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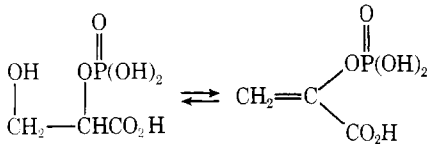
Enolase Catalyzed β,γ-α,β Isomerization of 2-Phospho-3-butenoic Acid to (Z)-Phosphoenol-α-ketobutyrate†

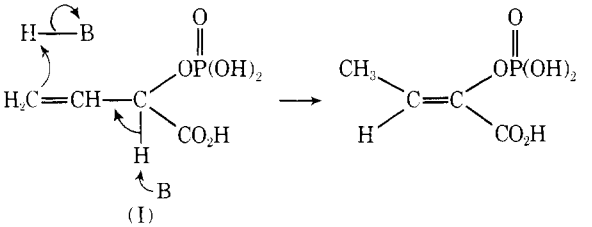
Jonathan Appelbaum and JoAnne Stubbe*

ABSTRACT: 2-Phospho-3-butenoic acid was synthesized and found to be a substrate for both yeast and rabbit muscle enolase (EC 4.2.1.11). Enolase catalyzes the isomerization of 2-phospho-3-butenoic acid to (Z)-phosphoenol-α-ketobutyrate, a β,γ-α,β isomerization. Nuclear magnetic resonance studies on the product indicate only one isomer is formed. This reaction provides indirect evidence in further support of a carbanion intermediate for the enolase reaction. 2-Phospho-3-butenoic acid is also a good competitive inhibitor of both yeast and rabbit muscle pyruvate kinase (EC 2.7.1.40).

Enolase (EC 4.2.1.11, phosphopyruvate hydratase) is a highly specific enzyme catalyzing the dehydration of D-glycerate-2-phosphate (PGA)[1] to phosphoenolpyruvate (PEP):



In connection with our studies on the active site of pyruvate kinase, we prepared a racemic mixture of 2-phospho-3-butenoic acid (I). This compound (I) was found to be a substrate for both yeast and rabbit muscle enolase. Thus enolase catalyzes a β,γ-α,β isomerization: 2-phospho-3-butenoic acid to (Z)-phosphoenol-α-ketobutyrate:



(I)

The equilibrium of this reaction, however, appears to lie very far to the right as no reverse reaction was detectable. Nuclear magnetic resonance (NMR) studies of the product indicate that only one isomer, (Z)-phosphoenol-α-ketobutyrate, is formed during the enolase-catalyzed reaction.

Mechanistic studies to date (Dinovo and Boyer, 1971; Shen and Westhead, 1973; Mildvan et al., 1973) seem to indicate that enolase catalyzes a nonconcerted dehydration reaction. The mechanism probably involves proton abstraction, C-O bond cleavage, and product release, the relative rates being pH dependent.

The mechanism of the β,γ-α,β isomerization probably involves abstraction of the α hydrogen by a base at enolase's active site followed by protonation of the terminal carbon and product release. Thus, our work indirectly supports the partially substantiated hypothesis (Wold, 1971) that there exists a base at enolase's active site which is capable of removing a very nonacidic C-2 proton to form a carbanion intermediate at pH 7.4. The pH studies, furthermore, implicate a histidine as the base which may be involved in proton abstraction.

Material and Methods

Infrared spectra were measured on a Perkin-Elmer infrared spectrophotometer, Model 237. Ultraviolet spectra and kinetic studies were run on a Cary 14. Proton nuclear magnetic resonance spectra were determined on a Perkin-Elmer R-12 using tetramethylsilane or 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal standards. Melting points are uncorrected. Microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. In studies involving pH changes, measurements were made directly on the reaction mixtures immediately after assay with a Beckman Expandomatic Model SS2 pH meter fitted with an Arthur A. Thomas combined electrode 4094-L15.

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[1] Abbreviations used are: PGA, D-glycerate-2-phosphate; PEP, phosphoenolpyruvate; CH_3-PEP, (Z)-phosphoenol-α-ketobutyrate; CH_2-PEP, α-(dihydroxyphosphinylmethyl)acrylic acid; F-PEP, (Z)-phosphoenol-3-fluoropyruvate; DSS, sodium 3-(trimethylsilyl)propanesulfonic acid.

Rabbit muscle and yeast enolase were purchased from Calbiochem Corporation and had specific activities of 10 and 50 μmol per min per mg, respectively, at 25°, as determined by the increase in absorbance at 230 nm. Pyruvate kinase from rabbit muscle was also obtained from Calbiochem Corporation and was found to have a specific activity of 160 μmol per min per mg by the coupled assay procedure (Tietz and Ochoa, 1958). Phosphoenolpyruvate monocyclohexylammonium salt was synthesized by the modified procedure (Clark and Kirby, 1966; Stubbe and Kenyon, 1972). (*Z*)-Phosphoenol-α-ketobutyrate was prepared as described previously (Stubbe and Kenyon, 1971). Diphenylphosphoryl chloride and acrolein were purchased from Aldrich Chemical Company. All other materials were purchased in the highest purity available from commercial sources.

Experimental Section and Results

2-Hydroxy-3-butenoic acid (vinyl glycolate) was prepared by the published procedure (Glattfeld and Hoen, 1935). It was purified by distillation in vacuo, bp 98–102° (1 mm), and crystallized in the receiver flask during purification. The overall yield from acrolein was 20%, mp 44–45°.

Preparation of Methyl 2-Hydroxy-3-butenoate (Methyl Vinyl Glycolate). To a 100-ml round-bottom flask fitted with dropping funnel, magnetic stirrer, and drying tube were added 5 g (0.05 mol) of 2-hydroxy-3-butenoic acid and 60 ml of methanol. The reaction vessel was cooled in an ice bath. Freshly distilled thionyl chloride, 3.6 ml (0.05 mol), was added dropwise to the reaction mixture over a period of 5 min. The reaction was then allowed to warm to room temperature over a period of 3 hr.

The methanol and any excess thionyl chloride were removed in vacuo. The product was distilled in vacuo, 70–75° (15 mm), yielding 4.6 g of product, 80% yield. The nuclear magnetic resonance spectrum in $CDCl_3$ showed peaks at δ 4.2 (3 H, singlet), 5.2 (1 H, doublet, J_{HCH} = 6 Hz), and 5.5–6.8 (3 H, multiplet).

Preparation of Methyl 2-Diphenylphospho-3-butenoate (Methyl Diphenylphosphovinyl Glycolate). Methyl 2-hydroxy-3-butenoate (2.00 g, 0.017 mol) was placed in a 50-ml round-bottomed flask equipped with $CaCl_2$ drying tube, stirrer, and 20 ml of anhydrous pyridine (distilled from BaO and stored over KOH). The flask was cooled in an ice bath. Diphenyl chlorophosphate (4.06 ml, 0.0175 mol) was then added dropwise over a period of 10 min. The reaction was stirred at 4° in the cold room for 24 hr. During this period a white solid precipitated from solution and did not appear to redissolve. After 24 hr, 3 drops of H_2O were added to the mixture to destroy the excess phosphorylating agent. The pyridine was then removed in vacuo. The oily residue was dissolved in 50 ml of $CHCl_3$ and extracted successively with 20 ml of H_2O, 20 ml of 1 N HCl, 20 ml of saturated $NaHCO_3$, and 20 ml of H_2O. The $CHCl_3$ layer was dried over $MgSO_4$ and the solvent, after gravity filtration, was removed in vacuo. The oily residue was a light orange brown color (5.9 g, 95% yield). The nuclear magnetic resonance spectrum was taken in $CDCl_3$ and showed peaks at δ 3.8 (3 H, singlet), 5.35–6.60 (4 H, multiplet), and 7.4 (10 H, broad doublet with broad base).

Dealkylation of Methyl 2-Diphenylphospho-3-butenoate. Methyl 2-diphenylphospho-3-butenoate, 8.6 mmol (3.00 g), was stirred with 17.2 mmol of 0.4 *M* LiOH for 1 day. The H_2O layer was then extracted with ether to remove any unreacted starting material. The H_2O was then removed in vacuo. The product isolated was characterized as the cyclohexylammonium salt of the monophenyl ester of the desired compound, mp 143–144°. The nuclear magnetic resonance spectrum (D_2O, DSS) showed peaks at δ 1.10–2.30 (broad multiplet), 4.75–6.05 (4 H, multiplet), and 7.3 (5 H, broad doublet with broad base).

In most cases the 2-monophenylphospho-3-butenoate was not isolated, but redissolved in a minimal amount of H_2O and passed through a Dowex 50 H^+ ion exchange column (2.2 × 14 cm). The acidic fraction was collected and allowed to stand at room temperature for 2.5 days. The solution was then neutralized with cyclohexylamine to pH 7 and the solvent was removed in vacuo. The solid residue was then dissolved in a minimal amount of hot methanol to which a small amount of anhydrous ether was added. The compound crystallized to give 3.03 g (87% yield) of the desired compound, mp 180.5–181.5°. The nuclear magnetic resonance spectrum (D_2O, DSS) showed peaks at δ 1.10 to 2.35 (broad multiplet), 5.45–6.5 (3 H, multiplet), and 5.0–5.35 (1 H, doublet of doublets). Anal. Calcd for $C_{16}H_{33}N_2O_6P$: C, 50.52; H, 8.74; N, 7.36; Found: C, 50.66; H, 8.80; N, 7.28.

Enzyme Assay. All assays were performed at 25°. The assay solution for yeast enolase contained 0.05 *M* Tris-HCl (pH 7.4), 1 m*M* $Mg(OAc)_2$, 0.01 m*M* EDTA, and varying amounts of 2-phospho-3-butenoic acid. The assay solution for the muscle enolase was the same except that it also contained 0.4 *M* KCl. In a 1-ml assay solution 4 μg of yeast enolase or 4–8 μg of rabbit muscle enolase was used. Initial velocities were obtained from an increase in absorbance of analog or PGA at 230 nm or decrease in absorbance of PEP at 230 nm. Extinction coefficients at 230 nm were determined in their corresponding assay mixtures and found to be: PEP, 3098 M^{-1} cm^{-1}; CH_3-PEP, 3293 M^{-1} cm^{-1}; 2-phospho-3-butenoic acid, not visible above 220 nm. The maximum velocities and Michaelis constants were obtained from Lineweaver–Burk plots and relative rates and K_m values are reported in Table I.

The assay solution for rabbit muscle pyruvate kinase contained 1.7×10^{-3} *M* ADP, 3.4×10^{-3} *M* $MgSO_4$, 1×10^{-4} *M* NADH, 0.1 *M* KCl, 0.05 *M* Tris (pH 7.5) with 2×10^{-3} *M* PEP, and either 5×10^{-3} *M* or 2×10^{-3} *M* 2-phospho-3-butenoic acid. For inhibition studies the analog was incubated for 5 min with 0.6 μg of pyruvate kinase and 45 μg of lactate dehydrogenase prior to addition of PEP which initiated the reaction. The decrease in absorbance of NADH at 340 nm was followed spectrophotometrically.

Table I: Activity of Enolase with 2-Phospho-3-butenoate.

Analog Tested[a]	V_{max} (μmol per min per mg)[b]	% Relative Rate (PGA = 100%)	K_m ($M \times 10^4$)
PGA with rabbit muscle enolase	10	100	0.8[c]
2-Phospho-3-butenoate with rabbit muscle enolase	0.1	1	3.81
PGA with yeast enolase	50	100	1.5[c]
2-Phospho-3-butenoate with yeast enolase	0.04	0.08	3.52

[a] Measured following increase in absorbance at 230 nm (see text). [b] The average of several determinations. [c] Wold (1971).

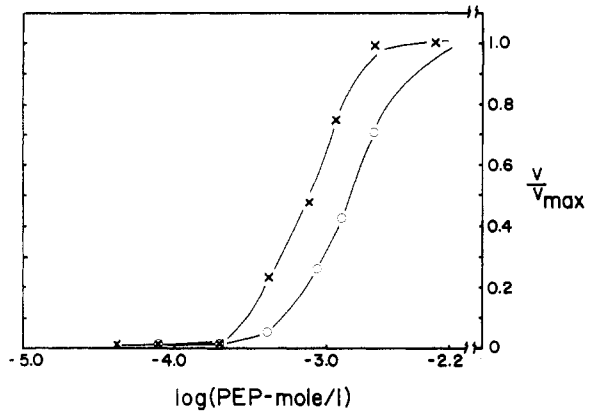


FIGURE 1: Inhibition of yeast pyruvate kinase by 2-phospho-3-butenoic acid. Reactions were carried out in 0.05 M potassium phosphate (pH 6.0), 30 mM $MgSO_4$, 5×10^{-3} M ADP, 1×10^{-4} M NADH, 10 μg of pyruvate kinase, and 45 μg of lactate dehydrogenase. (x) No inhibitor present; (○) 5×10^{-3} M 2-phospho-3-butenoate present.

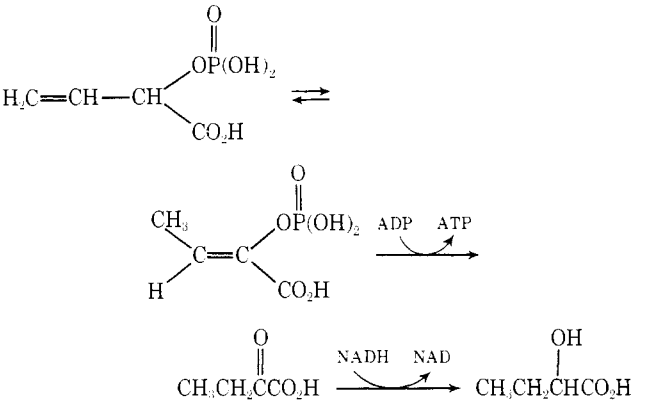
Lineweaver-Burk plots showed 2-phospho-3-butenoic acid to be a competitive inhibitor of pyruvate kinase with $K_i = 2.35 \times 10^{-3}$ M.

Yeast pyruvate kinase isolated by the procedure of Wieker and Hess (1972) had a specific activity of 25 μmol per min per mg of protein. Attempts to further purify the yeast enzyme by Cibracon Blau affinity chromatography failed (Röschlau and Hess, 1972). A typical assay mixture contained 0.05 M potassium phosphate buffer (pH 6.0), 30 mM $MgSO_4$, 5×10^{-3} M ADP, 1×10^{-4} M NADH, and 10 μg of pyruvate kinase and 45 μg of lactate dehydrogenase. As in the rabbit muscle case the inhibitor was incubated 5 min with the entire assay mixture prior to reaction initiation by addition of PEP. Inhibition data are presented in a plot of v/V_{max} vs. log [PEP] in Figure 1. This plot indicates that in the presence of 5×10^{-3} M 2-phospho-3-butenoate, the apparent K_m value for PEP is increased from 6.7×10^{-4} to 1.34×10^{-3} M.

Product Studies Using Polyethylenimine Cellulose Thin Layer Ion Exchange Chromatography (Randerath and Randerath, 1964; Rowley and Kenyon, 1974). 2-Phospho-3-butenoate was incubated in the standard assay mixture with 30 μg of enolase. Aliquots were taken at fixed intervals and spotted on polyethylenimine thin layer ion exchange plates. The plates were then developed in 0.3 M NaCl. The compounds were detected with a phosphomolybdate spray (Hanes and Isherwood, 1949; Rowley and Kenyon, 1974). Upon development, one could observe a decrease in the concentration of 2-phospho-3-butenoate with a concomitant increase in the concentration of (*Z*)-phosphoenol-α-ketobutyrate. R_f values of 2-phospho-3-butenoate and (*Z*)-CH_3-PEP were 0.12 and 0.33, respectively.

Identification, by Nuclear Magnetic Resonance Spectroscopy, of (Z)-Phosphoenol-α-ketobutyrate as the Product of the Enolase-Catalyzed Reaction. Yeast enolase (0.25 ml or 0.195 g) was desalted by passing it through a Sephadex G-25 column which had been preequilibrated with a solution containing 0.05 M Tris (pH 7.4), 1.0 mM $Mg(OAc)_2$ and 1×10^{-5} M EDTA. The protein was isolated and diluted to 50 ml with the same buffer which contained 0.076 g of 2-phospho-3-butenoate. The reaction was then allowed to proceed for 24 hr. The H_2O was then removed in vacuo and passed through a Dowex 50 cation exchange column (2.2 × 8 cm). The acidic fraction was collected and the solvent removed in vacuo. The solid residue was then exchanged twice with D_2O and the NMR spectrum taken. The spectrum was identical with that of an authentic sample of (*Z*)-phosphoenol-α-ketobutyrate. Only one isomer was detectable indicating that the solution is at least 90% *Z* isomer. Similar results were obtained with rabbit muscle enolase.

Use of Coupled Assay with Pyruvate Kinase and Lactate Dehydrogenase to Identify the Product of the Enolase Reactions. 2-Phospho-3-butenoic acid (2×10^{-3} M) was incubated in 0.05 M Tris (pH 7.4), 0.1 M KCl, 3.4×10^{-3} M $MgSO_4$, 1.7×10^{-3} M ADP, and 1.0×10^{-4} M NADH with 34 μg of rabbit muscle enolase (mixture A) for 5 min. Pyruvate kinase (62 μg) and lactate dehydrogenase (45 μg) were then added to the reaction vessel. The reaction was followed by a decrease in absorbance at 340 nm. Two control experiments were performed to ensure that this decrease in absorbance was due to product of the enolase-catalyzed reaction. (1) The same assay mixture A minus enolase was incubated for 5 min. Upon addition of pyruvate kinase and lactate dehydrogenase no decrease in absorbance was observed. Upon addition of enolase the reaction commenced almost immediately. (2) Mixture A was again incubated for 5 min at which time lactate dehydrogenase was added. No decrease in absorbance was observed. Upon addition of pyruvate kinase, decrease in absorbance at 340 nm commenced immediately. These data imply that 2-phospho-3-butenoic acid is converted to phosphoenol-α-ketobutyrate which is a substrate for pyruvate kinase. CH_3-PEP is converted by pyruvate kinase to α-ketobutyrate which is a substrate for lactate dehydrogenase.



Study of α,β-β,γ Isomerization. CH_3-PEP (2×10^{-3} M) was incubated with 80 μg of both yeast and rabbit muscle enolase in their respective assay solutions. No decrease in absorbance at 230 nm was observed after 1 hr (0.1-mm path-length cells were used). This experiment indicates that the isomerization reaction's equilibrium lies very far to the right.

Effect of pH. The effect of pH on the rate of the rabbit muscle and yeast enolase catalyzed reactions with 2-phospho-3-butenoic acid as substrate was studied. The system consisted of 0.05 M imidazole buffer (pH 6.02–7.77) or 0.05 M Tris buffer (pH 8.0–9.0), and 0.4 M KCl, 0.01 mM EDTA, varying concentrations of 2-phospho-3-butenoic acid, and ~4 μg of enolase. All the velocities were measured in μmol/min and were corrected for the effect of pH and metal ions on the molar extinction coefficient of (*Z*)-phosphoenol-α-ketobutyrate. The pH curve for these reactions is indicated in Figure 2. In further studies of pH effects, the variations of V_{max} and K_m were obtained. A plot of V_{max} against pH is shown in Figure 3. Since V_{max} is $K[E_0]$ and is

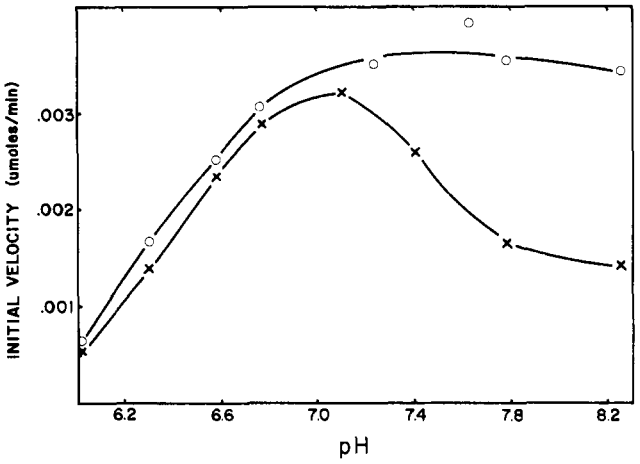


FIGURE 2: The pH dependence of the initial velocity in μmol/min of the enolase reaction, catalyzed by the yeast enzyme (x) and the muscle enzyme (O). Each value is the average of two determinations. The 1-ml reaction mixture contained 0.05 *M* buffer, 1.0 m*M* $Mg(OAc)_2$, 0.4 *M* KCl, 0.01 m*M* EDTA, 2×10^{-3} *M* 2-phospho-3-butenoic acid, and ~4 μg of enzyme.

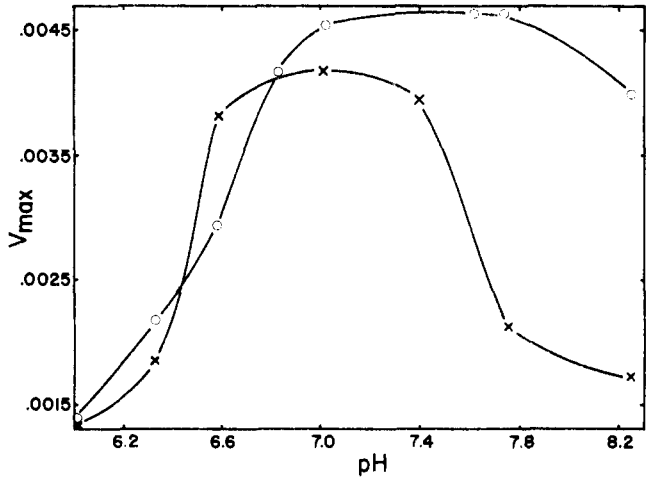


FIGURE 3: The pH dependence of the maximal initial velocity of the enolase reaction, catalyzed by the yeast enzyme (x) and the muscle enzyme (O). The individual values of V_{max} were obtained from Lineweaver-Burk plots. The 1-ml reaction mixture contained 0.05 *M* buffer, 1.0 m*M* $Mg(OAc)_2$, 0.4 *M* KCl, 0.01 m*M* EDTA, varying amounts of 2-phospho-3-butenoic acid, and ~4 μg of enzyme.

independent of substrate concentration, any change in V_{max} may signify a change in the enzyme–substrate complex. To obtain more information on change of the dissociation constant of a group at the active site, pK_m (the negative log of the Michaelis constant) is plotted against pH according to the procedure of Dixon (1953). Figure 4 gives the result showing a break in the curve at pH 7.10.

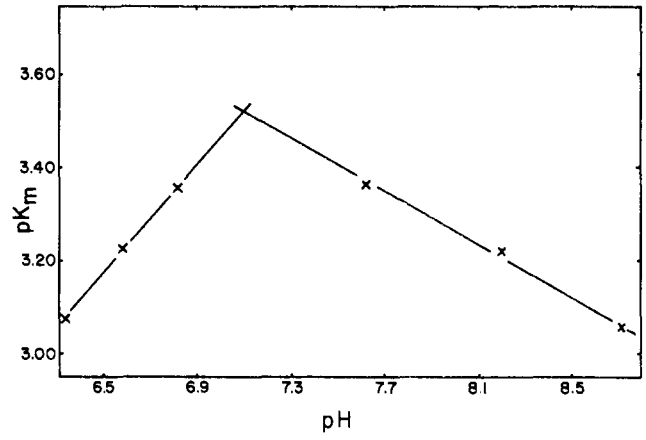


FIGURE 4: Variation of negative logarithm of the Michaelis constant (pK_m) with pH for the enolase reaction catalyzed by rabbit muscle. The K_m values were obtained from Lineweaver–Burk plots as described in Figure 3.

Discussion

Although a number of compounds have been tested as substrates for the enolase reaction, only three have been found: D-erythronate-3-P, $K_m = 3 \times 10^{-4}$ *M* (Wold, 1971); CH_2-PEP, $K_m = 2.5 \times 10^{-4}$ *M*; and F-PEP, $K_m = 2.0 \times 10^{-5}$ *M* (Stubbe and Kenyon, 1972). These compounds undergo a dehydration–hydration reaction. We have synthesized a new substrate analog of enolase, 2-phospho-3-butenoic acid, which undergoes a β,γ–α,β isomerization.

The K_m value for 2-phospho-3-butenoate is $\simeq 3 \times 10^{-4}$ *M*, very similar to the normal substrate PGA (Table I). This is not surprising in light of the work performed by Wold and Ballou (1957) and Nowak and Mildvan (1970), who found that both D- and L-phospholactate had K_i values of 4×10^{-4} *M*. Thus it appears that one may replace the hydroxymethyl group (CH_2OH) of the normal substrate with a vinyl group (CH_2=CH—) and not drastically alter the affinity of the enzyme for the substrate.

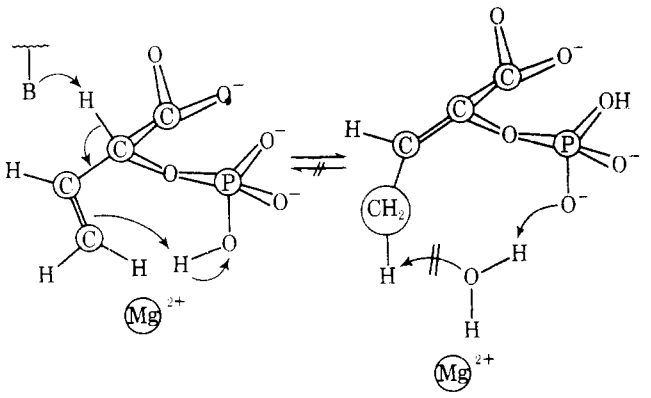
The V_{max} of the reaction with 2-phospho-3-butenoate, however, is greatly reduced. The analog reacts $1/100$ the normal rate with rabbit muscle enolase and $1/1000$ the normal rate with yeast enolase. Thus, the hydroxymethyl group is not necessary for binding or abstraction of C-2 hydrogen, although it is essential for the normal dehydration reaction to occur.

The product of the enolase-catalyzed isomerization is (*Z*)-phosphoenol-α-ketobutyrate. The nature of the product was established using polyethylenimine ion exchange chromatography and the coupled assay with pyruvate kinase and lactate dehydrogenase. CH_3-PEP is a known substrate for pyruvate kinase (Bondinell and Sprinson, 1970; Stubbe and Kenyon, 1971; and Woods et al., 1970).

The stereochemistry of the product was established by NMR spectroscopy. Only one isomer was produced by the enolase-catalyzed reaction. The NMR spectrum of this product was superimposable on that of an authentic sample of (*Z*)-phosphoenol-α-ketobutyrate prepared independently. It should be pointed out, however, that if the *E* isomer is present in less than 10% of the concentration of the *Z* isomer that one could probably not detect it by NMR spectroscopy.

Formation of *Z* isomer may be explained if the vinyl group on the D isomer of 2-phospho-3-butenoate lies in the same region of the active site as the hydroxylmethyl group. Perhaps the π cloud from the vinyl group may interact with the coordination sphere of the metal ion (Mildvan et al., 1973). After product is formed the CH_3-PEP is not aligned properly to be hydrated and the phosphate group acting as a general base is not strong enough to abstract a C-3 proton from the product. This would account for the fact that the reverse reaction α,β–β,γ isomerization does not appear to occur.

Further light may be shed on the mechanism of isomerization if resolution of the racemic 2-phospho-3-butenoate was accomplished. Because only D-2-phospho-3-glycerate is



a substrate in the normal enolase-catalyzed dehydration reaction, it would be expected that only the D isomer of the analog would undergo isomerization. Work is under way to effect this resolution.

The mechanism therefore probably involves abstraction of nonacidic C-2 proton by a base at enolase's active site followed by concomitant or stepwise protonation of C-3 and product release. This work is consistent with data reported to date (Mildvan et al., 1973; Dinovo and Boyer, 1971; Shen and Westhead, 1973) supporting a carbanion intermediate.

Because C-2 proton removal is a preequilibrium step with PGA (Dinovo and Boyer, 1971) and because the V_{max} for 2-phospho-3-butenoic acid is so much slower than the dehydration step, an alternative mechanism may be possible. A conjugate acid at the active site of enolase may protonate the double bond prior to C-2 proton abstraction. This alternative, which seems chemically unlikely, could be checked by incubation of the complete reaction mixture with T_2O followed by reisolation of unreacted substrate to look for tritium incorporation.

In an attempt to shed light on the nature of the base which may be involved in C-2 proton abstraction at enolase's active site, pH studies were carried out. Figure 2 indicates a much broader pH optimum for the rabbit muscle enolase catalyzed reaction than for that with the normal substrate, PGA (Holt and Wold, 1961). On the other hand, Figure 2, pH studies of yeast enolase with 2-phospho-3-butenoic acid, indicate a pH optimum quite similar to that found with the normal substrate PGA (Wold and Ballou, 1957). Replotting the data as V_{max} (K[E_0]) vs. pH indicates that the increase in rates from pH 6.0 to pH 6.8 may in fact be due to changes in the protonation state of the enzyme. If, in fact, the increase in rate in the first part of the activity curve was due solely to the ionization of substrate, one would expect V_{max}, independent of the concentration of the substrate, to remain constant.

In a further attempt to measure the dissociation constant of a group at enolase's active site which may be involved in catalysis, we plotted pK_m vs. pH (Figure 4). This plot shows a break in the curve at pH 7.1 and may indicate the pK_a of a group dissociating at the active site. These studies thus implicate an imidazole residue as a possible base involved in C-2 proton abstraction. This conclusion is supported by indirect evidence presented by Malmström and Westlund (1956) when studying the effect of pH on Zn^{2+} binding and by Spring (1970) when studying the tartronic acid semialdehyde phosphate–enolase complex interactions. One must be wary, however, of interpreting pH studies in that many processes are occurring simultaneously: substrate as well as enzymes undergo dissociations, enzymes undergo conformational changes and even possibly denaturation.

Finally, 2-phospho-3-butenoic acid may be of interest as a starting point for the study of other PEP-requiring enzymes. It is a fair competitive inhibitor of pyruvate kinase from rabbit muscle, $K_i = 2.3 \times 10^{-3}$ M, and also inhibits yeast pyruvate kinase.

Since O'Connell and Rose (1969) reported the irreversible inhibition of enolase by glycidol phosphate, it was hoped that 2-phospho-3-butenoate would be a starting point for synthesis of other PEP analogs which may be used as affinity labels. Synthesis of the epoxide or bromo derivatives of 2-phospho-3-butenoate may prove of interest. Work directed toward this end is in progress.

Acknowledgments

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