Mechanism of Action of Enolase: Effect of the β-Hydroxy Group on the Rate of Dissociation of the α-Carbon–Hydrogen Bond†

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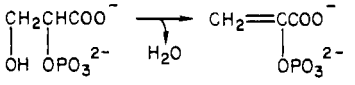
ABSTRACT: Rabbit muscle enolase catalyzes the elimination of H_2O from 2-phosphoglyceric acid (PGA) as well as a rapid exchange of the substrate α hydrogen with solvent protons. The elimination reaction proceeds through a carbanion mechanism, i.e., substrate α-proton abstraction occurs prior to OH departure [Dinovo, E. C., & Boyer, P. D. (1971) *J. Biol. Chem. 246*, 4586–4593]. We have now examined the effect of replacing the β-OH group by other substituents on the rate of enzyme-catalyzed α-proton abstraction. When the β-OH group is replaced by Cl or F, HCl and HF elimination occurs with yeast and muscle enolase. Replacement of the β-OH group by Cl (3-chloro-2-phosphoglyceric acid) reduces rate of abstraction of the α hydrogen $\sim 10^3$-fold. The elimination of HCl shows a V_{max} isotope effect of 8.5 (pH 7.8) with muscle enolase and 7 with yeast enolase. HCl elimination proceeds by a carbanion mechanism as evidenced by (1) a small secondary isotope effect (0–2%) with 3-chloro-2-phospho[3-$^2H_2$]glyceric acid and (2) a small leaving group effect, i.e., elimination of HCl is slower than elimination of HF. To further assess the contribution of the β-OH group to the rate of abstraction of the α proton, we determined the rate of exchange of the α hydrogen with solvent protons for substrates in which the $-CH_2OH$ group was replaced by H–, CH_3–, and CH_3CH_2–. Muscle enolase, in the presence of Mg^{2+}, catalyzes the exchange of the α proton of phosphoglycolate (β-OH group of PGA replaced by H). The rate of this exchange is 5×10^5-fold slower than with PGA. The exchange of the α hydrogen of phosphoglycolate does not show a deuterium isotope effect. This is in contrast to PGA where the exchange of the α hydrogen shows a deuterium isotope effect of 6 [Dinovo, E. C., & Boyer, P. D. (1971) *J. Biol. Chem. 246*, 4586–4593]. No detectable exchange was observed with phosphoglycolate in the presence of yeast enolase. With the other substrate analogues, no exchange is catalyzed by yeast or muscle enolase. The exchange of the α proton of these substrates must therefore be at least 10^6-fold slower than with PGA. The yeast and muscle enzyme catalyze the exchange of the α hydrogen of tartronic acid semialdehyde phosphate $CHOCH(PO_3H^{2-})CO_2^-$. The exchange proceeds $\sim 3 \times 10^3$-fold slower than with the normal substrate with the muscle enzyme. However, substrate dissociation from the enzyme is probably rate determining for the exchange reaction. The data show that the β-OH group has a large effect on the dissociation of the α-C–H bond, although C–H bond cleavage and departure of the OH group are not concerted. We propose that the β-OH group specifically interacts with the active site. The energy resulting from this interaction is utilized to align the molecule optimally at the active site for α-proton abstraction and to bring about a conformational change which facilitates release of the α proton from the enzyme.

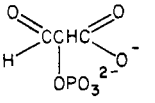
Enolase catalyzes the elimination of water from PGA[1] to form PEP (eq 1). This reaction is intriguing from a mech-



(1)

PGA PEP

anistic point of view, since it involves the abstraction of relatively nonacidic proton and the elimination of an OH^- group which is a poor leaving group. The mechanism of action of rabbit muscle enolase and yeast enolase has been investigated (Dinovo & Boyer, 1971; Shen & Westhead, 1973). It was concluded that the elimination reaction is a two-step process involving, initially, the abstraction of the α hydrogen as a proton to form a carbanion and then the elimination of the OH group (E1cB mechanism). Since enolase operates through a carbanion mechanism, one would expect that enolase can catalyze the abstraction of the α proton of substrate analogues which do not have a leaving group in the β position, for instance, phospholactate. This proton abstraction should result in the exchange of the substrate analogue α proton with isotopic solvent protons. Exchange of the abstracted proton is expected since enolase catalyzes a rapid exchange of the α proton of PGA with solvent protons (Dinovo & Boyer, 1971). Available evidence, however, does not support the expectation that enolase can catalyze the exchange of the α hydrogen of substrate analogues which do not have a leaving group in the β position. NMR studies have been done (Nowak & Mildvan, 1970) of the interaction of phospholactate with enolase. These experiments were carried out in ^2H_2O, and no exchange of the α proton was observed. These studies were, however, not primarily carried out to detect exchange reactions, and low exchange rates could have easily escaped detection. More surprising is the observation that incubation of enolase with tartronic acid semialdehyde phosphate (I, TSP) in ^3H_2O does not lead to ^3H incorporation (Spring & Wold, 1971a,b). The α proton of tartronic acid semialdehyde phosphate is considerably more acidic than that of PGA. The failure to observe



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enolase-catalyzed exchange with solvent protons of the α hydrogen of PGA analogues, which do not have a leaving group

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[1] Abbreviations used: PGA, phosphoglyceric acid; TSP, tartronic acid semialdehyde phosphate; PEP, phosphoenolpyruvic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; FT, Fourier transform; $NaDodSO_4$, sodium dodecyl sulfate; ADP, adenosine 5′-diphosphate.

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in the β position, seemed inconsistent with the carbanion mechanism. We, therefore, undertook a more detailed investigation of exchange reactions catalyzed by enolase as well as elimination reactions with substrates other than PGA. The results reported here show that PGA can catalyze the exchange of the α proton of phosphoglycolate, a PGA analogue which cannot undergo an elimination reaction, as well as tartronic acid semialdehyde phosphate. The enzyme also catalyzes the elimination of HCl[2] and HF from chloro- and fluorophospholactate. These reactions are slow. It is concluded that the β-OH group of PGA makes a surprisingly large contribution to the abstraction of the substrate α hydrogen.

Materials and Methods

Infrared spectra were taken on a Perkin-Elmer infrared spectrometer, Model 237. Ultraviolet spectra and kinetic studies were run on a Cary 15 or Beckman DU with a Gilford recording spectrophotometer. Proton nuclear magnetic resonance spectra were determined on a Varian A-60 MHz or a Bruker FT WH-90 MHz spectrometer by using tetramethylsilane or 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal standards. Melting points are uncorrected. The ^3H was counted on a Packard Tri-carb scintillation spectrometer with Bray's scintillation fluid.

Rabbit muscle and yeast enolase (EC 4.2.1.11) were purchased from Calbiochem Corp. and had specific activities of 30 and 80 μmol/(min mg), respectively, at 25 °C. Rabbit muscle enolase was also isolated by the procedure of Westhead (1966) and had a specific activity of 86 μmol/(min mg). Pyruvate kinase from rabbit muscle was also obtained from Calbiochem Corp. and was found to have a specific activity of 160 μmol/(min mg) by the coupled procedure of Tietz & Ochoa (1958). Lactate dehydrogenase and calf intestinal alkaline phosphatase were purchased from Sigma Chemical Co. and found to have specific activities of 560 and 1.0 μmol/(min mg) at 27 °C, pH 10.4, respectively. Phosphoenolpyruvate monocyclohexylammonium salt was synthesized by a modified procedure (Clark & Kirby, 1966; Stubbe & Kenyon, 1972). Diphenylphosphoryl chloride and diazole were purchased from Aldrich Chemical Co. Fluoropyruvate and phosphoglyceric acid were purchased from Sigma Chemical Co. Glycoaldehyde phosphate diethyl acetal was purchased from Calbiochem Corp. All other materials were purchased in the highest purity available from commercial sources. T_2O (1 Ci/mL) was obtained from New England Nuclear. $NaBD_4$ was purchased from Merck Sharp and Dohme.

Synthesis of 3-Chloro-2-phospholactic Acid and 3-Chloro-2-phospho[2-^2H]lactic Acid. Chloropyruvate was prepared by the procedure of Cragoe & Robb (1973) and was isolated as the sodium salt. The chloropyruvate was then reduced with $NaBH_4$ or $NaBD_4$ by the procedure of Walsh et al. (1973). After treating the crude mixture with CH_3OH–HCl overnight to hydrolyze any borate esters (Creighton & Rose, 1976), the chlorolactate or deuterated chlorolactate was then isolated on Dowex-1-Cl (with 0.015–0.03 N HCl gradient. This procedure separates chlorolactate, from dichlorolactate, from lactic acid.

Chlorolactate (0.003 mol, 0.368 g) was placed in 25 mL of CH_3OH in a 50-mL round-bottom flask fitted with a $CaCl_2$ drying tube and placed in an ice bath. To this solution, $SOCl_2$ (0.003 mol, 0.357 g, 0.216 mL) was added dropwise. The reaction was then allowed to warm to room temperature over a period of 4 h. At this time, the solvent was removed in vacuo to give 0.002 mol of methyl chlorolactate: bp 78 °C (2 mm) (67% yield); NMR ($CDCl_3$) δ 3.83 (3 H, s), 4.58 (1 H, t, J = 4 Hz), and 3.87 (2 H, d, J = 4 Hz); NMR ($CDCl_3$) (for $CH_2ClCDOHCO_2CH_3$) δ 3.87 (s) and 3.83 (3 H, s). Alternately, chlorolactate in CH_3OH–ether (1:1) was esterified by the CH_2N_2 method (Boer & Backer, 1963).

Methyl chlorolactate (0.002 mol) was placed in 10 mL of $CHCl_3$ in an ice bath. The $CHCl_3$ was extracted with H_2SO_4, dried over $CaCl_2$ and distilled prior to use. Diphenyl chlorophosphate (0.522 g, 0.44 mL, 0.002 mol) and dry pyridine (0.158 g, 0.002 mol) were added to this solution dropwise with stirring. The reaction was allowed to proceed for 12 h at 4 °C. The solvent was removed in vacuo. The residue was redissolved in 50 mL of $CHCl_3$ and extracted successively with 15 mL of H_2O, 1 N HCl, and H_2O, and the $CHCl_3$ layer was dried over $MgSO_4$. NMR ($CDCl_3$) showed some unreacted starting material and a 75% yield of the desired compound: δ 3.68 (3 H, s), 3.86 (2 H, d, J = 4 Hz), 5.33 (1 H, dt, J_{POCH} = 9 Hz, J_{CHCH} = 4 Hz), and 7.308 (10 H, br d); NMR ($CDCl_3$) [for $CH_2ClCD[OPO(OPh)_2]CO_2CH_3$] δ 3.68 (3 H, s), 3.86 (2 H, s), and 7.3 (10 H, br d).

The above compound was placed in 20 mL of HOAc with 10% by weight PtO_2. The hydrogenation was run at atmospheric pressure for 5–8 h. The catalyst was then removed by filtration through Supercel, and the HOAc was removed in vacuo. The residue showed no remaining phenyl groups. NMR (D_2O) was as above without phenyl resonance at δ 7.30.

The methyl chlorophospholactate was placed in 10 mL of H_2O, and removal of the methyl ester was followed by NMR (a decrease in the CO_2CH_3 peak and an increase in the CH_3OH peak). The reaction was complete after 9 h at 75 °C. The solution was cooled and then titrated to pH 9 with cyclohexylamine. The solvent was removed in vacuo and the solid residue recrystallized from CH_3OH–ether to give a 50% yield of the desired product. The chlorophospholactate could also be purified on Dowex-1-Cl with an HCl gradient. Chlorophospholactate (500 mg) was placed on a 1.5 × 20 cm Dowex-1-Cl column. The column was washed to neutrality with H_2O and then washed with 300 mL of 0.015 N HCl to remove the P_i. The column was then washed with 250 mL of 0.03 N HCl to remove any unphosphorylated chlorolactate, and finally 30 15-mL fractions were collected. The product appeared in tubes 15–21. NMR (D_2O) [for $CH_2ClCH[OPO(OH)_2]$-CO_2H] gave δ 1.00–2.00 (br m), 3.73 (2 H, d, J = 3.5 Hz), and 4.95 (1 H, dt, J_{POCH} = 9 Hz, J_{CHCH} = 3.5 Hz); NMR (D_2O) [for $CH_2ClCD(OPO_3^{2-})CO_2H$] δ 3.73 (s).

Chlorophospholactate can also be purified by electrophoresis at pH 3.5 (H_2O–pyridine–HOAc, 760:4:40) and 3.0 kV for 14.0 min (migration from origin value 8.5 cm) (PGA; $7^5/_{16}$ cm). The spots were detected with phosphomolybdate spray (Hanes & Isherwood, 1949; Fiske & Subbarow, 1925). Chlorophospho[2-^2H]lactate was purified by identical procedures and had the same R_f migration value on electrophoresis. All of the PGA analogues described are racemic mixtures.

Preparation of 3-Chlorophospho[3-$^2H_2$]lactate and 3-Chlorophospho[3-$^1H_2$]lactate by Identical Procedures. Pyruvate and [3-$^2H_3$]pyruvate were prepared from acetic acid and [3-$^2H_3$]acetic acid by the procedure of Calvin (1949). The pyruvate was then chlorinated by the procedure of Cragoe & Robb (1973). The chloropyruvate was then reduced with $NaBH_4$ as described by Walsh et al. (1973): NMR (D_2O) [for $CD_2ClCH(OH)CO_2H$] δ 4.62 (s); NMR (D_2O) [for $CH_2ClCH(OH)CO_2H$] δ 3.87 (2 H, d, J = 4 Hz) and 4.62 (1 H, t, J = 4 Hz). The compounds were then esterified with

[2] Wold (1975) reported the preparation of chlorophospholactate and that this compound underwent very slow enolase-catalyzed dehydrohalogenation.

CH_2N_2 (Boer & Backer, 1963): NMR ($CDCl_3$) [for $CD_2ClCH(OH)CO_2CH_3$] δ 3.83 (3 H, s), 4.58 (1 H, broad shifts as a function of concentration), and 4.60 (1 H, s); NMR for $CH_2ClCH(OH)CO_2CH_3$ as described above. The compounds were then phosphorylated as described previously to give an 85% yield of methyl chloro(diphenylphospho)lactate: NMR ($CDCl_3$) [for $CD_2ClCH[OPO(OPh)_2]CO_2CH_3$] δ 3.687 (3 H, s), 5.30 (1 H, d, J_{POCH} = 9 Hz), and 7.28 (10 H, br d); NMR ($CHCl_3$) [for $CH_2ClCH[OPO(OPh_2)]CO_2CH_3$] δ 3.68 (3 H, s), 3.86 (2 H, d, J = 4 Hz), 5.33 (1 H, dt, J_{POCH} = 9 Hz, J_{CHCH} = 4 Hz), and 7.30 (10 H, br d). The phenyl groups were removed by catalytic hydrogenation to yield methyl chlorophospholactate: NMR (D_2O) [for $CD_2ClCH(OPO_3^{2-})CO_2CH_3$] δ 3.75 (3 H, s) and 5.04 (1 H, d, J_{POCH} = 9 Hz); NMR (D_2O) [for $CH_2ClCH(OPO_3^{2-})CO_2CH_3$] δ 3.75 (3 H, s), 3.80 (2 H, d, J = 4 Hz), and 5.04 (1 H, dt, J_{POCH} = 9 Hz, J_{CHCH} = 4 Hz).

The compounds were then deesterified by heating in H_2O at 70 °C for 10 h to give the desired products isolated as the cyclohexylammonium salts. The compounds were purified by Dowex-1-Cl as described above to give the tricyclohexylammonium salts of chlorophospho[3-$^2H_2$]- and chlorophospho[3-$^1H_2$]lactate: mp 148–150 °C (loss of CO_2); NMR (D_2O) [for $CD_2ClCH(OPO_3)CO_2$] δ 4.95 (d, J = 9 Hz) and 1.00–2.00 (br m); NMR (D_2O) [for $CH_2ClCH(OPO_3)CO_2$] δ 3.73 (2 H, d, J = 3.5 Hz), 4.95 (1 H, dt, J_{POCH} = 9 Hz, J_{CHCH} = 3.5 Hz), and 1.00–2.00 (br m due to cyclohexylamine protons).

Preparation of Fluorophospholactate. Fluoropyruvate (Sigma) was reduced to fluorolactate by a modification of the procedure of Walsh et al. (1973). Fluoropyruvate (212 mg, 0.002 mol) was suspended in 15 mL of CH_3OH in a 25-mL round-bottom flask. A 10-equiv excess of $NaBH_4$ was then added to the solution at room temperature. The reaction was allowed to proceed for 2 h at room temperature, during which time the solid suspension turned clear. The excess $NaBH_4$ was destroyed by acidifying to pH 7.0 with 2 N HCl. The fluorolactate was then purified on Dowex-1-Cl as described for chlorolactate. The acid was then esterified with diazomethane or $SOCl_2$–CH_3OH procedures. Both methods gave similar NMR spectra whose peaks were not clearly assignable.

The phosphorylation is carried out as described for the chloro analogue and gives a product: NMR ($CDCl_3$) δ 3.73 (3 H, s, ester), 4.73 (2 H, dd, J_{CHF} = 47.5 Hz, J_{CHCH} = 3.5 Hz), 5.27 (1 H, m), and 7.28 (10 H, d).

The phenyl groups were removed by catalytic hydrogenation, and the methyl ester was removed by heating for 12.5 h at 65 °C. The resulting product was purified on Dowex-1-Cl and isolated as the tricyclohexylammonium salt: NMR (D_2O) δ 4.79 (dd, J_{CHF} = 46.5 Hz, J_{CH-CH} = 2.8 Hz) and 5.0 (multiplet under H_2O peak and extending to either side).

Preparation of Phospho[^2H]glycolate. For the synthesis of diphenyl oxalate, phenol (56.4 g, 0.6 mol) and oxalyl chloride (25.5 mL, 0.3 mol) were placed in 1500 mL of anhydrous ether in a 21-mL round-bottom flask cooled in an ice bath. A solution of 114 mL of triethylamine (distilled from naphthyl isocyanate to remove primary and secondary amines) in 70 mL of anhydrous ether was then added dropwise to the above solution over a period of 1 h. A white solid fell from solution almost immediately after commencement of addition. The entire mixture was then refluxed for 3.5 h. The triethylamine hydrochloride and most of the product were filtered with a Büchner funnel. To the filter cake was added 100 mL of H_2O. The triethylamine hydrochloride dissolved, but the desired product did not. Then the desired product was filtered by suction and 72 g (75% yield) was isolated: mp 132–138 °C.

Diphenyl oxalate (14.4 g, 0.06 mol) was placed in 600 mL of acetone in a 1-L round-bottom flask, fitted with a dropping funnel. Potassium acetate (4.8 g, 0.03 mol) in 50 mL of H_2O was added dropwise over a period of 10 min. The reaction was allowed to stir for 12 h at room temperature.

Almost immediately after the addition of KOAc was complete, a white crystalline solid began to fall from solution. At the end of the 12-h period, a white solid was filtered by suction to give an 88% yield of potassium phenyl oxalate.

Phenyl oxalate (1.026 g, 0.005 mol) was placed in 10 mL of dimethylformamide with 50 mL of H_2O in a 50-mL round-bottom flask. To this solution was added $NaBH_4$ or $NaBD_4$ (0.089 g, 0.005 mol). The solution was then heated for 4 h at 70 °C and was allowed to stir overnight at room temperature. The solution was acidified to pH 4 with concentrated HCl to destroy excess $NaBH_4$. The solution was made basic by addition of $NaHCO_3$ and the phenol was extracted with ether. The bicarbonate layer was reacidified to pH 1.5 with HCl and was continuously extracted with ether for 24 h. The ether was dried over $MgSO_4$ and the solvent removed in vacuo to give 285 mg (80% yield) of glycolate and [$^2H_2$]glycolate: NMR (D_2O) (for $HOCH_2CO_2H$) δ 4.18 (s); for $HOCD_2CO_2H$, no NMR. Glycolate (2-H_2, 2-D_2) was then esterified with CH_2N_2 (Boer & Backer, 1963) to give a 95% yield of methyl glycolate: NMR ($CDCl_3$) (for $HOCH_2CO_2CH_3$) δ 3.78 (3 H, s), 4.20 (2 H, s), and 5.02 (broad peak); NMR (for $HOCD_2CO_2CH_3$) δ 3.78 (3 H, s) and 5.78 (broad peak).

Methyl glycolate was then phosphorylated by the anhydrous pyridine method described above to give an 80% yield of methyl (diphenylphospho)glycolate: NMR ($CDCl_3$) [for $CH_2[OP(OPh)_2]CO_2CH_3$] δ 3.72 (3 H, s), 4.73 (2 H, d, J_{POCH} = 11 Hz), and 7.28 (10 H, s); NMR [for $CD_2[OP(OPh)_2]CO_2CH_3$] δ 3.72 (3 H, s) and 7.275 (10 H, s). The phenyl groups were removed by catalytic hydrogenation to give the desired product: NMR (D_2O) [for $CH_2(OPO_3^{2-})CO_2CH_3$] δ 3.78 (3 H, s), 4.56 (2 H, d, J_{POCH} = 10 Hz); NMR (D_2O) [for $CD_2(OPO_3^{2-})CO_2CH_3$] δ 3.78 (3 H, s). The methyl ester was removed by saponification at room temperature by the procedure described by Hartman & Wold (1967): NMR (D_2O) [for $CH_2(OPO_3^{2-})CO_2^-$] δ 4.99 (d) and 1.00–2.00 (m); NMR [for $CD_2(OPO_3^{2-})CO_2^-$] δ 1.00–2.00 (br m); mp 162–164 °C dec. Both phospho[^{1}H]- and phospho[^{2}H]-glycolate were purified by Dowex-1-Cl chromatography with HCl elution as described above. This procedure removes P_i. The purity of these compounds was established by using electrophoresis and paper chromatography. Electrophoresis at pH 3.5 [H_2O–pyridine–HOAc, 760:4:40] and 3.0 kV for 13 min indicated that both the phospho[^{1}H]- and phospho-[^{2}H]glycolate migrated 8.5 cm from the origin. In this system, P_i migrated 7.3 cm from the origin and was not observed. Both ^{1}H and ^{2}H compounds comigrated on Whatman 3 MM paper chromatography in butanol–HOAc–H_2O (7:2:5) with an R_f value of 0.26.

Synthesis of Tartronic Acid Semialdehyde Phosphate (TSP) and Characterization. Synthesis of TSP was carried out by a modification of the procedure of Spring & Wold (1971a,b): NMR (D_2O) δ 4.45 (1 H, dd, J_{POCH} = 9.34 Hz, J_{CHCH} = 6.67 Hz), and 5.21 (1 H, d, J = 6.67 Hz). The NMR data of the final product were obtained by diluting the isolated TSP solution 50-fold and placing it on a 1 × 3 cm Dowex-1-Cl column. The column was washed with 10 mL of D_2O and eluted with 6 mL of 0.01 M imidazole–DCl, pD 7.5, and 0.5

M KCl. Fractions (1 mL) were collected, and most of the product eluted in tubes 2 and 3. From each tube, 50 μL of the contents was assayed to determine the phosphate content. The most concentrated samples contained 1×10^{-2} M TSP. The NMR was taken on Bruker 90-MHz FT spectrometer and indicated hydrated aldehyde.

The solution was then made basic with 0.1 N NaOD containing 10^{-3} M Mg^{2+}: NMR (D_2O) [for $^{-}OCH{=}C(OPO_3^{2-})CO_2^{-}$] δ 7.15 (d, J = 1.67 Hz).

Glycolaldehyde phosphate diethyl acetal was converted to the aldehyde by a procedure described by Calbiochem Corp. using Dowex-50-H^+. This reaction was run in D_2O and gave a product with the NMR spectrum δ 5.10 (1 H, t, J = 4.67 Hz) and 3.74 (2 H, dd, J_{POCH_2} = 7.34 Hz, J_{CHCH} = 4.67 Hz). This compound showed no aldehyde peak downfield.

Enzyme Assays. All assays were performed at 25 °C. The assay solution for yeast enolase typically contained 0.10 M Tris-HCl (pH 7.8), 5 mM $Mg(OAc)_2$, 0.01 mM EDTA, 0.4 M KCl, and varying concentrations of substrates and inhibitors, 10^{-4}–4×10^{-2} M. The assay solution for the muscle enzyme was the same except that the solution contained 0.1 M imidazole–HCl (pH 7.0) and 1 mM $Mg(OAc)_2$. In 1 mL of assay solution 0.005–0.1 mg of enolase was used. Initial velocities were obtained by measuring the increase in absorbance of the PGA analogue at 230 nm. The following extinction coefficients at 230 nm were determined in their corresponding assay mixtures: PEP, 3098 cm^{-1}, and PGA analogues, 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. K_i values were obtained by plotting $1/V$ vs. I and by Lineweaver–Burk plots and are reported in Table III.

Exchange Study with Substrate Analogues Which Do Not Undergo Elimination. A typical solution contained 8 IU yeast enolase, 4×10^{-2} M substrate, 0.1 M Tris-HCl (pH 7.5), 0.4 M KCl, 0.005 M $Mg(OAc)_2$, T_2O (sp act. = 2×10^5 cpm/μmol), and 0.5–1.0 mg of enolase. The reaction was allowed to proceed for 8–16 h and then stopped by heating the solution for 60 s at 100 °C. After removal of ^3H_2O by bulb to bulb distillation, the residue was redissolved in H_2O. The H_2O was again removed by bulb to bulb distillation. The residue was placed on a 1 × 14 cm Dowex-1-Cl column and washed until no more radioactive material eluted (~150 mL). The column was eluted with 0.015 N HCl (~60 mL) until the effluent was no longer radioactive. The substrate was eluted with 0.1 N HCl, and 2-mL fractions were collected. The compound was eluted in tubes 9–13.

The fractions were assayed as follows: (1) 0.5 mL of each fraction was used for determination of radioactivity in 10 mL of Bray's solution for 10 min and (2) 0.5 mL of each fraction was treated with alkaline phosphatase, followed by phosphate determination.

TSP reactions were carried out as described above for 2–4 h, except that all reactions were run at 0 °C. The reaction was stopped by addition of 100 μL of 1 M EDTA (pH 7.9) (since heat destroys TSP). Modification of the workup procedure was required due to the instability of TSP. PGA (3 mg) carrier was added to the reaction mixture followed by 20 μL of 1 M $NaBH_4$. The reaction was allowed to proceed for 15 min at ice-bath temperature. Acetone (20 μL) was added to destroy the excess $NaBH_4$. The solution was worked up as described above. In all cases, controls were used which contained TSP and no enzyme.

Exchange Studies with Chlorophospholactate. A typical assay mixture contained final concentrations of 2×10^{-2} M chlorophospholactate, 0.10 M Tris, pH 7.4, 0.4 M KCl, 0.01 mM EDTA, 1 mM $MgSO_4$, 20 mM ADP, 20 mM NADH, and T_2O (sp act. = 1.0×10^5 cpm/atom; 50 μg of pyruvate kinase and 50 μg of lactate dehydrogenase) in a final volume of 0.5 mL. The reaction was begun by addition of 0.50–1.0 mg of enolase and allowed to proceed at room temperature for 4–16 h. The reaction was stopped by heating the mixture 60 s at 110 °C. The solution was then lyophilized twice by bulb to bulb distillation and the residue redissolved in 20 mL of H_2O and placed on a 1 × 10 cm Dowex-1-formate column. The column was washed with H_2O until no more radioactive material emerged, and the products were then eluted with a linear gradient, 250 mL of H_2O, pH 6.4, and 250 mL of 1 M NH_4 formate, pH 7.4. Lactate was assayed by the procedure of Horn & Bruns (1956) and by counting 100 μL of each 4-mL fraction collected. PGA was assayed by two methods: (1) with yeast enolase and (2) with phosphomolybdate assay for inorganic phosphate after hydrolysis with alkaline phosphatase at pH 9.8. ADP was determined spectrophotometrically from the A_{260nm}. No radioactivity was found in PGA.

Table I: Kinetic Constants for Elimination Reactions Catalyzed by Yeast and Rabbit Muscle Enolase

$RCH(OPO_3^{2-})$-COO^-, R =	K_m (M)		V_{max} (min^{-1})	
	rabbit	yeast	rabbit	yeast
CH_2Cl[a,b]	2.0×10^{-3}	6.2×10^{-3}	2.0	5.7
CH_2Cl,[a] α-^2H	2.0×10^{-3}	6.2×10^{-3}	0.235	0.80
CH_2F[a]	6.7×10^{-4}	5.0×10^{-4}	8.6	12.85
CH_2OH	8×10^{-5}	1.0×10^{-4}	1200	3790

[a] All kinetic constants were determined on a racemic mixture of substrate. [b] All kinetic assays were performed under the following conditions for both yeast and muscle enolase: 0.1 M Tris (pH 7.8), 5 mM $MgSO_4$, 0.4 M KCl, and 0.01 mM EDTA where an increase in A at 230 nm was monitored. Reaction rates were also monitored by the coupled assay procedure of Tietz & Ochoa (1958) using 1.7 mM ADP, 0.15 mM NADH, 60 units of lactate dehydrogenase, and 46.5 units of rabbit muscle pyruvate kinase.

Results

Reaction of Chlorophospholactate and Fluorophospholactate with Enolase. When chlorophospholactate was added to either yeast or rabbit muscle enolase, pyruvate kinase, lactate dehydrogenase, and NADH (see Table I for reaction conditions), a decrease in the absorption of NADH was observed. No change in absorption was seen when either enolase or chlorophospholactate was omitted. This suggested that enolase catalyzes the elimination of HCl from chlorophospholactate to product PEP.[2] The following additional experiments were performed to confirm the conversion of chlorophospholactate to PEP. (1) The reaction was carried out on a 1-mL scale under standard assay conditions as described above, in the presence of pyruvate kinase. Pyruvate formed under these conditions was isolated as the 2,4-dinitrophenylhydrazone derivative (Seligson & Shapiro, 1952). The R_f of this derivative was the same as that of authentic pyruvate-2,4-dinitrophenylhydrazone on Whatman 3 MM paper chromatography with 1 N $NaHCO_3$, 1-butanol (2:1, R_f 0.28), and 1-butanol–ethanol–0.5 N NH_4OH (7:1:2, R_f 0.46). (2) A reaction mixture consisting of 40 mM chlorophospholactate under standard assay conditions was incubated with 0.10 mg of yeast enolase and was examined by FT NMR. A time-dependent loss of chlorophospholactate protons was seen accompanied by a concomitant increase in PEP vinyl protons.

Kinetic constants for chlorophospholactate and fluorophospholactate are summarized in Table I. For comparison, data for PGA are also given. Since the reaction rate of the

Table II: Determination of Secondary Isotope Effects for the Conversion of 2-Chlorophospho[3-$^2H_2$]lactate to PEP[a]

	secondary isotope effect	
	k_H/k_D	k_H/k_D (av)
yeast enzyme		
run 1	1.00, 1.06, 1.00, 1.07, 1.00	1.03
run 2	1.00, 1.05, 0.98, 1.03, 1.00	1.01
muscle enzyme	1.03, 0.95, 1.00, 1.11, 1.02	1.02

[a] Substrate concentration 4×10^{-2} M. Assay conditions were as described in Table I.

halophospholactate is low, one must consider the possibility that a contaminating enzyme is responsible for the activity observed with chlorophospholactate. The following considerations make this a remote possibility. (1) Both yeast and muscle enzyme, isolated by independent procedures, act on chlorophospholactate. (2) The experiments in Table I were repeated with homogeneous muscle enzyme which we isolated. The same results as with the commercial enzyme were obtained. (3) Chlorophospholactate was found to competitively inhibit the conversion of PGA to PEP with the yeast and muscle enzyme, with K_i values of 1.5×10^{-3} M and 5.0×10^{-3} M, respectively. These K_i values are close to the independently determined K_m values obtained from the dehydrohalogenation reaction (Table I).

We also examined the rate of loss of chloride from 3-chlorophospho[2-^{2}H]lactate to determine whether a primary isotope effect occurs. Kinetic parameters for 3-chlorophospho[2-^{2}H]lactate are given in Table I. Both the deuterated and nonisotopic compounds were prepared by identical procedures and purified by both ion-exchange chromatography and paper electrophoresis. The results in Table I show that a relatively large V_{max} deuterium isotope effect is observed; $k_H/k_D = 8.5$ for muscle enzyme and 7.3 for yeast enzyme. In both cases, the K_m values remained unchanged.

Since a large isotope effect occurs in dehydrohalogenation of 3-chlorophospholactate, one would not expect enzyme-catalyzed exchange of the substrate α H with solvent protons. An experiment was carried out to test this point. Enolase and 3-chlorophospholactate were allowed to react in [^{3}H]H_2O (2×10^5 cpm/μmol). The reaction was allowed to proceed until 40% of substrate had reacted. On reisolation of substrate, no 3-chlorophospho[α-^{3}H]lactate could be detected, i.e., the rate of exchange must be less than 1×10^{-4} min^{-1}. In this experiment, which also contained pyruvate kinase and lactate dehydrogenase as a trap, the reaction mixture was also analyzed for [α-^{3}H]PGA. This compound could have arisen through hydration of the enzyme-bound PEP, which is formed from 3-chlorophospholactate. No [^{3}H]PGA was detected, i.e., its rate of formation was less than 1×10^{-4} min^{-1}.

Secondary Isotope Effect. Experiments were also carried out to establish whether a secondary isotope effect is observed when the enzyme acts on 3-chlorophospho[3-$^2H_2$]lactate. The deuterated and nondeuterated compounds were synthesized and purified by the same procedure. A significant secondary isotope effect would be expected for the concerted process, but no isotope effect, or only a small effect, is expected for the E1cB mechanism. The results, summarized in Table II, show that no isotope effect occurs.

Exchange of the α Hydrogen of PGA Analogues Catalyzed by Yeast and Rabbit Muscle Enolase. A number of substrate analogues were incubated with enolase in [^{3}H]H_2O and ^{3}H incorporation was determined. The results are summarized in Table III. Enolase-catalyzed ^{3}H exchange was observed

Table III: ^{3}H Exchange Reactions Catalyzed by Yeast and Muscle Enolase Substrate

$RCH(OPO_3^{2-})COO^-$, R =	K_i or K_m	metal	K for exchange (min^{-1})
	Yeast Enolase[a]		
H	7.1×10^{-4} [f]	Mg^{2+}	$<1 \times 10^{-4}$
H	2×10^{-4} [b]	Mn^{2+}	7.5×10^{-4}
CH_3	3.95×10^{-4} [b]	Mg^{2+}	$<1 \times 10^{-4}$
CH_3CH_2	3.4×10^{-4}	Mg^{2+}	$<1 \times 10^{-4}$
CHO	1.4×10^{-5} [c]	Mg^{2+}	(0.0475)[d]
		Mn^{2+}	(0.26)[d]
CH_2OH	1.0×10^{-4} [c]	Mg^{2+}	385
	6.7×10^{-6} [b]	Mn^{2+}	
	Muscle Enolase[a]		
H		Mg^{2+}	3.2×10^{-4}
H	7.5×10^{-5} (30 °C) [b]	Mn^{2+}	1.9×10^{-3}
CH_3	3.5×10^{-4} [e]	Mg^{2+}	$<1 \times 10^{-4}$
CH_3CH_2	7.3×10^{-3}	Mg^{2+}	$<1 \times 10^{-4}$
CHO	1.2×10^{-5}	Mg^{2+}	(0.062)[d]
CH_2OH	8.0×10^{-5} [c]	Mg^{2+}	175
	1.6×10^{-5} [b]	Mn^{2+}	

[a] Reaction conditions for exchange studies with both yeast and muscle enolase: 0.04 M analogue, 0.1 M Tris (pH 7.5), 5 mM $Mg(OAc)_2$ or 0.5 mM $MnCl_2$, 0.01 mM EDTA, 0.5–1.0 mg of enolase, ^3H_2O (sp act. = 2×10^5 cpm/μmol); T = 25 °C. Reactions with TSP were carried out at 4 °C, since this compound is unstable at 25 °C. Rates were extrapolated to 25 °C. [b] Nowak & Mildvan, 1970. [c] Hartman & Wold, 1967. [d] All exchange studies were carried out at 4 °C, but values reported are extrapolated to 25 °C by studying rate variation with temperature for PGA. [e] Wold & Barker, 1964. [f] Cardenas & Wold, 1968.

with phosphoglycolate and tartronic acid semialdehyde phosphate. Exchange reactions with other substrate analogues, if they occur, are below the limits of our detection. In all cases where exchange occurs, the rate of exchange was more rapid in the presence of Mn^{2+} than in the presence of Mg^{2+}.

All of the exchange studies reported in Table III were done with commercially available rabbit muscle and yeast enolase. Because the exchange rate with phosphoglycolate was very slow, the possibility that exchange is due to a contaminating enzyme must be considered. We, therefore, repeated the exchange studies with rabbit muscle enolase which we isolated and purified to homogeneity by the procedure of Westhead (1966). The specific activity of this muscle enzyme was 86 IU, and the protein was homogeneous by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970). Exchange studies carried out with this enzyme preparation and phosphoglycolate gave results identical with those reported in Table III.

The effect of pH on the rate of exchange of tritium into the phosphoglycolate was also investigated. With yeast enolase, no exchange (exchange rate $< 1 \times 10^{-4}$ min^{-1}) was observed at pH 6.9, 7.5, and 9.2. With the rabbit muscle enzyme, the exchange in the presence of Mg^{2+} was 1.1×10^{-4} min^{-1} at pH 6.5 (0.1 M imidazole buffer) and 4.0×10^{-4} at pH 8.5 (0.1 M Tris buffer). The rate of exchange with yeast enzyme in the presence of Mn^{2+} was unaffected by buffer concentration. The concentration of the imidazole buffer was varied from 0.005 to 0.15 M at pH 7.15.

Isotope Effect on the α-Proton Exchange. The rate of exchange of the α proton of PGA catalyzed by muscle enolase shows a deuterium isotope effect of 6. We, therefore, examined the deuterium isotope effect on the exchange rate of the α proton of phosphoglycolate. Phospho[α-$^2H_2$]glycolate was incubated with muscle enolase in [^{3}H]H_2O under the conditions of Table III. The rate of ^{3}H incorporation from the solvent was unaffected by deuterium substitution.

Characterization of TSP. NMR spectroscopy showed that within experimental detection, TSP exists in solution entirely

as the hydrated species $CH(OH)_2CH(OPO_3^{2-})CO_2^-$, i.e., no aldehydic proton is detectable. Adjustment of the pH to 12.0 with NaOD in the presence of 5 mM Mg^{2+} results in generation of a new NMR spectrum consistent with the enolate formation of TSP [$^-OCH{=}C(OPO_3^{2-})CO_2^-$]. The NMR spectrum at high pH supports the enolate structural assignment (Hartman & Wold, 1967; Spring & Wold, 1971a,b) based on UV spectroscopy.

Our NMR observations raise the question as to whether hydrated TSP or the aldehydic form is bound by enolase. If the aldehydic species is the inhibitor, then the K_i for TSP has been underestimated at least 20-fold.

Design of Potential Enolase Inactivators. Enzymes which can catalyze proton abstractions are particularly suitable targets for suicide inactivators (Maycock & Abeles, 1976). We therefore synthesized a number of PGA analogues [$RCH(OPO_3H^-)COO^-$] which might act as suicide inactivators for enolase. The compounds synthesized were 2-phospho-3-butynoate (R = CH≡C–), 2-phospho-3-chloro-3-butenoate (R = CH=CCl–), and 2-phospho-3,3,3-trifluoropropionate (R = CF_3–).[3] While both R = HC≡C– and R = CH_2=CCl– were competitive inhibitors of yeast enolase (K_i = 3.3×10^{-3} M and 1.1×10^{-2} M, respectively), no time-dependent inactivation of enolase by these analogues was observed. Furthermore, if allene, $H_2C{=}C{=}C(OPO_3)CO_2$, was generated by enolase and dissociated from the active site, one might be able to detect it by UV or NMR spectroscopy. No allene was detectable in either case. Finally, R = CF_3 does not appear to bind to yeast or muscle enolase at concentrations as high as 10^{-2} M.

Discussion

The dehydration of PGA, catalyzed by enolase, proceeds through a carbanion mechanism, i.e., the abstraction of the α hydrogen as a proton occurs independently of the departure of the β-OH group (Dinovo & Boyer, 1971). Nevertheless, as the experiments presented here show, the β-OH group makes an important contribution to the abstraction of the α proton. V_{max} for HCl elimination from 3-chlorophospholactate (muscle enzyme) is 6×10^2 times slower than V_{max} for H_2O elimination from PGA, although Cl^- is a much better leaving group than OH^-. The difference in V_{max} actually underestimates the effect of replacing OH by Cl on the rate of C–H bond dissociation, since dissociation of the C–H bond is rate determining for elimination from 2-chlorophospholactate (V_{max} deuterium isotope effect) but not from PGA. Consequently, the rate of dissociation of the α-C–H bond is reduced 10^3–10^4-fold by replacement of OH by Cl.

The lower rate of α-proton abstraction from 3-chlorophospholactate as compared to PGA could be due to a difference in mechanism. Elimination of HCl could be a concerted process, whereas elimination of H_2O from PGA proceeds by a carbanion mechanism. However, our data are inconsistent with a concerted mechanism and suggest a carbanion mechanism for HCl elimination from PGA. For a concerted mechanism, where the β carbon changes hybridization from sp^3 to sp^2 in the transition state, a secondary isotope effect is expected. A theoretical maximum of 35% per deuterium atom has been calculated (Streitwieser et al., 1958). However, in practice, isotope effects ranging from 0 to 18% (Smith & Bourns, 1974; Cook, et al., 1974, and references cited therein; Thomas & Stirling, 1977) have been seen. The low secondary isotope effect observed for the conversion of 3-chlorophospho[3-$^2H_2$]lactate to PEP suggests that the β carbon has not acquired significant sp^2 character in the transition state.

Further evidence against the concerted mechanism comes from our studies of the leaving group effect, F^- vs. Cl^-. If carbon–halogen bond cleavage were to occur in the transition state, one would expect the elimination of F^- to proceed at a slower rate than the elimination of Cl^- since the carbon–fluorine bond is stronger than the carbon–chlorine bond (Saunders & Cockerill, 1973). For instance, in the phenylethyl halide system ($Ph–CH_2–CH_2X$), in which the elimination of HX is thought to proceed through a concerted process, F^- is eliminated 70-fold slower than Cl^-. In fact, in all cases of concerted elimination reactions, recently reviewed (Saunders, 1976), F^- leaves 70–10^3 times more slowly than Cl^-. We have found that the conversion of 3-fluorophospholactate to PEP proceeds somewhat faster than that of 3-chlorophospholactate. A rate acceleration by F might be expected for a carbanion mechanism since F would enhance the acidity of the α hydrogen more than would Cl. Thus, the leaving group effect as well as the secondary isotope effect suggests an E1cB mechanism for the dehydrohalogenation reaction.

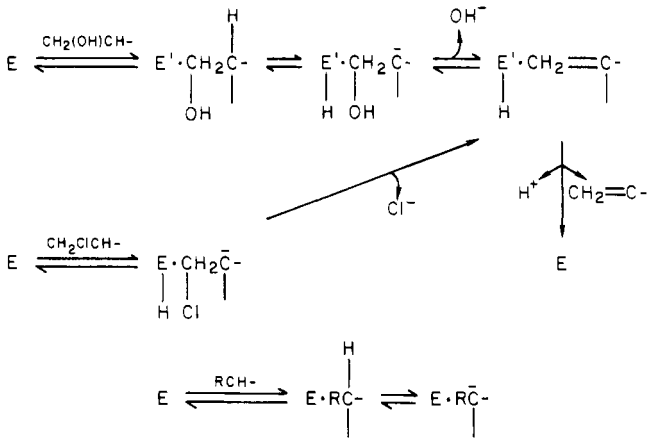
The slower rate of reaction of 2-chlorophospholactate cannot be due to steric effects since the covalent radius of Cl is nearly equal to that of OH and F is considerably smaller. The rate difference can also not be attributed to inductive effects, since F will enhance the acidity of the α hydrogen more than will OH. Therefore, some specific interaction of the OH group with the active site, possibly hydrogen bond formation or complexation with the metal (Nowak & Mildvan, 1970), must greatly facilitate the abstraction of the substrate α hydrogen. The energy of this interaction could be used for the precise alignment of the substrate at the active site to bring about substrate distortion or an enzyme conformational change. One might speculate that the active site of the enzyme is designed to bind substrates where the β carbon and possibly also the α carbon have considerable sp^2 character. The binding energy of the β-OH group is required (Jencks, 1975) to obtain proper alignment of substrates which do not have a β-sp^2 carbon. The requirement of a β-sp^2 carbon for favorable interaction with the active site could be the basis for the tight binding (K_i = 1.2×10^{-5}) of tartronic acid semialdehyde phosphate (I), i.e., tartronic acid semialdehyde phosphate would then be a transition state analogue (Spring & Wold, 1971a,b).

The effect of the β-OH group of PGA on the rate of cleavage of the α-C–H bond was also examined by measuring the rate of exchange with solvent tritons of the α hydrogen of PGA analogues, which do not have a leaving group in the β position. The result (Table III) shows that the effect of the β-OH group on the rate of exchange of the α hydrogen is at least as large as on the rate of carbon–hydrogen bond dissociation, measured by the elimination reaction. The rate of ^3H exchange from ^3H_2O into the α position of PGA catalyzed by the muscle enzyme (Mg^{2+}) is 5×10^5 times faster than ^3H exchange into phosphoglycolate. No ^3H exchange was detected into phosphoglycolate with yeast enolase (Mg^{2+}), nor was any exchange detected with either enzyme with other substrate analogues (CH_3– and CH_3CH_2–). The rates of these latter exchange reactions must, therefore, be at least 10^6-fold slower than the exchange of ^3H into PGA.

The rate constant for the exchange of the α proton of phosphoglycolate is a lower limit for the abstraction of the α hydrogen, since the exchange of the α hydrogen of phosphoglycolate with solvent tritons does not show a deuterium isotope

[3] We would be happy to supply detailed synthetic procedures upon request.

Scheme I: Enzyme Conformational Changes during Catalysis[a]



[a] $CH_2(OH)CH$-, PGA; CH_2ClCH-, chlorophosphate; RCH- where R = H-, CH_3-, or CH_3CH_2-.

effect. This is in contrast to the reaction with the normal substrate, PGA, where the exchange of the substrate α hydrogen with solvent protons shows a deuterium isotope effect of 6 (Dinovo & Boyer, 1971). Thus, the rate-determining step for α-hydrogen exchange is different for PGA and phosphoglycolate. Presumably, although this was not experimentally determined, the other substrate analogues which do not have a β leaving group fall in the same category as phosphoglycolate. It is likely that with the PGA analogues (R = H, CH_3, CH_2CH_3) a rate-determining protein conformational change is required for the enzyme-bound proton exchange with solvent protons. If this interpretation is accepted, it follows that a conformational change induced by the β-OH group of PGA not only affects the rate of abstraction of the α proton but also the rate of exchange of the abstracted proton with solvent.

The conclusion, reached above, that the OH group is required to facilitate release of the abstracted α proton from the enzyme appears inconsistent with the results obtained from our studies of the elimination of HCl from chlorophospholactate, a substrate which does not have a β-OH group. In that reaction, dissociation of the C–H bond, but not proton release from the enzyme, is rate determining. In Scheme I, we present a model which eliminates this contradiction. According to this model, there are two forms of the enzyme, E and E′. Conversion of E to E′ is facilitated by PGA and PEP. Exchange of the enzyme-bound proton from E′ is rapid and slow from E. PGA analogues which do not have an OH group in the β position always react with the E form of the enzyme from which hydrogen release is slow. Elimination of HCl from 3-chlorophospholactate occurs on the E form of the enzyme. This elimination leads to PEP which then facilitates conversion of E → E′. The proton can then be released rapidly from E′ so that proton release does not limit the rate of elimination of HCl from 3-chlorophospholactate.

A conformational change subsequent to substrate binding was also proposed by Lane & Hurst (1974). These authors investigated the kinetics of the interaction of PEP and PEP analogues with yeast enolase. A fast unimolecular step was spectroscopically detected. This step was attributed to substrate distortion subsequent to the formation of an enzyme–substrate complex.

Tartronic acid semialdehyde falls in a different category than the PGA analogues discussed above. The α hydrogen is considerably more acidic and it has an oxygen substituted in the β position which may interact with the enzyme analogously to the –OH group of PGA, and, if it is bound as the enolate, it resembles PEP. In terms of Scheme I, it is very likely that tartronic acid semialdehyde binds to E′. The rate of exchange of the α proton is considerably faster than that of other PGA analogues (0.1 min^{-1}). Available data suggest that the rate of the exchange reaction is not limited by the rate of α-proton abstraction. The kinetics of the reaction of tartronic acid semialdehyde with yeast enolase have been measured (Lane & Hurst, 1974). The first-order rate constants for the reaction (eq 2) are k_2 = 11.4 min^{-1} and k_{-2} = 0.6 min^{-1}.

$$E \underset{k_{-1}}{\overset{k_1(TSP)}{\rightleftharpoons}} E{\cdot}TSP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}TSP(enolate) \qquad (2)$$

The rate constants for the on and off rate (k_1 and k_{-1}) are very fast. Since the rate constant for the exchange reaction which we have determined is 0.1 min^{-1}, it is very likely that k_{-2} is rate determining for the exchange reaction.

The requirement of an –OH substituent in the β position of the substrate for α-proton abstraction probably also contributes to the failure of the putative suicide inactivators. The action of these inactivators is based on the abstraction, by the enzyme, of a proton from the α position of the inactivator. In the absence of a β-OH substituent, this process would be too slow for significant inactivation to occur during the course of the experiment.

The data presented here show that the interaction of the β-OH group of PGA with the active site has a substantial effect on the rate of abstraction of the α hydrogen as well as the rate of release of the protein-bound proton after abstraction. The question then arises whether this is generally the case for H_2O eliminations involving the cleavage of a relatively nonacidic carbon–hydrogen bond. Unfortunately, little information bearing on this subject is available. For instance, it is not known whether fumarase catalyzes the elimination of HCl from chlorosuccinate or the exchange of succinate protons. The rate of hydrogen exchange in the absence of a leaving group has been studied with *S*-adenosylhomocysteinase (Palmer & Abeles, 1979). This enzyme catalyzes the oxidation of the 3′ position of adenosine to a carbonyl group and subsequently the elimination of H_2O from the 4′,5′ position. It also catalyzes the exchange of the 4′ hydrogen with solvent protons more rapidly than the elimination of H_2O. When the enzyme acts on 5′-deoxyadenosine, exchange of the C-4′ hydrogen occurs 1.2×10^3 times slower than the exchange with adenosine. Evidence was presented which indicated that the 5′-OH group of adenosine plays an important role in properly aligning the substrate at the active site.

Some information concerning the effect of the β-OH group on α-H cleavage is available for H_2O elimination reactions involving pyridoxal phosphate enzymes. For instance, γ-cystathionase catalyzes H_2O elimination from serine, HCl elimination from chloroalanine, and the exchange of the α hydrogen of alanine with solvent protons. The rate of HCl elimination from β-chloroalanine is ~250 times faster than the conversion of serine to pyruvate.[4] The rate of exchange of the α proton of alanine with solvent deuterons proceeds at 25% of the rate of the conversion of serine to pyruvate (Washtein et al., 1977). Rat liver serine dehydratase catalyzes elimination of HCl from β-chloroalanine at nearly the same rate as the elimination of H_2O from serine.[5] Tryptophan synthase ($\alpha_2\beta_2$) catalyzes the conversion of β-chloroalanine and serine to pyruvate. The rate of pyruvate formation from

[4] C. Fearon and R. H. Abeles, unpublished experiments.
[5] E. Borcsok and R. H. Abeles, unpublished experiments.

serine is fourfold faster than from chloroalanine (it is not certain that these rates represent V_{max}) (Kumagai & Miles, 1971). It is clear then that at least for some pyridoxal phosphate reactions, the β-OH group does not play a major role in the abstraction of the α hydrogen. One might speculate that elimination reactions involving relatively nonacidic hydrogen, such as the α hydrogen of PGA, require a much more precise alignment of the substrate at the active site than elimination reactions from substrates with more acidic hydrogens (pyridoxal phosphate dependent reactions). For the more demanding reactions, the additional energy provided by the interaction of the OH group with the active site is required.

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