example of a PEP analogue which inhibits PEP carboxylase without possessing both a phosphate group and a carboxylate group.

Variation of PEP Carboxylase Activity as a Function of pH in the Presence of **1**

The effect of **1** on the activity of PEP carboxylase is pH-dependent. At pH 6.8, **1** activates the enzyme, while above pH 7.8 it inhibits the enzyme. Cooperativity in binding of PEP to PEP carboxylase has been observed for the maize enzyme (*45*). The enzyme shows hyperbolic kinetics in the presence of magnesium ion at pH 8 (*46, 47*). At pH 7, however, the kinetics are sigmoidal (*48*). The allosteric nature of PEP carboxylase is not well understood. The allosteric behavior arises from binding to a remote site (*45*).

The maize enzyme is tetrameric (*36*) and therefore the activation of PEP carboxylase by **1** at low pH may be due to intersubunit contacts. At pH 6.8, **1** may bind the active site of the enzyme, inducing a conformational change which causes activation. At higher pH, **1** is a more potent inhibitor of PEP carboxylase. On going from pH 7.8 to 8.8 the K_i value changes from 2.2 to 0.3 mM. In addition, the inhibition kinetics change from competitive to linear mixed-type. **1** exists as 69 and 96% in the tetraanionic form at pH 7.8 and 8.8, respectively. The results are consistent with the dianionic form of the phosphonate binding to the enzyme more tightly than the monoanionic form. This may be due to the doubly ionized phosphonate moiety of **1** coordinating the Mg(II) in the active site more strongly at pH 8.8 (*32*). The phosphate group of PEP is usually coordinated to the divalent metal ion in PEP-metabolizing enzymes (*49–51*). Binding of the phosphonate moiety of **1** to the metal ion in place of the phosphate group may also account for the inability of **1** to act as a substrate. The linear mixed-type inhibition kinetics observed at pH 8.8 probably arise from the binding of **1** to other subunits of the enzyme (thereby forming an ESI complex) or at a site other than the active site.

Pyruvate Kinase

Pyruvate kinase (EC 2.7.1.40) catalyzes the combined enolization and phosphorylation of pyruvate to yield phosphoenolpyruvate in a reaction with ATP. In the reverse reaction, which is the normal metabolic direction, the phosphate of PEP is transferred to the terminal phosphate of ADP, which must be bound nearby since the enzyme utilizes a direct displacement mechanism. Therefore, if the analogues cannot bind in the alignment necessary for the substrate to react, they may be blocked by the residues which bind ADP. Without detailed structural information on this enzyme, further analysis is inconclusive.

EPSP Synthetase

Enol pyruvyl shikimate-3-phosphate synthetase (EPSP synthetase) is a key enzyme in aromatic amino acid biosynthesis in plants and is the target of action of commercially important herbicides (*52*). Preliminary studies of **1** and **2** as inhibitors of this enzyme reveal that they bind but not as strongly as materials which are commercially useful herbicides (*53*).

Stereoelectronic Implications

The stereoelectronic difference of the phosphonate monoester and the carboxylate is illustrated in Fig. 2. The arrow indicates the direction of the bond from the

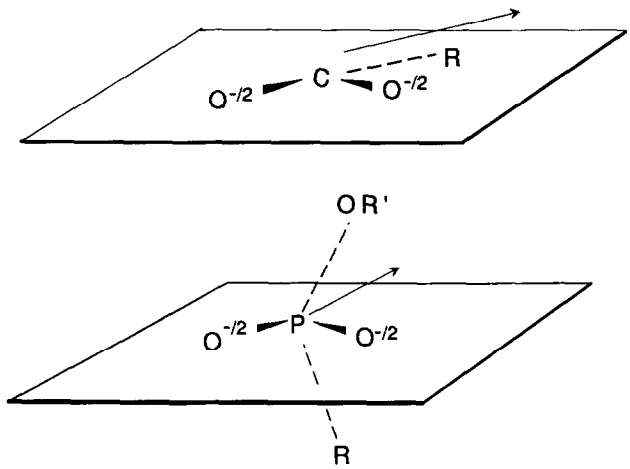


FIG. 2. Stereoelectronic differences of carboxylate and phosphonate monoanions, Front and rear views of each are shown with resulting alignment of alkyl chain (indicated by arrow) where contact between delocalized anion of ligand and enzymic delocalized cation defines geometry of interaction.

functional group to the enolphosphate substituent with electrostatic factors holding the two anionic oxygen centers (each with a charge of $-1/2$) aligned. The third bond from the carboxylate is in the plane and bisecting the angle defined by the contact of the two oxygen atoms and the central carbon. The phosphonate anion is tetrahedral so that the third and fourth ligands are in a plane that is the perpendicular bisector of the plane containing the two anionic centers and the phosphorus atom. The neutral groups on phosphorus are approximately 54° above and below the plane. Thus the interaction of the enol phosphate group with the protein will be at significantly different positions in **1** and PEP.

CONCLUSION

These studies demonstrate that analogues in which the carboxylate group of PEP is replaced by phosphonic or methyl phosphonic groups elicit diverse responses from enzymes which utilize PEP. The parent phosphonate analogue of PEP, **1**, is a competitive inhibitor of enolase and PEP carboxylase. The lack of reactivity of **1** as a substrate for these two enzymes is likely due to stereoelectronic factors. Pyruvate kinase is not inhibited by the phosphonate analogues. It is likely that alignment with the acceptor is critical and this is built into the binding site.

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