Origin of the Slow-Binding Inhibition of Aldolase by D-*glycero*-Tetrulose 1-Phosphate (D-Erythrulose 1-Phosphate) from the Comparison with the Isosteric Phosphonate Analog

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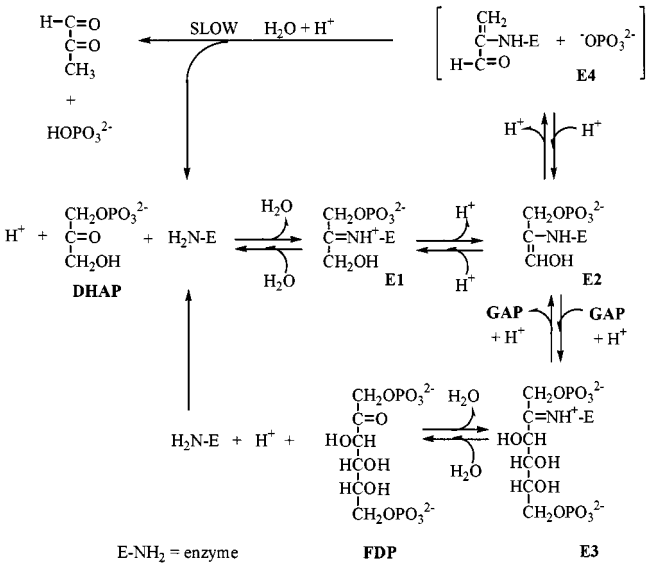
The mechanistic reaction pathway for the slow-binding inhibition of rabbit muscle aldolase by D-*glycero*-tetrulose 1-phosphate (D-erythrulose 1-phosphate) was investigated through the use of its phosphonomethyl isoster **4** which was synthesized for this study. The latter is not a substrate nor a slow-binding inhibitor but interferes in the enzyme-catalyzed reaction with the substrate fructose 1,6-diphosphate in a competitive manner. It was found that phosphonate **4** forms an iminium ion with aldolase and undergoes subsequent α-proton abstraction to form an enamine intermediate. We show from these results that enzyme slow-binding inhibition by D-erythrulose 1-phosphate is consistent with a phosphate β-elimination reaction through the enamine intermediate. This mechanism takes into account the stereochemical features known for aldolase, the parallel between enzyme activity recovery and phosphate release after action of D-erythrulose 1-phosphate, and also the same reaction from dihydroxyacetone phosphate.

Fructose 1,6-diphosphate aldolases reversibly cleave the fructose 1,6-diphosphate (FDP) into two triose phosphates: dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). There are two distinct classes of aldolases: Class I aldolases proceed by Schiff base formation with the substrate, while class II require a metal ion as cofactor and do not form a Schiff base.[1] The ordered multistep reaction catalyzed by class I aldolases, typified by rabbit muscle aldolase, shown in Scheme 1 (**E1**: iminium ion or Schiff base; **E2**: enamine; **E3**: iminium ion), is supported by several experimental corroborations.[2] There is also some evidence that the enzyme rate is limited by the release of products in both directions.[3] Additionally, in the absence of GAP, the unusual reversible covalent complex, enamine-aldehyde plus phosphate intermediate **E4**, is made possible by the slow aldolase-catalyzed conversion of DHAP into methylglyoxal and orthophosphate.[4] However, the concentration of this intermediate could not exceed 6% of the total amount of DHAP bound to aldolase.[2d]

Other data suggest that class I aldolases prefer the (*S*) configuration at C-3 for their substrates.[5] Accordingly, D-*glycero*-tetrulose 1-phosphate (D-erythrulose 1-phosphate), having the (*R*) configuration at this carbon atom, is not a substrate but interestingly leads to a time-dependent inhibition (slow-binding)[6] of aldolase by formation of a stable enzyme−ligand intermediate,[7] whereas the L derivative is a substrate.[5c,7] We report the comparative mechanism/inhibition studies of aldolase from rabbit muscle by D-erythrulose 1-phosphate and phosphonate isoster **4** (Scheme 2); the stabilization of an intermediate analogous to **E4**, for the phosphate derivative, is consistent with the observed inhibition. This result is of interest for both a better knowledge of the class I aldolases nontypical mechanistic pathway, and the design of a new class of inhibitors for these enzymes.[8]



Scheme 1. Reaction catalyzed by aldolase and mechanistic pathway

Results and Discussion

When aldolase (35 μM subunits) is incubated in the presence of D-erythrulose 1-phosphate monosodium salt (16 mM) in D_2O (pD = 7.2), the reaction monitored by ^{31}P-NMR spectroscopy indicates the slow formation of inorganic phosphate from the inhibitor catalyzed by the enzyme with a turn-over number $k = 0.0022$ min^{-1}.[9] In control, where β-glycerophosphate aldolase (an active-site modified enzyme, see Experimental Section) was used instead of the

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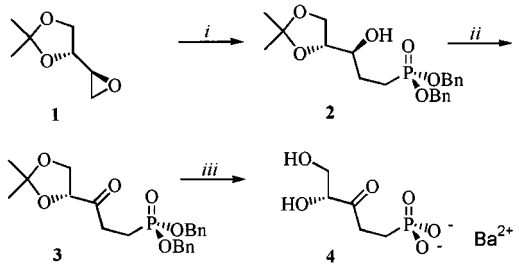
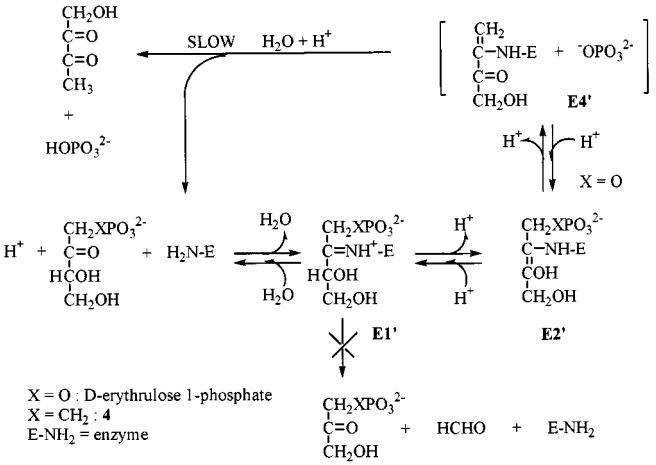
native enzyme, no inorganic phosphate formation was observed; this suggests that the reaction does occur at the enzyme active site. The value of the turn-over number for aldolase-catalyzed conversion of D-erythrulose 1-phosphate to inorganic phosphate, is the same within the error of these experiments as the the rate constant of 0.0041 min^{-1}[7] for the slow recovery of enzyme activity from an enzyme-inhibitor complex.



Scheme 2. Synthetic scheme for the synthesis of phosphonate **4**: (i) $(BnO)_2P(O)CH_3$, BuLi, $Et_2O{\cdot}BF_3$, THF, −70°C, 63%; (ii) DCC, pyridine/DMSO, CF_3CO_2H, benzene, 84%; (iii) a) H_2, Pd/C, $MeOH/H_2O$, b) $Ba(OH)_2$, 90%

To gain additional insight into the inhibition mechanism, phosphonomethyl analog **4** of D-erythrulose 1-phosphate was synthesized (Scheme 2) starting from (2*R*,3*S*)-3,4-epoxy-1,2-*O*-isopropylidenebutane-1,2-diol (**1**).[10] The ring opening of the starting epoxide by dibenzylmethanephosphonate anion catalyzed by $BF_3{\cdot}OEt_2$[11] afforded dibenzyl (3*S*,4*R*)-3,4,5-trihydroxy-4,5-*O*-isopropylidenepentyl phosphonate **2**. Oxidation of the latter compound according to the Pfitzner-Moffat method[12] yielded dibenzyl (4*R*)-4,5-dihydroxy-4,5-*O*-isopropylidene-3-oxopentyl phosphonate (**3**). Removal of the benzyl protecting group by catalytic hydrogenation concomitant with ketal hydrolysis, provided (4*R*)-4,5-dihydoxy-3-oxopentylphosphonic acid (**4**), isolated as barium salt.

Phosphonate **4** (monosodium salt), is not a time-dependent inhibitor nor a substrate for aldolase but inhibits the enzyme-catalyzed reaction competitively with a K_i value of 110 μM. The Schiff base intermediate **E1′** (Scheme 3; X = CH_2) was evidenced by sodium tetrahydroborate treatment[2a−2c,13] of the aldolase−**4** complex leading to a significant level of irreversible inactivation of the enzyme.[14] The reaction performed with the parent phosphate indicates that this latter compound also forms a reductible intermediate with the enzyme.[14] Aldolase catalyzes the exchange of the (*pro-S*) hydrogen atom at C-3 of DHAP with solvent water through enamine intermediate **E2** (Scheme 1).[2e−2h] The enzyme (1 mg mL^{-1}) was able to catalyze the H/D exchange in D_2O (pD = 7.2) of the hydrogen atom at the corresponding carbon atom of **4** (16 mM); indeed this hydrogen atom occupies the equivalent position of the (*pro-S*) hydrogen atom in the substrate, the reaction being followed by ^{1}H-NMR spectroscopy (CHOH peak; δ = 4.43).[15] The observed exchange rate value v = 0.144 mmol·min^{-1}·$unit^{-1}$ is close to that determined with the phosphonomethyl analog of DHAP (v = 0.18 mmol·min^{-1}·$unit^{-1}$).[13] To provide additional evidence for the formation of enamine **E2′** (Scheme 3; X = CH_2), this intermediate was also characterized by oxidation by hexacyanoferrate(III),[2j−2l] dihydroxy-2,3-butadione phosphate being expected as an oxidation product. The initial rates of oxidation were of zero-order in hexacyanoferrate(III) and depended on the concentration of **4** in accordance with Michaelis-Menten kinetics. The values of k'_{cat} and K'_m for this reaction were determined by fitting the kinetic data to the Michaelis-Menten equation using a nonlinear regression program and were found to be 37 min^{-1} and 87 μM, respectively.[16] The latter correlates with the K_i value determined above. Thus, phosphonate **4** has a dissociation constant value similar to those of DHAP (60 μM[13]) and D-erythrulose 1-phosphate (75 μM[7]) with aldolase, within fast equilibria.



Scheme 3. Proposed mechanism for aldolase inhibition by D-erythrulose 1-phosphate and **4**

These results account for the fast formation of intermediates **E1′** and **E2′** (Scheme 3; X = CH_2) catalyzed by aldolase with the phosphonate **4** and without observable decomposition by retro-aldolization from intermediate **E1′**. As it can be argued that D-erythrulose 1-phosphate reacts in a similar manner to its phosphonate isoster, these resulting intermediates **E1′** and **E2′** (Scheme 3; X = O) have also to be in fast equilibrium with free enzyme and D-erythrulose 1-phosphate; therefore, both intermediates cannot be directly associated with the slow-binding inhibition process of the enzyme. On the other hand, the different inhibition patterns observed between these two compounds are consistent with the possibility for the parent phosphate to lead to a stabilized intermediate enamine-hydroxy ketone plus phosphate **E4′**, analogous to **E4**, formed from the enamine intermediate **E2′** (Scheme 3; X= O) with a limiting first-order rate constant of 0.28 min^{-1} previously determined.[7] This finding is reminiscent of the nonenzymatic phosphate β-elimination reaction of DHAP through an enediolate intermediate, similar in structure to intermediate **E2** (Scheme 1).[17] Subsequently, intermediate **E4′** undergoes the observed slow release of inorganic phosphate concomitant with the recovery of the enzyme activity (k = 0.0041 min^{-1}), while the phosphonate isoster cannot do so. This parallel, and also the fact that the inhibition can be reversed, indicates that the covalent adduct present in **E4′** is hydrolyzed and

suggests that inorganic phosphate release and enzyme activity recovery are both under the control of slow conformational change of the enzyme. It turns out that the stereochemistry at C-3 controls two different mechanistic pathways with the substrate erythrulose 1-phosphate: (i) with the D derivative, the formation of an enamine-hydroxy ketone thermodynamically more stabilized by extra hydrogen bonds into the enzyme active site than the equivalent aldehyde **E4** formed from DHAP (no more than 6% of the total DHAP bound to the enzyme),[2d] can account for the observed inhibition; (ii) as indicated above, the L derivative is substrate.[5c,7] In other words: (i) the time-dependent inhibition of aldolase by D-erythrulose 1-phosphate is consistent with the slow transformation of the resulting enamine into the new complex **E4′** (Scheme 3; X = O); (ii) in the absence of GAP, the formation of complex **E4′** is quantitative from **E2′** whereas this process occurs to a low extent from DHAP-enamine intermediate **E2**; (iii) the decomposition of this complex **E4′** leads to a second time-dependent process, the recovery of the enzyme activity, which parallels the slow inorganic phosphate release rate. As a conclusion, D-erythrulose 1-phosphate can also be regarded as a slow alternative substrate for the minor and nontypical methylglyoxal synthase activity of aldolase.

Experimental Section

General: FDP, NADH, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, rabbit muscle aldolase were purchased from Boerhinger Mannheim, DHAP (lithium salt) from Sigma. Aldolase (10 U/mg) was dialyzed overnight against the appropriate buffer prior to use. For isotope exchange and inorganic phosphate formation studies aldolase was freed of triose-phosphate isomerase activity as previously described.[18] β-Glycerophosphate aldolase derivative was obtained by sodium tetrahydroborate treatment of a solution of aldolase and DHAP as previously described.[19] The enzymatic activity of β-glycerophosphate aldolase was 1% of the initial value. L-Erythrulose 1-phosphate was obtained by aldol reaction beetween DHAP (formed in situ) and formaldehyde catalyzed by rabbit muscle FDP aldolase.[20] Chemicals and solvents were reagent grade. − The NMR spectra were recorded in $CDCl_3$ or D_2O with a Bruker AC80 (80 MHz ^{1}H NMR), a Bruker AC200 (200 MHz ^{1}H NMR, 50 MHz ^{13}C NMR and 81 MHz ^{31}P NMR) or a Bruker ARX400 (400 MHz ^{1}H NMR and 162 MHz ^{31}P NMR) spectrometer. All chemical shifts are reported in ppm with respect to TMS for ^{1}H- and ^{13}C-NMR spectra and H_3PO_4 for ^{31}P-NMR spectra as internal standards. − Elementary analyses were performed by the Ecole Nationale de Chimie de Toulouse, France.

Activity Assays: Aldolase activity was measured using a coupled assay system by monitoring NADH oxidation at 340 nm, with detection by a Perkin−Elmer Lambda 2 spectrophotometer thermostated at 25°C.[21] Assays were initiated by the addition of substrate (FDP, 1 mM final concentration) to a final volume of 1 mL of solution containing aldolase made up in triethanolamine buffer (100 mM triethanolamine/HCl, pH = 7.6, 50 mM NaCl, 1 mM EDTA), 0.42 mM NADH and coupling enzymes (10 μg/mL glycerol-3-phosphate dehydrogenase; 1 μg/mL triose-phosphate isomerase). The aldolase concentration was determined spectrophotometrically from $\varepsilon_{280} = 0.91$ mL mg^{-1} cm^{-1}.[22] Inhibition constant value (K_i) for **4** was determined on the basis of double-reciprocal plots; initial rates were measured at different substrate concentrations (10, 20, 40 and 100 μM FDP) with four different inhibitor concentrations (50, 100, 200 and 300 μM); aldolase concentration was 50 nM in subunits.

Orthophosphate Formation from D-Erythrulose 1-Phosphate Catalyzed by Aldolase: The inorganic phosphate formation rate in D_2O catalyzed by aldolase was followed by ^{31}P-NMR spectroscopy. In a typical run, aldolase (0.70 mg) was added to D-erythrulose 1-phosphate (16 mM) in D_2O (0.5 mL final volume) pre-equilibrated in the spectrometer probe (25°C). The pD = 7.2 of the solution, adjusted using small quantities of concentrated NaOD or DCl solutions, was measured before and after each kinetic run. At intervals of 24 h after the addition, ^{31}P-NMR spectra were taken. The series of spectra shows the progress, consistent with a zero-order kinetic, of D-erythrulose 1-phosphate peaks (δ = 2.43 and 3.22, hydrate and ketone forms, 30% and 70%, respectively) which are diminished in size with respect to the increase of inorganic phosphate peak (δ = 0.9). Inorganic phosphate was evidenced by ^{31}P-NMR spectroscopy by adding Na_2HPO_4 to the solution under study. In controls where the enzyme was omitted or replaced by β-glycerophosphate aldolase derivative, or L-erythrulose 1-phosphate used instead of the D isomer, no inorganic phosphate formation was detected.

Reduction by Sodium Tetrahydroborate: Sodium tetrahydroborate treatments of the enzyme-inhibitor (or substrate) complex were performed according to a previously described technique.[13][23]

Isotope Exchange and Rate Measurements: The deuteration rate of **4** in D_2O catalyzed by aldolase was monitored by ^{1}H-NMR spectroscopy (CHOH peak; δ = 4.43), as described previously.[13][15] In a typical run, solid aldolase (0.5 mg) was added to **4** (16 mM) in D_2O (0.5 mL) pre-equilibrated in the spectrometer probe. No additional buffer was used. The pD = 7.2 of the solution, adjusted using small quantities of concentrated NaOD or DCl solutions, was measured before and after each kinetic run. At timed intervals after the addition, 64 scans (lasting about 1.5 min) were taken and transformed separately. In a control run without enzyme, no deuteration was observed.

Carbanion Assay: The carbanion (or enamine) can be oxidized by a variety of oxidants, for example, hexacyanoferrate(III). The rate of hexacyanoferrate(III) reduction was monitored by the decrease in A_{420} difference between sample and reference cuvettes as described previously.[2k,13] In a typical run, both cuvettes (final volume 1 mL) contained 1 mM hexacyanoferrate(III) in triethanolamine buffer (0.1 M, 50 mM NaCl, pH = 7.6) and 0.03 mg of aldolase (0.19 μM); in addition, the sample cuvette contained **4** (10−400 μM). After 5 min of preincubation, the enzyme was added to start the reaction and the decrease in absorbance was measured within the first 60 s at 25°C. The molar activity (k'_{cat}) of aldolase for oxidation of **4** by hexacyanoferrate(III) and the substrate concentration at which the initial rate of hexacyanoferrate(III) reduction is half-maximal (K'_m, which is a measure of aldolase affinity for **4**), were determined by fitting the data to the Michaelis-Menten equation.

Dibenzyl (3*S*,4*R*)-(3,4,5-Trihydroxy-4,5-*O*-isopropylidenepentyl)phosphonate (2): (2*R*,3*S*)-3,4-Epoxy-1,2-*O*-isopropylidenebutane-1,2-diol (**1**) was synthesized as previously described.[10] A 1.6 M solution of *n*BuLi in hexane (7.3 mL, 11.6 mmol) was added dropwise to a stirred solution of dibenzylmethylphosphonate (3.2 g, 11.6 mmol) in dry THF (60 mL) at −80°C under nitrogen. After 30 min of stirring, the mixture was added dropwise to a stirred solution of **1** (0.56 g, 3.89 mmol) in dry THF (40 mL) at −80°C. After 15 min of stirring, $BF_3{\cdot}Et_2O$ (1.4 mL, 11.6 mmol) was slowly

introduced. The reaction mixture was stirred for 2 h at −70 °C, then overnight at room temperature. Saturated NH_4Cl solution (20 mL) was added, the solvents were removed under reduced pressure and the remaining residue was dissolved in 100 mL of ethyl acetate. The organic solution was washed with brine, dried with $MgSO_4$, then concentrated and purified by flash chromatography (CH_2Cl_2/MeOH, 96:4) to provide **2** as a colourless oil (1.03 g, 63%). − $[\alpha]_D^{25}$ = +15.28 (*c* = 1.25 mg/mL, EtOH). − IR (neat): $\tilde{\nu}$ = 1235 cm^{-1}. − ^1H NMR ($CDCl_3$): δ = 1.32 (s, 3 H), 1.37 (s, 3 H), 1.7−2.1 (m, 4 H), 3.15 (1 H, D_2O exchangeable), 3.6−4.0 (m, 4 H), 5.0 (m, 4 H), 7.34 (s, 10 H). − ^{13}C NMR ($CDCl_3$): δ = 22.5 (d, $^1J_{CP}$ = 141 Hz), 25.3, 26.0, 26.6, 66.0, 67.4, 71.6 (d, $^3J_{CP}$ = 12 Hz) 78.3, 109.2, 128.4, 128.6, 136.3. − ^{31}P NMR ($CDCl_3$): δ = 34.5. − $C_{22}H_{29}O_6P$: calcd. C 62.86, H 6.9, O 22.86; found C 63.0, H 6.8; O 22.7.

Dibenzyl (4*R*)-(4,5-Dihydroxy-4,5-*O*-isopropylidene-3-oxopentyl)phosphonate (3): To a stirred mixture of dicyclohexylcarbodiimide (0.74 g, 3.6 mmol) in dry pyridine (0.2 mL, 2.5 mmol) or dry benzene (20 mL) was added dropwise **2** (0.5 g, 1.2 mmol) in dry DMSO (2.5 mL) under nitrogen. Trifluoroacetic acid (0.1 mL, 1.2 mmol) was added and the mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure and the remaining residue dissolved in 60 mL of ether. The suspension (dicyclohexylurea) was filtered and the filtrate washed with brine (3 × 30 mL). The organic layer was dried with $MgSO_4$ and concentrated under reduced pressure. The residue was chromatographed (CH_2Cl_2/MeOH, 96:4) to yield **3** as a colourless oil (0.41 g, 82.4%). − $[\alpha]_D^{25}$ = +18.36 (*c* = 3.65 mg/mL, EtOH). − IR (neat): $\tilde{\nu}$ = 1720, 1240 cm^{-1}. − ^1H NMR ($CDCl_3$): δ = 1.35 (s, 3 H), 1.40 (s, 3 H), 1.90−2.25 (m, 2 H), 2.70−2.90 (m, 2 H), 3.80−4.45 (m, 3 H), 5.0 (m, 4 H), 7.30 (s, 10 H). − ^{13}C NMR ($CDCl_3$): δ = 19.2 (d, $^1J_{CP}$ = 145 Hz), 25.0, 26.0, 31.8, 66.5, 67.5, 80.0, 111.0, 128.0, 128.5, 136.2, 208.2 (d, $^3J_{CP}$ = 14 Hz). − ^{31}P NMR ($CDCl_3$): δ = 32.6. − $C_{22}H_{27}O_6P$: calcd. C 63.15, H 6.46, O 23.0; found C 63.4, H 6.3, O 22.8.

(4*R*)-(4,5-Dihydroxy-3-oxopentyl)phosphonic Acid, Barium Salt (4): To a suspension of Pd/C (10%, 100 mg) in a solution of water/MeOH (1:1, 10 mL) was added **3** (0.38 g, 0.9 mmol). The mixture was degassed and hydrogenated for 12 h at atmospheric pressure. The catalyst was filtered off and water (20 mL) was added to the filtrate. The pH value was adjusted to 7.6 with saturated $Ba(OH)_2$. The solution was freeze-dried, and the residue dissolved in 5 mL of distilled water. The suspension was discarded by centrifugation, barium salt **4** was precipitated by addition of ethanol (15 mL) and the resulting mixture was kept at 0 °C for 3 h. The salt was collected by centrifugation, washed twice with ethanol (80%, then absolute), diethyl ether and dried in vacuo to yield **4** (0.27 mg, 90%). − $[\alpha]_D^{25}$ = +16.0 (*c* = 3.50 mg/mL, H_2O). − IR (KBr): (nu)tilde = 1706, 1234 cm^{-1}. − ^1H NMR (D_2O): δ = 1.60−2.05 (m, 2 H), 2.70−2.90 (m, 2 H), 3.80−4.05 (m, 2 H), 4.5 (m, 1 H). − ^{13}C NMR (D_2O): δ = 24.6 (d, $^1J_{CP}$ = 134 Hz), 36.1, 65.3, 80.3, 216.2 (d, $^3J_{CP}$ = 12 Hz). − ^{31}P NMR (D_2O): δ = 23.2. − $C_5H_9BaPO_6$: calcd. C 18.0, H 2.7, O 28.8; found C 18.2, H 2.5, O 28.6.

Acknowledgments

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