Synthesis of 3-arsonopyruvate and its interaction with phosphoenolpyruvate mutase

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3-Arsonopyruvate was prepared in four steps from glycine. The arsenic–carbon bond was formed by a Meyer reaction between alkaline arsenite and 2-bromo-3-hydroxy-2-(hydroxymethyl)propionic acid; the 3-arsono-2-hydroxy-2-(hydroxymethyl) propionic acid formed was oxidized with periodate to give 3-arsonopyruvate. This proves to be an alternative substrate for phosphoenolpyruvate mutase, giving pyruvate, which was assayed using lactate dehydrogenase. The K_m is 20 μM, similar to that observed for the natural substrate phosphonopyruvate (17 μM), whereas the $k_{cat.}$ of 0.01 s^{-1} was much lower than that for phosphonopyruvate (58 s^{-1}). Arsonopyruvate competitively inhibited the action of the mutase on phosphonopyruvate.

INTRODUCTION

The main biosynthetic reaction for forming C–P bonds is the intramolecular rearrangement of phosphoenolpyruvate (PEP), H_2O_3P-O-C(=CH_2)-COOH, to phosphonopyruvate, H_2O_3P-CH_2-C(=O)-COOH, catalysed by PEP mutase (EC 5.4.2.9). This enzyme has been isolated and characterized from *Tetrahymena pyriformis* [1,2] and *Streptomyces hygroscopicus* [3]. Its equilibrium constant is about 2000 in favour of PEP [4]. The active enzyme is a dimer, and the monomer possesses 290 residues [5]. The amino-acid sequence of the enzyme from *T. pyriformis* [5] has significant sequence similarity to that of carboxyphosphonoenolpyruvate (CPEP) mutase from *S. hygroscopicus* (30% identity over a 180-residue overlap) [6]. This is another enzyme known to catalyse the formation of a C–P bond; it rearranges and decarboxylates carboxyphosphonoenolpyruvate, HOOC-P(O)(OH)-O-C(=CH_2)-COOH, to (hydroxyphosphinoyl)pyruvate, H-P(O)(OH)-CH_2-CO-COOH [7,8]. The configuration at phosphorus is retained in the rearrangement catalysed by PEP mutase, which suggests that the reaction proceeds via a phosphoenzyme [9–11]. McQueney et al. [11] have shown that thiophosphonopyruvate is a substrate for PEP mutase with similar $k_{cat.}$ and K_m values to those of phosphonopyruvate, which suggests that nucleophilic attack at phosphorus is not the rate-limiting step. Seidel and Knowles [12] have evaluated a range of compounds as inhibitors of PEP mutase. From their results they have proposed a mechanism in which the rate-limiting step is the translocation of the enzyme-bound phospho group from one end of the enolpyruvate anion to the other.

Enzymes that act on phosphorus compounds often act similarly on arsenic analogues, e.g. adenylate kinase [13,14] and RNA polymerase [15]. We have now prepared the arsenical analogue of phosphonopyruvate, arsonopyruvate, and studied its interaction with PEP mutase. Arsonopyruvate binds well to PEP mutase, presumably because of its similarity in geometry and charge to phosphonopyruvate, although it is only a poor substrate.

MATERIALS AND METHODS

Enzymes

Lactate dehydrogenase (LDH; EC 1.1.1.27) was supplied by Sigma as a solution in 50% glycerol and 10 mM sodium phosphate buffer, pH 7.5. One unit of LDH is defined as the amount of enzyme required to reduce 1 μmol of pyruvate to lactate/min at pH 7.5 at 37 °C. PEP mutase was isolated by the procedure of Seidel et al. [5].

Chemicals

Reagents and solvents were of reagent grade and were purchased from Sigma, Aldrich, BDH and Lancaster Synthesis Ltd. NADH was purchased from Boehringer-Mannheim.

Methods

Paper electrophoresis and the detection of arsonates on paper was carried out as described by Adams et al. [14]. The mobilities given are relative to that of arsenate. Primary amines were detected by spraying papers with 0.25% ninhydrin in acetone and heating.

^{1}H-NMR spectra were recorded on a Bruker wide-bore AM400 spectrometer. Chemical shifts were measured relative to tetramethylsilane. Samples were stored in a desiccator. Water was removed by repeated additions of ^2H_2O and evaporation under reduced pressure. Where required NaO^2H was used to adjust the pH of the sample.

^{13}C-NMR spectra (^{1}H-coupled) were recorded on a Bruker wide-bore AM400 spectrometer operating at 100.6 MHz or on a Bruker AC250 spectrometer operating at 62.9 MHz. For samples dissolved in ^2H_2O, NaO^2H was used to adjust the pH. Chemical shifts were expressed relative to trimethylsilylpropionic acid or 1,4-dioxane.

A_{340} was recorded with a Pye–Unicam PU 8800 UV/visible spectrophotometer.

Abbreviations used: PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase.

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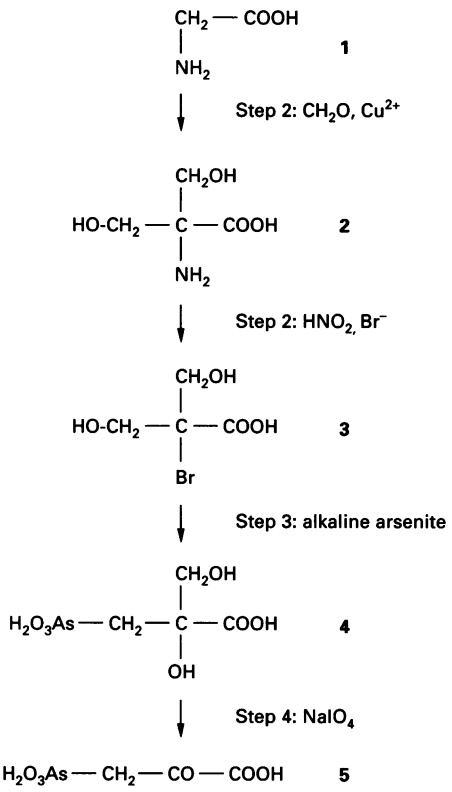

Scheme 1 The route of synthesis of 3-arsonopyruvic acid (5)

The course of step 3 is explained by the transitory formation of an epoxide.

Synthesis of 3-arsono-2-hydroxy-2-(hydroxymethyl)propionic acid (Scheme 1, steps 1–3)

2-(Hydroxymethyl)serine (**2**) was prepared by the method of Otani and Winitz [16]. Step 2 was carried out by adding a solution of $NaNO_2$ (2.07 g; 30 mmol) in water (10 ml) over 1 h to a stirred solution of compound (**2**) (2.68 g; 20 mmol) in a 1:1 solution of water and 48% HBr (15 ml) at 0 °C. The solution was stirred for a further 1 h, diluted with water and tested for the absence of compound (**2**) with ninhydrin. Dowex 50 (XW8 resin; H^+ form) was added and the mixture was stirred for 1 h at 60 °C. The reaction mixture was passed through a column of Dowex 50 (XW8 resin; H^+ form) and the eluate evaporated to dryness. Water was added and the solution re-evaporated several times to remove HBr, to give a crude sample of 2-bromo-3-hydroxy-2-(hydroxymethyl)propionic acid (**3**) as a thick yellow oil.

For step 3, the bromoacid (**3**) was dissolved in water (10 ml) and added slowly to a stirred solution of As_4O_6 (4 g; 40 mmol of As) and NaOH (6.4 g; 160 mmol) in water (15 ml). After 2 h at room temperature, the pH of the reaction mixture was adjusted to 5.0 with acetic acid, and the unchanged arsenite that precipitated as arsenic(III) oxide was removed by filtration. The filtrate was passed through a column of Dowex 50 (XW8 resin; H^+ form) to remove Na^+ cations, and washed through with water (200 ml). The eluate was concentrated and electrophoresis at pH 3.5, 100 V/cm for 20 min, showed two compounds of mobilities 0.52 and 0.67. The reaction mixture was applied to a column (30 cm × 2 cm) of Amberlite GC 400 (acetate form). The column was washed successively with 200 ml each of water, aq. 5% acetic acid, aq. 10% acetic acid, aq. 5% formic acid, aq. 7.5% formic acid and with 500 ml of aq. 10% formic acid. The fractions were evaporated to dryness, water was added and re-evaporated several times to remove acetic and formic acids. Electrophoresis of the acetic acid and formic acid washes at pH 3.5, 100 V/cm, for 20 min showed that the compound of mobility 0.52 was eluted with 7.5% formic acid. Evaporation to dryness gave a thick oil which on titration with 0.1 M NaOH showed the presence of one acidic group with a pK_a of 3.5, consistent with 2,3-dihydroxy-2-(hydroxymethyl)propionic acid. This was confirmed by ^{13}C NMR (^2H_2O; chemical shifts were measured relative to an internal dioxane standard) δ: 65.2 (t, J_{CH} 144.1 Hz, CH_2O^2H); 81.0 (s); 179.5 (s, COO^2H). The arsenate-positive compound of mobility 0.67, believed to be the product 3-arsono-2-hydroxy-2-(hydroxymethyl)propionic acid (**4**), was eluted with 10% formic acid and gave a colourless solid on evaporation to dryness, which on titration with NaOH indicated the presence of an arsonic acid. It was recrystallized from water/acetone [0.9 g; 18% with respect to 2-(hydroxymethyl)serine]. Elemental analysis gave: C, 21.8; H, 3.1%. The free acid $C_4H_9AsO_7$ requires C, 19.7; H, 3.7%, but the values observed are in better agreement with those calculated for the cyclic arsonic ester $C_4H_7AsO_6$ of C, 21.3; H, 3.1%. ^1H-NMR (^2H_2O) spectra were recorded at pH 4.25 and pH 13.0. The spectrum at pH 4.25 gave δ: 3.78 (1 H, d, J_{gem} 11.5 Hz, CH_AH_B-O^2H), 3.75 (1 H, d, J_{gem} 11.5 Hz, CH_AH_B-O^2H), 2.965 (1 H, d, J_{gem} 14.1 Hz, CH_AH_BAs) and 2.87 (1 H, d, J_{gem} 14.1 Hz, CH_AH_BAs). The spectrum at pH 13.0 gave δ: 3.75 (1 H, d, J_{gem} 11.5 Hz, CH_AH_B-O^2H), 3.70 (1 H, d, J_{gem} 11.5 Hz, CH_AH_B-O^2H), 2.25 (1 H, d, J_{gem} 14.1 Hz, CH_AH_BAs) and 2.23 (1 H, d, J_{gem} 14.1 Hz, CH_AH_BAs). The downfield shift for the CH_2-As group on the addition of acid is consistent with that reported by Adams et al. [14]. ^{13}C NMR (^2H_2O, pH 10.35) δ: 42.4 (t, J_{CH} 133.5 Hz, CH_2As), 69.6 (t, J_{CH} 146.2 Hz, CH_2O^2H), 78.9 (s), 182.0 (s, COO^2H). The assignments were confirmed by comparison with ^{13}C-NMR spectra of arsonoacetic and arsonopropionic acids.

Synthesis of 3-arsonopyruvic acid (Scheme 1, step 4)

A solution of $NaIO_4$ (0.9 g; 4.2 mmol) in water (10 ml) was added dropwise to a stirred solution of the diol (**4**) (1 g of the cyclic ester; 4.4 mmol) in water (10 ml). After a further 5 min, ethanol (100 ml) was added to precipitate the $NaIO_3$ which was removed by filtration. The ethanol was removed from the filtrate by concentration under reduced pressure below 30 °C to minimize the breakdown of arsonopyruvate. The solution was then freeze-dried to give arsonopyruvate (**5**) as a red solid which was stored at 4 °C. Electrophoresis of a solution of this solid at pH 2.0 for 25 min at 5 kV showed that the arsonopyruvate (mobility 1.5) was contaminated with a small amount of diol (**4**), arsenate and a trace of arsonoacetate (mobility 0.6).

A solution of impure arsonopyruvate was loaded on to a column of DEAE-cellulose (Whatman DE 52) (35 cm × 1 cm) equilibrated with a solution of 0.1 M pyridine and 0.5 M acetic acid buffer, pH 3.4. The column was washed with 300 ml of this buffer and 3 ml fractions were collected. The fractions were analysed for the presence of arsenate or arsonates by the method of Ben-Yoseph et al. [17], which is based on the ability of arsenates and arsonates to diminish the A_{450} of a buffered acidic solution of Fe^{3+} and N_3^- by chelating Fe^{3+} cations. Fractions 20–25 were pooled together as were fractions 39–60. These were passed through a column of Dowex 50 (XW8 resin; H^+ form) to remove the pyridine, concentrated by rotary evaporation at 30 °C and analysed by electrophoresis at pH 2.0 for 30 min at 5 kV. Fractions 20–25 contained the diol (**4**) and arsenate.

Fractions 39–60 contained predominantly arsonopyruvate (5) and some arsenate, presumably formed by breakdown as the arsonopyruvate came off the column and was desalted. The arsenate amounted to under 10%, as judged by a ratio of 1:0.09 of pyruvate released on heating to that already present before heating (item 2 of the characterization below). These fractions were then evaporated several times with the addition of water at 30 °C to remove acetic acid and stored as an aqueous yellow solution at 4 °C.

Characterization of arsonopyruvate

(1) Step 4 of the synthesis was repeated by adding a solution of $NaIO_4$ (0.09 g) in water (2 ml) to a solution of the diol (4) (0.1 g of the cyclic ester) in water (2 ml). To part of the reaction mixture was added an equal volume of 7% $NaBH_4$ solution. Electrophoresis at pH 2.0 showed that the presumed arsonopyruvate of mobility 1.5 was converted into a compound with the same mobility (0.4) as arsonolactate [18]. Addition of excess $NaIO_4$ to the reaction mixture converted the compound with mobility 1.5 into one of mobility 0.6, which co-migrated with arsonoacetate. This confirmed that the compound with mobility 1.5 was arsonopyruvate.

(2) To estimate the amount in a solution, the amount of pyruvate was assayed with LDH/NADH before and after boiling the sample of arsonopyruvate for 20 min on a water bath in a sealed Eppendorf tube. Boiling for 20 min was sufficient to break down all of the arsonopyruvate in the sample, as further heating did not increase the amount of pyruvate released. The assay mixture contained 100 mM sodium phosphate buffer, pH 7.4 (1 ml), 7.5 mg/ml NADH solution (0.02 ml) and test solution (0.02 ml) (before or after heating). The assay was initiated by the addition of LDH (4680 enzyme units/ml; 0.005 ml). The consumption of NADH was monitored by the decrease in A_{340} at 21 °C, until the reading was constant. The amount of arsonopyruvate present was calculated from the difference in pyruvate released on heating and that present before heating.

(3) The ^{13}C-NMR spectrum (H_2O, ^2H_2O inner lock, referenced to 1,4-dioxane at $\delta = 67.3$) of the crude reaction mixture obtained after the removal of $NaIO_4$, concentration to remove ethanol, and addition of water and concentration gave δ_C: 44.9 (t, J_{CH} 139.4 Hz, CH_2-As), 92.3 [t, J_{CH} 4.4 Hz, $C(OH)_2$] and 173.4 (s, COOH) for arsonopyruvate with a hydrated carbonyl group, together with a peak for the by-product, hydrated formaldehyde, δ_C: 82.6 (t, J_{CH} 164.3 Hz).

(4) Arsonopyruvate was assayed using PEP mutase and LDH/NADH as described below, except that tenfold more PEP mutase was added (0.88 mg) to ensure that all of the arsonopyruvate was rapidly converted into pyruvate.

Stability of arsonopyruvate

Examination of periodate oxidation of the diol at pH 8.0 and 10.0 by electrophoresis showed low concentrations of arsonopyruvate and high amounts of arsenate when compared with the oxidation at pH 2.0 and 3.5.

To study the effect of pH on its stability, a solution of impure arsonopyruvic acid (6 mg/ml) was incubated at pH 5.5 and 11.6 (pH adjusted with NaOH) at room temperature. The solution of arsonopyruvic acid alone had a pH of 2.0, and its stability was also examined. The solutions were assayed for arsonopyruvate using LDH/NADH assay, described in the characterization (2) above, after 30 min, 4 h and 20 h.

Assays with PEP mutase

The assay buffer contained 100 mM Hepes, pH 7.5, 10 mM magnesium acetate and 10 mM potassium acetate. The assay mixture (total volume 1.065 ml) contained assay buffer (1 ml), 7.5 mg/ml NADH solution (0.02 ml) and LDH (0.005 ml) at 23 °C. Arsonopyruvate solution (0.02 ml) was added and the consumption of pyruvate was monitored at 340 nm. When all of the pyruvate had been consumed and the A_{340} was constant, PEP mutase (0.088 mg; 2.775 nmol; 0.02 ml) was added and the change in A_{340} monitored. Arsonopyruvate concentrations in the assay ranged from 8.8 to 141 μM. Michaelis constants K_m and catalytic constants $k_{cat.}$ for it with the enzyme were determined from the initial-velocity data using Lineweaver–Burk, Eadie–Hofstee and non-linear regression analysis [19]. Similar conditions were used for measuring activity with phosphonopyruvate as substrate and arsonopyruvate as an inhibitor. The quantity of PEP mutase was increased to 0.88 mg when the assay was used to determine the total concentration of arsonopyruvate in a sample from the total pyruvate released when the reaction had ceased.

RESULTS

Stability of arsonopyruvate

The assay of pyruvate released by LDH was used to assess the stability of arsonopyruvate at a range of pH values at room temperature. Arsonopyruvate was most stable at the extreme pH values of 2.0 and 11.6 with less than 10% decomposition over 20 h. It was much less stable at intermediate pH values, with complete breakdown to pyruvate in 20 h at pH 5.5.

Arsonopyruvate as a substrate for PEP mutase

Arsonopyruvate proved to be a substrate. The Eadie–Hofstee plot ($r = 0.975$) (Figure 1) gives a K_m of 20.2 μM and a $k_{cat.}$ of 0.0095 s^{-1} for it. These values are in excellent agreement with those calculated from the Lineweaver–Burk plot (K_m 20.4 μM and $k_{cat.}$ 0.0095 s^{-1}; $r = 0.990$) and non-linear regression analysis (K_m 21.9 μM and $k_{cat.}$ 0.0099 s^{-1}, $r = 0.994$). The $k_{cat.}/K_m$ values

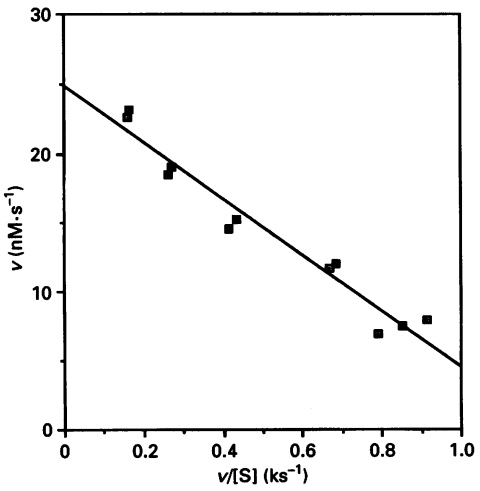

Figure 1 Eadie–Hofstee plot for the dependence of the rate of arsonopyruvate breakdown by PEP mutase on substrate concentration

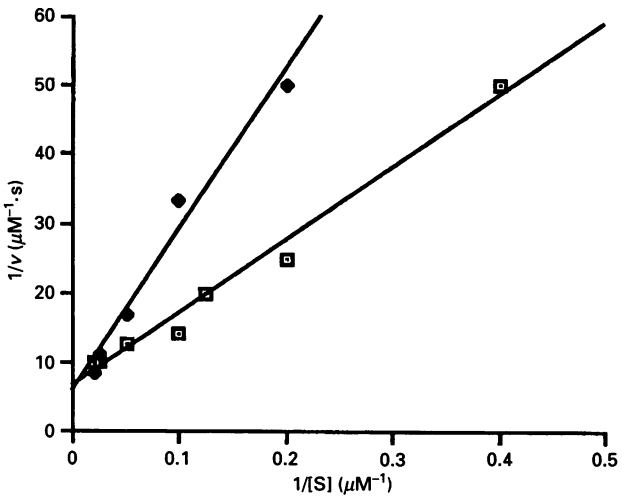

Figure 2 Arsonopyruvate inhibition of PEP mutase

The points show the results obtained in the absence (⊡) and presence (◆) of 40 μM inhibitor (arsonopyruvate). [S], concentration of phosphonopyruvate. As the lines were fitted to these double-reciprocal plots, they give only rough estimates of the kinetic parameters, namely a K_m of 16 μM for phosphonopyruvate and a K_i of 27 μM for arsonopyruvate.

were 470, 467 and 452 $M^{-1} \cdot s^{-1}$ respectively for the three methods. The corresponding steady-state parameters with phosphonopyruvate as substrate with the same enzyme preparation were K_m 17 μM, $k_{cat.}$ 58 s^{-1} and $k_{cat.}/K_m$ 3.4×10^6 $M^{-1} \cdot s^{-1}$. The experiments with phosphonopyruvate were carried out at 30 °C and those with arsonopyruvate at 23 °C.

Arsonopyruvate as a competitive inhibitor of PEP mutase

In case the slow action of PEP mutase on arsonopyruvate might have been due to a contaminating enzyme, arsonopyruvate was tested as an inhibitor for the enzyme at a concentration of 40 μM with 2.5–50 μM phosphonopyruvate as substrate. Competitive inhibition was seen (Figure 2). Our data give a rough indication of K_i as 27 μM, not inconsistent with the value of 20 μM for the K_m it exhibited as a substrate.

DISCUSSION

Synthesis of arsonopyruvate

Three recent attempts in our laboratory to prepare arsonopyruvate had failed. Kamal [20] attempted the oxidation of tripropyl arsonolactate, using dicyclohexylcarbodi-imide with anhydrous orthophosphoric acid in dimethyl sulphoxide [21], but only arsenate was observed. Ali and Dixon [22] attempted the transamination of arsonoalanine using identical conditions to those employed by Sparkes et al. [23] in the transmination of 3-phosphonoalanine to phosphonopyruvate with glyoxylate as amino group acceptor and Cu^{2+} and pyridine as catalysts, but arsenite was released. Finally, Ali [24] treated bromopyruvic acid with alkaline arsenite, but arsonopyruvic acid was not formed.

These results suggested that arsonopyruvate might be too unstable to exist, owing to the ability of the 2-oxo group to accept electrons and thus facilitate cleavage of the C–As bond by attack of water on the arsenic atom. Lacoste et al. [25], however, then showed that arsonoacetaldehyde, produced by an enzymic transamination, had a half-life of 5 h at pH 8.5. They also generated arsonoacetaldehyde in solution by treating 2,3-dihydroxypropylarsonic acid with $NaIO_4$ (1 mol/mol). They failed to isolate a solid sample because of its partial breakdown to arsenate on handling. Nevertheless, the solution could be used to synthesize arsonoalanine by a Strecker synthesis [22,24].

These results suggested that the preparation of arsonopyruvate should be possible and we decided to attempt it by oxidizing the diol, 3-arsono-2-hydroxy-2-(hydroxymethyl)propionic acid, with periodate. This diol was prepared (Scheme 1) in three steps from glycine. The step forming the As–C bond was a Meyer [26] reaction of 2-bromo-3-hydroxy-2-(hydroxymethyl)propionic acid with alkaline arsenite. The reaction is explained by the fact, emphasized by Tsivgoulis et al. [27], that Meyer reactions with 2-haloalcohols proceed via epoxides; the epoxide is opened by attack of the arsenite at the less hindered position. Arsonopyruvate was then generated in solution by the periodate oxidation of the diol (Scheme 1, step 4) and was partially purified by ion-exchange chromatography.

The low yield (18%) of the diol may be explained by the appearance of some 2,3-dihydroxy-2-(hydroxymethyl)propionic acid. This could arise both during the diazotization (step 2), where water might compete with bromide in replacing the amino group, and in the Meyer reaction (step 3) in which the postulated epoxide could be opened by hydroxide.

Stability of arsonopyruvate

Arsonopyruvate is stable in very acidic and alkaline solutions, but at the intermediate pH of 5.5 it broke down completely in 20 h. Clark et al. [28] proposed that any molecule of the general formula $(RO)_2P(O)$-X-Y-Z is a potential phosphorylating agent if the electrons of the P–X bond can be accommodated on Z. Thus the electron-withdrawing carbonyl group of arsonopyruvate can facilitate its hydrolysis, in the way proposed for the hydrolysis of phosphonoacetaldehyde [29] and phosphonopyruvate [30], and also applicable to arsonoacetaldehyde. The high stability in acid may be due to the fact that water is too poor a nucleophile to attack even the electrophilic $-AsO_3H_2$ group, and a nucleophile such as hydroxide or the $-O^-$ groups of another arsonopyruvate molecule is required to do so. The stability at pH 11.6 can be attributed to the decreased electrophilicity of the $-AsO_3^{2-}$ group.

Arsonopyruvate as a substrate of PEP mutase

The enzyme-catalysed reaction with phosphonopyruvate is monitored using pyruvate kinase/ADP and LDH/NADH to detect the formation of PEP. If arsonopyruvate is a substrate for PEP mutase, then as arsenate esters and anhydrides are extremely unstable, the arsono–enzyme intermediate or arsonoenolpyruvate, if formed, would be expected to hydrolyse spontaneously. In either case, pyruvate is the expected product; we therefore monitored the enzyme-catalysed reaction using only LDH/NADH, after establishing that the inclusion of pyruvate kinase and ADP in the assay mixture did not have any effect on the reaction. Arsonopyruvate was shown to be a substrate for PEP mutase with a K_m of 20 μM, which is comparable with that of the natural substrate phosphonopyruvate (17 μM). This indicates that it binds well to the active site of the enzyme. The $k_{cat.}$ with arsonopyruvate (0.01 s^{-1}) is, however, about 6000 times less than that with phosphonopyruvate (58 s^{-1}). The fact that arsonopyruvate competitively inhibited the action of PEP mutase on phosphonopyruvate supports our contention that the hydrolysis seen when it was added to the enzyme is indeed due to its behaviour as a substrate. The good binding but impaired turnover

of arsonopyruvate with PEP mutase is consistent with the formation of an arsono–enzyme intermediate and the enolpyruvate anion in the active site. The arsono–enzyme intermediate may hydrolyse spontaneously and the enolate anion may dissociate only slowly from the active site of the enzyme. Alternatively, the arsono–enzyme intermediate may be inaccessible to solvent and this may make its hydrolysis rate-limiting. With phosphonopyruvate, the enzyme transfers the phospho group to the oxygen of the enolate anion forming PEP, which binds poorly to the enzyme (K_i, 0.35 mM) [12] and therefore dissociates immediately from the active site.

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