Interaction of Inhibitors with Phosphoenolpyruvate Mutase: Implications for the Reaction Mechanism and the Nature of the Active Site†

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ABSTRACT: The active site and mechanism of action of the enzyme phosphoenolpyruvate mutase have been probed using substrate and intermediate analogues as inhibitors of the mutase-catalyzed reaction. Smaller anions (*e.g.* sulfite, nitrate, phosphinate, and bicarbonate) are noncompetitive inhibitors of the mutase, while larger anions in the complementary series (sulfate, phosphonate, phosphate) inhibit competitively. Combining oxalate, an intermediate analogue that is a potent inhibitor of the mutase (K_i = 25 μM), with small, noncompetitive inhibitor anions results in synergistic inhibition of the mutase, suggesting that the combined presence of oxalate and anion creates a "bimolecular transition-state analogue". The phosphoenolpyruvate (PEP) mutase genes from *Tetrahymena* and *Streptomyces* are known, and these enzymes share significant amino acid sequence similarity to the isocitrate lyase gene from *Ricinus*. Despite their seeming structural unrelatedness to the substrates of PEP mutase, several isocitrate analogues are good inhibitors, suggesting that isocitrate lyase and PEP mutase are evolutionarily related. An active-site model has been developed that is in accord with the data presented, which are consistent with a mechanism involving the intermediacy of a phosphoenzyme.

The enzyme phosphoenolpyruvate mutase (EC 5.4.2.9) catalyzes the interconversion of phosphoenolpyruvate (PEP) and phosphonopyruvate (Figure 1) and is thought to be responsible for the formation of the carbon–phosphorus bond in essentially all naturally-occurring phosphonates[1] (Bowman et al., 1988; Seidel et al., 1988; Hidaka et al., 1989b). The equilibrium lies well toward PEP [K_{eq} (pH 7.5) $\approx$ 2000 (Seidel, 1992)] and the reaction is driven in the physiological direction by the decarboxylation of phosphonopyruvate catalyzed by a different enzyme (Barry et al., 1988). The gene encoding PEP mutase has been cloned from *Tetrahymena* and overexpressed in *Escherichia coli* (Seidel et al., 1992), and the stereochemical outcome of the reaction at phosphorus has been determined (Freeman et al., 1989; Seidel et al., 1990; McQueney et al., 1991). The finding that the mutase-catalyzed reaction proceeds with overall retention of the configuration at phosphorus rules out a concerted mechanism due to the constraints of orbital symmetry (Woodward & Hoffmann, 1970), but is consistent with several other mechanistic possibilities. Indeed, the fundamental question of exactly *how* the phosphorus–carbon bond of phosphonates is synthesized has remained unanswered. On the basis of enzymological precedent and chemical reasonableness, it has been argued that the most likely mechanism involves a phosphoenzyme intermediate, as shown in Figure 2.

Substrate, intermediate, and transition-state analogues have long been useful in elucidating enzyme mechanisms (Walsh, 1979). Binding affinities of substrate and intermediate analogues can provide information about an enzyme's active site, and the credibility of a particular mechanism can often be assessed by the use of transition-state analogues, which are expected to bind tightly to the enzyme. This expectation is based on the tenet that enzymes have evolved to bind preferentially to the transition states of the reactions that they catalyze, as this is the only way to achieve a rate acceleration (Pauling, 1946, 1948; Fersht, 1985).

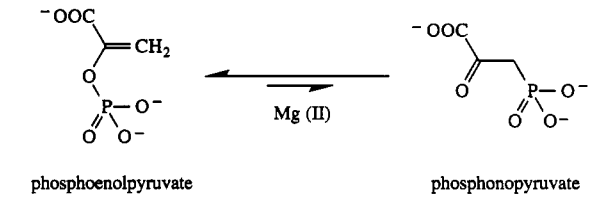

FIGURE 1: The reaction catalyzed by PEP mutase.

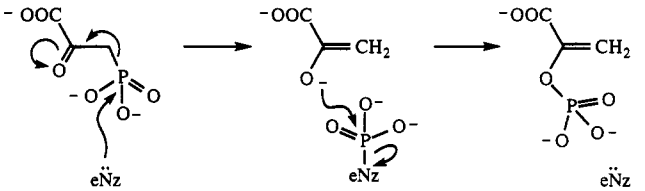

FIGURE 2: A proposed mechanism for PEP mutase.

We have used these approaches to probe the PEP mutase-catalyzed reaction, focusing particularly on the possible mechanism that involves a phosphoenzyme intermediate (Figure 2). This mechanism is presented in more detail in Figure 3, which includes the presumed transition states. The mutase-catalyzed reaction requires a divalent metal ion, the role of which is currently unknown. The transition states for the phospho group transfer steps are expected to involve pentacoordinate phosphorus, with the three equatorial oxygens of the trigonal bipyramid lying in a plane. The intermediate in the reaction is the pyruvoyl enolate bound to the phosphoenzyme. With this putative mechanism in mind, the binding of substrate, intermediate, and transition-state analogues to the mutase has been examined.

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[1] One of the P–C bonds in the *phosphinate* herbicide bialaphos owes its existence to a related enzyme, carboxyphosphoenolpyruvate (CPEP) mutase (Hidaka et al., 1989a; Hidaka et al., 1990), while the formation of the other P–C bond is catalyzed by an apparently unrelated enzyme that has recently been characterized (Kamigri et al., 1992).

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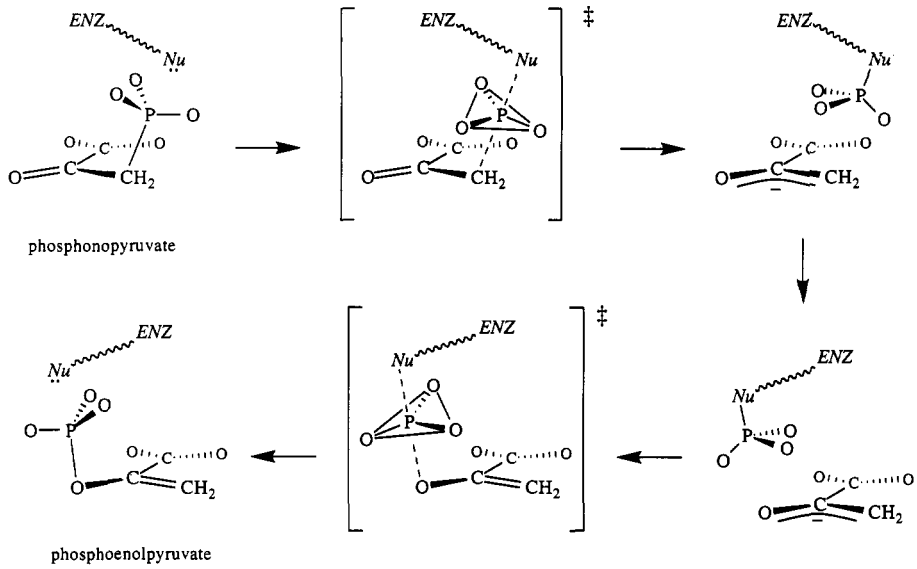


FIGURE 3: Intermediates and transition states along the "phosphoenzyme" pathway. The divalent cation and some double bonds have been omitted for clarity.

EXPERIMENTAL PROCEDURES

Phosphonopyruvate. Triethyl phosphonopyruvate was prepared according to the method of Coutrot et al. (1978) and was deprotected with trimethylsilyl bromide (Lancaster Synthesis Ltd., Windham, NH). The product was purified as described by Seidel et al. (1990). The phosphonopyruvate thus obtained was quantitated by three methods: (a) derivatization to the semicarbazone according to the method of Anderson et al. (1984) ($\Delta\epsilon_{253nm}$, 10 000); (b) total phosphate assay after treatment with 70% (w/v) perchloric acid (Chen et al., 1956); and (c) enzyme assay with PEP mutase-pyruvate kinase/ADP-lactate dehydrogenase/NADH, following the decrease in NADH absorbance ($\Delta\epsilon_{340nm}$, 6220; Horecker & Kornberg, 1948). The three methods gave mutually consistent results.

Other Compounds. D- and L-2-phosphomalate were prepared using a modification of the procedure of Nowak & Mildvan (1970) for D- and L-phospholactate. Phosphonoacetaldehyde was a kind gift from Dr. H. B. F. Dixon (University of Cambridge). All other compounds were from Fisher (Fair Lawn, NJ), Sigma (St. Louis, MO), Fluka (Ronkonkoma, NY), or Aldrich (Milwaukee, WI).

Reexamination of k_{cat} and K_m for the Mutase-Catalyzed Reaction. Phosphoenolpyruvate mutase activity was determined by using phosphonopyruvate as substrate and measuring the rate of phosphoenolpyruvate (PEP) formation by following A_{233nm}, for which $\Delta\epsilon = 1500$ at pH 7.5 (Bowman et al., 1990). The assay mixture contained 100 mM potassium HEPES buffer, pH 7.5, containing $Mg(OAc)_2$ (10 mM).

Inhibitor Assay. Compounds were tested using either the direct spectrophotometric assay described above or a coupled enzyme assay (with care taken to exclude interfering anions) when potential inhibitors had unacceptably high absorbances at 233 nm. When inhibition was observed using the coupled assay, the coupling efficiency was monitored to screen out inhibitors of the coupling enzymes. The coupled enzyme assay contained potassium HEPES buffer (100 mM), pH 7.5, $Mg(OAc)_2$ (10 mM), ADP (1.5 mM), NADH (160 μM), and a desalted pyruvate kinase/lactate dehydrogenase mixture (10 units of each).

Data Analysis. The initial velocity data obtained were analyzed using either the kinetics software package of the Hewlett-Packard Model 4582A or the Grafit software package (Erithacus Software, London, England). The data for double inhibition experiments ("bimolecular transition-state analogues") were fitted to eq 1 (Segel, 1975) for a combination

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[X]}{K_X} + \frac{[I][X]}{\beta K_I K_X}\right) + [S]\left(1 + \frac{[X]}{\alpha K_X}\right)} \quad (1)$$

of a noncompetitive inhibitor and a competitive inhibitor. In eq 1, α is the factor by which K_S changes when I occupies the enzyme and β is the interaction factor of the two inhibitors (see Results and Discussion). Data were fitted to eq 2 (Segel,

$$\frac{v}{V_{max}} = \frac{[S]}{K_S\left(1 + \frac{[I]}{K_I} + \frac{[X]}{K_X} + \frac{[I][X]}{\beta K_I K_X}\right) + [S]} \quad (2)$$

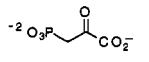
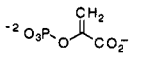
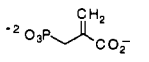
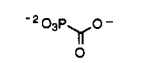
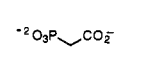
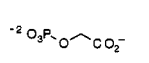
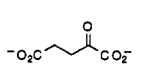
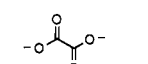
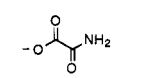
1975) for the combination of two competitive inhibitors. In eq 2, β is the interaction factor of the two inhibitors (see Results and Discussion).

RESULTS AND DISCUSSION

Reexamination of k_{cat} and K_m for the Mutase-Catalyzed Reaction. In our original determination of the kinetic parameters of PEP mutase, a coupled spectrophotometric assay was used (Seidel et al., 1988, 1992). These assay conditions contain components, notably sulfate and chloride, that were later found to be competitive and noncompetitive inhibitors of PEP mutase, respectively. The kinetic parameters (k_{cat} and K_m) were therefore redetermined using the direct spectrophotometric assay in an anionic buffer (potassium HEPES) without small inhibitory anions present. Upon reinvestigation, the values of the steady-state parameters were found to be k_{cat}, 150 s^{-1}; K_m, 10 μM; and k_{cat}/K_m, 1.5×10^7 M^{-1} s^{-1} [as compared to k_{cat}, 41 and 77 s^{-1} and K_m, 67 and 63 μM, determined previously (Seidel et al., 1988; Seidel et al., 1992), and k_{cat}, 24 s^{-1} and K_m, 20 μM, reported by McQueney et al. (1991)].

Substrate Analogues. To help define the structural requirements of the mutase active site, substrate analogues

Table 1: Substrate Analogues and Intermediate Analogues Tested as Inhibitors of PEP Mutase[a]

compd	K_i(mM)	compd	K_i(mM)	compd	K_i(mM)
 phosphonopyruvate (1)	0.010 (K_m)	 phosphoenolpyruvate (2)	0.35	 phosphonomethylacrylate (3)	7.4
 phosphonoformate (4)	0.18	 phosphonoacetate (5)	3.8	 2-phosphoglycolate (6)	2.6
 α-ketoglutarate (7)	0.90	 oxalate (8)	0.025	 oxamate (9)	> 5

[a] The following compounds were tested and found to have a $K_i > 5$ mM: itaconate (**10**), phosphonoacetaldehyde (**11**), carboxyethylphosphonate (**12**), D-malate (**13**), L-malate (**14**), glutarate (**15**), *trans*-glutaconate (**16**), succinate (**17**), fumarate (**18**), maleate (**19**), glyphosate (**20**), fosfomycin (**21**), and mesaconate (**22**).

Table 2: Inhibition of PEP Mutase by Anions

anion	K_i (mM)	αK_i (mM)	inhibitor type[a]
I^-	29	280	NC
Br^-	36	220	NC
Cl^-	87	160	NC
NO_3^-	1	0.6	NC
NO_2^-	2	6.0	NC
SO_4^{2-}	25		C
SO_3^{2-}	15	15	NC
VO_4^{3-}	1.1		C
PO_4^{3-}	21		C
HPO_3^{2-}	23		C
$H_2PO_2^-$	200	450	NC
$CH_3CO_2^-$			nd
HCO_3^-	14	14	NC
HCO_2^-	34	30	NC

[a] NC, noncompetitive; C, competitive; nd, no inhibition detected.

were examined for their ability to inhibit the mutase-catalyzed reaction in the direction of PEP synthesis. The results are summarized in Table 1.

Two of the more interesting compounds tested were PEP (**2**, product as inhibitor) and phosphonomethylacrylate (**3**), both of which are competitive inhibitors, as expected. Compared to phosphonopyruvate ($K_m = 10\ \mu M$) and PEP ($K_i \approx 350\ \mu M$), however, **3** binds very poorly ($K_i = 7.4$ mM). It is interesting that phosphonomethylacrylate binds so much more poorly than does PEP or phosphonopyruvate, even though phosphonomethylacrylate is a hybrid structure that closely resembles both PEP and phosphonopyruvate (Table 1). The mutase must therefore be able to distinguish the bridging oxygen from the bridging methylene group in each of the substrates. This is likely to be the result either of hydrogen bonding between the enzyme and the bridging oxygen (as has been suggested for the binding of glucose 1-phosphate to phosphoglucomutase; Ray et al., 1993; Knight et al., 1991; Sem & Cleland, 1990) or of metal coordination with the bridging oxygen (as has been postulated for the recognition of PEP by PEP carboxylase: Janc et al., 1992). In the latter case, which is especially relevant to the reaction catalyzed by PEP mutase, the metal, in coordinating with the bridging oxygen, is thought to act as a Lewis acid, stabilizing the high-energy pyruvoyl enolate and thus catalyzing the transfer of the phospho group. With PEP mutase, the fact that the methylene substitution in **3** *decreases* the binding affinity to such a large extent

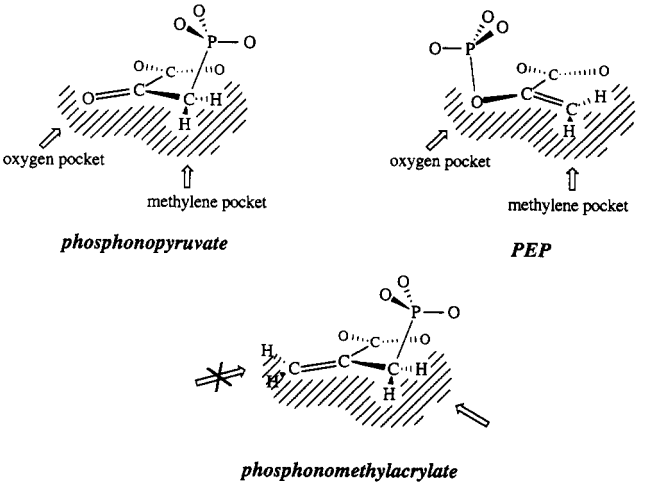


FIGURE 4: Substrate and substrate analogue data indicate that phosphonopyruvate and PEP have different binding modes at the active site of the mutase.

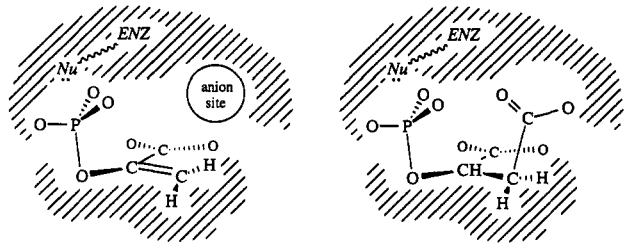
suggests that the active site allows binding of each substrate in only one orientation, as shown in Figure 4.

The question then arises as to how the phospho group migrates from one end of the enolate to the other (see Figure 3). Two possibilities can be imagined. First, through a conformational shift, a mobile, phosphorylated enzyme nucleophile could translocate the phospho group from the *C*-end to the *O*-end of the pyruvoyl enolate as shown in Figure 3. A second possibility involves a mobile enolate that moves, relative to a fixed phosphorylated enzyme nucleophile. The binding data for **1**–**3** (Table 1) are inconsistent with the latter model, which requires alternately oriented binding modes for the enolate. In this connection, it is interesting that thiophosphonopyruvate (phosphonopyruvate in which one of the peripheral phospho group oxygens has been replaced by sulfur) has the same k_{cat} as all-oxy phosphonopyruvate (McQueney et al., 1991). In essentially all other enzymatic examples, substitution reactions at phosphorus are slower with phosphorothioates than with their all-oxy counterparts (Knowles, 1980), and McQueney et al. (1991) have suggested that, in the case of PEP mutase, a nonchemical step (possibly a conformational shift) may be rate-limiting for the overall isomerization.

Anion Effects and the Active Site. As has been mentioned, certain anions are inhibitors of PEP mutase (Table 2), some being competitive inhibitors, while other act noncompetitively. In general, the larger anions are competitive, and the smaller anions are noncompetitive inhibitors. Thus phosphate ion (PO_4^{3-}) and phosphonate ion (HPO_3^{2-}) are competitive inhibitors, while phosphinate ion ($H_2PO_2^-$) is a noncompetitive inhibitor. It is not surprising to find that anions are competitive inhibitors, as the active site accommodates a trianionic substrate. For example, it was noted by Ray and Roscelli (1966) that a variety of anions were competitive inhibitors with respect to glucose 1,6-diphosphate activation of phosphoglucomutase. Noncompetitive inhibitors, however, must bind to both the unliganded enzyme and to the enzyme: substrate complex.

The fact that smaller anions are noncompetitive inhibitors is consistent with the model presented in Figures 3 and 4. PEP and phosphonopyruvate bind in different modes, with their phospho groups in different locations in the active site, so that when PEP is bound, for example, the locus that the phospho group of phosphonopyruvate would occupy if *that* substrate were bound will be unfilled (Figure 5A).

Intermediate Analogues. The reaction intermediate postulated in Figure 3 is comprised of the enolate of pyruvate



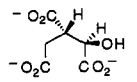
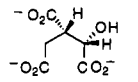
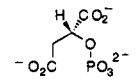
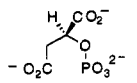
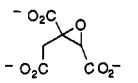
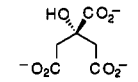
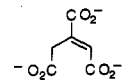
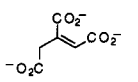
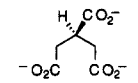
A B

FIGURE 5: (A) With PEP bound, there is a cavity where the phospho group of phosphonopyruvate would bind. (B) Phosphomalate conforms to the proposed shape of the PEP mutase active site.

Table 3: Inhibitor Synergy: Inhibition of PEP Mutase by Combinations of Anions and Oxalate

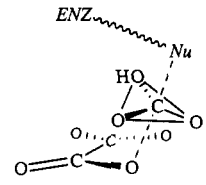
anion	β	x-fold increase (decrease) in affinity ($1/\beta$)
NO_3^-	0.05	20
NO_2^-	0.20	5
SO_3^{2-}	0.10	10
HPO_3^{2-}	2.0	(2)
$H_2PO_2^-$	0.14	7
HCO_3^-	0.05	20
HCO_2^-	0.08	13

Table 4: Isocitrate-like Molecules Tested as Inhibitors of PEP Mutase

compd	K_i(mM)	compd	K_i(mM)	compd	K_i(mM)
	1.7		1.1		1.3
D_S-isocitrate (**23**)		L_S-isocitrate (**24**)		D-phosphomalate (**25**)	
	0.27		0.60		4.5
L-phosphomalate (**26**)		(±) epoxycarballylate (**27**)		citrate (**28**)	
	1.4		> 5		> 5
trans-aconitate (**29**)		*cis*-aconitate (**30**)		tricarballylate (**31**)	

bound to the phosphorylated enzyme. Two possible analogues of the pyruvoyl enolate are oxalate and oxamate (**8** and **9**, Table 1). Oxalate mimics the charge distribution of the pyruvate enolate, while oxamate mimics the disposition of the methylene protons of the pyruvate enolate. When these two compounds were tested as inhibitors of PEP mutase, oxalate was found to be competitive, with $K_i = 25\ \mu M$, while oxamate binds poorly if at all ($K_i > 5$ mM). In fact, oxalate binds more tightly than any other analogue tested (Tables 1 and 4). These results are consistent with a literature report that pyruvate kinase (the mechanism of which is also thought to proceed via an intermediate pyruvoyl enolate) is also inhibited strongly by oxalate but not by oxamate (Reed & Morgan, 1974). These data are therefore also fully consistent with the phosphoenzyme mechanism (Figure 3).

"*Bimolecular Transition-State Analogues*". There are no obvious single-molecule mimics of the proposed transition states shown in Figure 3, but the results reported above suggest the possibility of synergistic binding of two species at the active site. Thus, oxalate may mimic the intermediate pyruvoyl enolate, and certain small oxyanions probably bind in the phospho group pocket(s) in the mutase active site. As shown in Figure 6, some of these oxalate/anion combinations could



oxalate + bicarbonate

FIGURE 6: Possible "bimolecular transition-state analogue" formed by combination of oxalate and carbonate (cf. Figure 3).

be effective "bimolecular" transition-state analogues, particularly in the case of planar anions.

If these combinations are in fact transition-state analogues, one might expect that the anion and oxalate should show concerted inhibition, also known as inhibitory synergy. Synergy is defined as an interaction *between* the two inhibitors on the enzyme such that the presence of one inhibitor decreases the dissociation constant of the other (and vice versa). Equation 1 is the equation for such a system containing a competitive inhibitor and a noncompetitive inhibitor ($V_{max} = k_{cat}E_{total}$). The variable β is the interaction factor of the two inhibitors. If $\beta > 1$, the binding of one inhibitor hinders the binding of the other ("antisynergy"); if $\beta = 1$, the binding of one inhibitor has no effect on the binding of the other; and if $\beta < 1$, the inhibitors bind synergistically. Equation 1 can be rearranged to arrive at a linear expression in the form of Dixon plots (Segel, 1975). At constant substrate concentration, plots of velocity versus the competitive inhibitor concentration, [I], at different fixed concentrations of the noncompetitive inhibitor X give lines that intersect on the x-axis for $\beta = 1$, lines that intersect below the x-axis for $\beta > 1$, or lines that intersect above the x-axis for $\beta < 1$ (synergy). The same holds for plots of velocity versus [X] at different fixed concentrations of I. An example of the Dixon plots obtained with data from combinations of oxalate and formate is shown in Figure 7. The lines intersect above the x-axis, indicating a value of $\beta < 1$ (inhibitor synergy). The value of β was found to be 0.08, showing that the presence of one inhibitor increases the affinity of the other by a factor of more than 12-fold. The β-values obtained from combinations of other anions with oxalate are shown in Table 3. The anions that are noncompetitive inhibitors all display synergistic inhibition with oxalate. For combinations of competitive inhibitors, the data obtained must be fitted to eq 2. The variable β again express the interaction factor between the two inhibitors. In accordance with the active site models presented above, phosphonate ion (HPO_3^{2-}), a competitive inhibitor, and oxalate hindered each other's binding and displayed antisynergism ($\beta = 2$). Sulfate or phosphate displayed no synergistic inhibition when combined with oxalate (data not shown). As another control, phosphonoformate (**4**), a nonplanar competitive inhibitor structurally related to oxalate, was tested and also showed no synergistic inhibition when combined with formate ion.

The planar anions nitrate and bicarbonate showed the greatest amount of synergistic inhibition, suggesting that the combinations of anions and oxalate do indeed mimic the transition state of the postulated phospho group transfer steps in the phosphoenzyme mechanism (Figure 3). The binding of two discrete species simultaneously in the active site of an enzyme will have a higher entropic cost compared to the binding of one species in the active site, not to mention any electrostatic penalty paid for bringing two negatively-charged species into close proximity. That the binding of the two

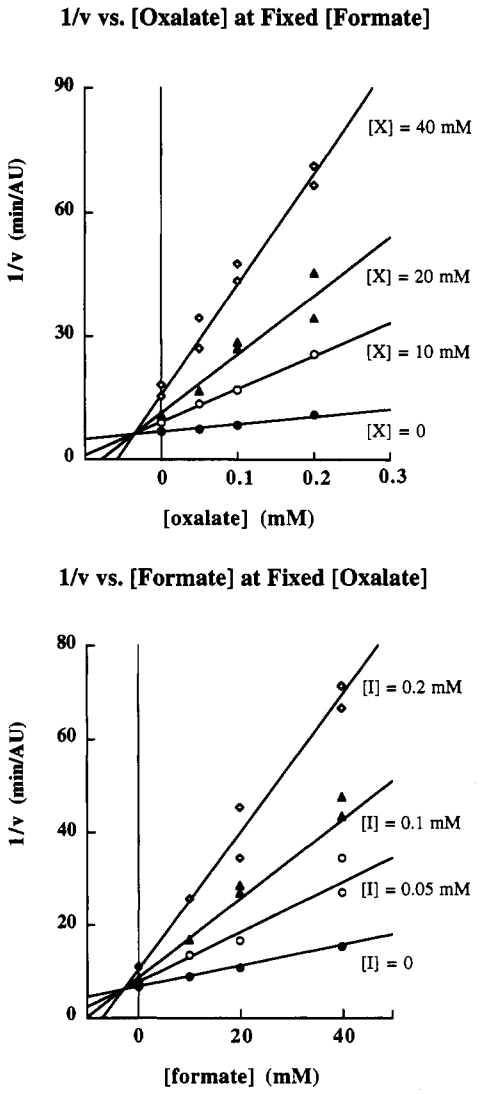


FIGURE 7: Dixon plots for combinations of the competitive inhibitor oxalate (I) and the noncompetitive inhibitor formate (X).

components (small anion and oxalate) to the mutase is maximal only when both are present suggests that these added energetic costs have been overcome by the interactive nature of the complex.

There is precedent for the type of synergistic inhibition reported here in studies on creatine kinase (Milner-White & Watts, 1971), arginine kinase (Buttlaire & Cohn, 1974), and adenylosuccinate synthetase (Markham & Reed, 1975). For example, creatine kinase is synergistically inhibited by combinations of ADP (a product of the reaction), creatine (a substrate for creatine kinase), and various anions (as with PEP mutase, nitrate was found to show the largest degree of synergy when combined with creatine and ADP). Milner-White and Watts (1971) postulated that the three species form a quaternary dead-end complex with the enzyme that mimics the transition state for the reaction. Similarly, Janc et al. (1992) have shown synergistic inhibition of PEP carboxylase by combinations of the two intermediate analogs oxalate and carbamyl phosphate.

Information from Sequence Alignments. As we have previously reported (Seidel et al., 1992), the derived amino acid sequence of the *Tetrahymena pyriformis* PEP mutase has significant similarity (Doolittle, 1986) to the gene sequence of carboxyphosphonoenolpyruvate mutase from *Streptomyces hygroscopicus* (Pollack et al., 1992; Hidaka et al., 1992a:

```
                                      10        20
T. pyr.  PEPM                  MLANSLKSFFSSTRKTTQLKNMIQSKDLS
S. hyg.  PEPM                MNATEQAANGDRGTTRSAGGR.RYLLHAPGAC
S. hyg.  CPEPM                       MAVTKAR--TFRELMNAPEIL

                          40        50        60
T. pyr.  PEPM  FIMEAHNGLSAAIVEETGFKGIWGSGLSISAAM-GVRDSNE
S. hyg.  PEPM  QL.GV.D....R.AVAE..EAL.A...CM.T.R-.....D.
S. hyg.  CPEPM VVPS.YDA...KVIQQA..PAVHMT.SGT..S.L.LP.LGF

                          80        90        100
T. pyr.  PEPM  ASYTQVLEVL-EFMSDRTKIPILLDGDTGYGNYNNARRLVK
S. hyg.  PEPM  ..W.EL.TLVGTMTDAVPGV.V.V........F.T...FAG
S. hyg.  CPEPM T.VSEQAIN.KNIVLTV-DV.VIM.A.A....AMSVW.ATR
Isocit.  Lyase                 -LK..IA.....F.GTTATVK.C.

                    120       130       140       150
T. pyr.  PEPM  KLEQRSIAGVCLEDKIFPKRNSLLDDGRQELAPINEFVAKI
S. hyg.  PEPM  RA.RVGA....F...VF..M..FFG..H.-...VA..CG..
S. hyg.  CPEPM EF.RVG.V.YH...QVN...CGH.EG--KR.ISTE.MTG..
Isocit.  Lyase LFVE.GA...HI..QSSVTKKCGHMA.K-V.VA.S.HINRL

                    160       170       180
T. pyr.  PEPM  KACKD--TQQDADFQVVARVEAFIAGWGLEEALKRAEAYRN
S. hyg.  PEPM  R.....--A.R.P..V....T..L.SKLPM....D..A..AE
S. hyg.  CPEPM E.AVE--ARE.E..TII..TD.-RESF..D...R.SRE.VA
Isocit.  Lyase V.ARLQFDVMGVETLL...TD.EA.NLIQSNVDT.D-

                    200       210       220
T. pyr.  PEPM  AGADAILMHS--KLKEPSEIEAFMKEWKNRSPVIIVPTNYH
S. hyg.  PEPM  .....LFI..--RMNT.QQ.AT..ER.EGST..L.A..T..
S. hyg.  CPEPM ....C.FLEAMLDVE.MKRVRDEIDAPLLANM.EGGK.PWL

                    240       250       260
T. pyr.  PEPM  TVPTDTFRKWGVNMVIWANHNMRACVSAMQETSRRIYEDES
S. hyg.  PEPM  .PSV.D.AAL.IAGC.....S...AFA..MRDVCQRIRTDR
S. hyg.  CPEPM .--.KELESI.Y.LA.YPLSGWM.AA.VLRKLFTELR.AGT

                          280       290       300
T. pyr.  PEPM  L------VNVEPKVAKVKEVFRLQGEDELKQADKKYL
S. hyg.  PEPM  GIYGIEDQVAPL.EIFGLFDYEGLEK..NCYTQAPD.AAVQG
S. hyg.  CPEPM TQKFWDDMGLKMSF.ELF...EYSKIS..EARFVRDQD
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FIGURE 8: Sequence alignment of the full *T. pyriformis* PEP mutase, *S. hygroscopicus* PEP mutase, and *S. hygroscopius* CPEP mutase genes as well as the partial *Ricinus communis* (Castor bean) isocitrate lyase gene (residues 169–267). Amino acids identical to the *T. pyriformis* sequence are marked by periods (.), and gaps are signified by a dash (–).

30% identity over a 180-residue overlap). Recently, Hidaka et al. (1992b) have reported the sequence of the PEP mutase gene from *S. hygroscopicus*, and this enzyme, not surprisingly, also bears significant similarity (50% identity over a 200-residue overlap) to the PEP mutase from *Tetrahymena*. In an early search of the GenBank and EMBL databases, the *Tetrahymena* gene sequence had also shown a lower score alignment match with the gene for isocitrate lyase, which we had attributed to happenstance. However, all three mutase sequences align with the *same* portion of the isocitrate lyase gene (the *Tetrahymena* PEP mutase gene shares 27% identity; the *Streptomyces* PEP mutase gene, 25% identity; and the *Streptomyces* CPEP mutase gene, 22% identity over a 98-residue overlap), which lessens the likelihood of coincidence. The sequence alignments with isocitrate lyase from Castor beans (*Ricinus communaris*) are illustrated in Figure 8.

The enzyme CPEP mutase (Hidaka et al., 1989a, 1990), an enzyme in the biosynthetic pathway to bialaphos, catalyzes a very similar rearrangement to that mediated by PEP mutase, and the sequence similarity between these two proteins is not surprising. But it is less obvious why the PEP mutase and isocitrate lyase genes might be related, as both the substrates and the type of reaction catalyzed appear to be quite dissimilar [though both enzymes likely generate and stabilize enol(ate) intermediates]. Yet on the basis that the mutase active site contains three anionic group binding pockets (two phospho group sites and one carboxylate site), one can imagine that isocitrate, with its three carboxylate groups, might map into the mutase active site quite well. It is certainly conceivable that isocitrate lyase has adapted the PEP mutase active site to catalyze a different reaction (or vice versa), resulting in the sequence similarity observed between the sequence of the two enzymes. Indeed, many of the residues that are common to the isocitrate lyase and PEP mutase genes are those that are conserved among all three mutases (see Figure 8).

Driven by these suggestive data, several compounds related to isocitrate were tested as inhibitors of PEP mutase. The results are summarized in Table 4. It is clear that some of the tricarboxylate molecules are fairly good inhibitors (a fact that one might not have guessed *a priori*), and it seems that all three charged groups play a role in binding (compare compounds **23** and **24** with **13**, **14**, **15**, and **17**, or **29** with **10**, **18**, and **22**). Following from these arguments, we synthesized D- and L-2-phosphomalate (**25** and **26**), which represent hybrids that share structural features of substrates of both PEP mutase and isocitrate lyase. As shown in Figure 5B, phosphomalate fits well into the postulated active site depicted in Figures 4 and 5A. L-Phosphomalate, the structure of which at first glance is quite unrelated to PEP, has an affinity for the enzyme comparable to that of the substrate PEP (270 μM vs 350 μM)! This finding lends support to the notion that the active sites of isocitrate lyase and PEP mutase may be evolutionarily related and is fully consistent with the proposed active site topography presented in Figures 4 and 5.

Conclusion. The data presented here are consistent with a mechanism for PEP mutase that involves a phosphoenzyme intermediate (Figure 3). While the evidence is not inconsistent with a dissociative mechanism involving a metaphosphate intermediate, arguments have been presented against the likelihood of metaphosphate intermediates in enzyme-catalyzed reactions (Knowles, 1980), and such intermediates are as yet unproven. These ambiguities notwithstanding, the inhibition studies described have painted a much more detailed picture of the active site of PEP mutase and have provided some insight into the mechanistic course of the reaction that it catalyzes.

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