J. Enzyme Inhibition, 1995, Vol. 8, pp. 255–259
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Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by HINARI on 12/16/11
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ENOLASE AND THE ARSONOMETHYL ANALOGUE OF 2-PHOSPHOGLYCERATE

SANGEETA CHAWLA† and HENRY B.F. DIXON*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, UK

(Received 18 July 1994)

(*RS*)-3-Arsono-2-(hydroxymethyl)propionic acid was synthesized by the action of alkaline arsenite on 3-bromo-2-(bromomethyl)propionic acid. It is a substrate for yeast enolase (EC 4.2.1.11) with a K_m of 6.5 mM (for 2-phospho-D-glycerate K_m = 0.08 mM). The catalytic constant of the enzyme with the arsonomethyl analogue is 230 times lower than with 2-phosphoglycerate.

KEY WORDS: Enolase, 2-phosphoglycerate, arsonomethyl analogue

INTRODUCTION

Enolase catalyses the reversible elimination of water from (*R*)-2-phosphoglycerate to form phosphoenolpyruvate (PEP). The mechanism of the reaction is interesting, since it involves the abstraction both of a rather nonacidic hydron and of OH^-, a poor leaving group. Lebioda and Stec[9] determined the crystal structure of the yeast enolase-Mg^{2+}-substrate complex to a resolution of 2Å and summarized earlier evidence that loss of H^+ preceded loss of OH^-. They located the phosphate group as hydrogen-bonded to the residue Arg-374.

One of the few analogues of PEP that is also a substrate for enolase is the compound in which the -O-PO_3H_2 group is replaced by -CH_2-PO_3H_2;[11,14] we now report the synthesis (Scheme 1) of the analogue of 2-phosphoglycerate in which the -O-PO_3H_2 group is replaced by -CH_2-AsO_3H_2; it also proves to be a substrate, although only a poor one. Several other enzymes act on arsonomethyl analogues of their natural susbtrates, e.g. phosphoglycerate kinase[1], adenylate kinase,[2] and RNA polymerase.[10]

MATERIALS AND METHODS

3-Bromo-2-(bromomethyl)propionic Acid

Diethyl bis(hydroxymethyl)malonate was made by the method of Block[4] by treating diethyl malonate with formaldehyde (Scheme 1, step 1). This was converted

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†Correspondence
*MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH

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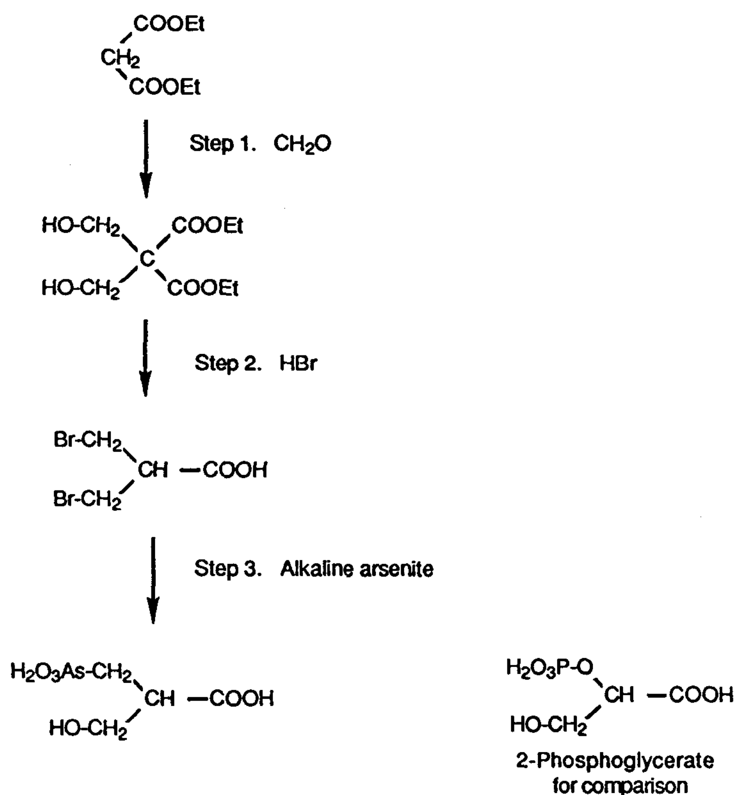

SCHEME 1 The synthetic route to 3-arsono-2-(hydroxymethyl)propionic acid.

(step 2) into 3-bromo-2-(brmomethyl)propionic acid by the method of Ferris,[7] as follows. Diethyl bis(hydroxymethyl)malonate (11 g) was dissolved in 83 ml of 48% HBr and the solution boiled under reflux for 6 h. The solution was concentrated on a rotary evaporator and then cooled on ice when the product crystallized. The crystals were filtered off on a sintered glass funnel and the filtrate boiled for a further 1 h to get a second crop of product. The product was recrystallized from boiling water.

3-Arsono-2-(hydroxymethyl)propionic Acid

This was synthesized (Scheme 1, step 3) by adding a solution of 3 g (75 mmol) of NaOH in 10 ml of water to 3.6 g (15 mmol) of solid 3-bromo-2-(bromomethyl)propionic acid and stirring until the solid dissolved. This solution was stirred while a solution of 1.95 g (15 mmol) of $NaAsO_2$ in 3 ml of water was added dropwise. The reaction mixture was incubated at 75°C for 1 h. Paper electrophoresis, performed with white spirit as coolant and the buffers of Ambler,[3] was used to assess the products of the

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reaction. Arsonates were detected as described for phosphonates[5] by staining with $FeCl_3$ and sulphosalicylic acid.[12] Electrophoresis at pH 3.5 revealed the presence of three 'arsenate-positive' spots running with mobilities 0.014, 0.18, 0.33 that of an arsenate marker. The compound with electrophoretic mobility 0.33 appeared to be the major product. The compound with mobility 0.18 came up slowly as a faint spot. The compound that appeared at the origin on electrophoresis (mobility 0.014) was shown to be arsenite by oxidizing it with hydrogen peroxide to yield material that migrated with the arsenate marker. The reaction mixture was acidified with glacial acetic acid to pH 5.0. The arsenite that precipitated as arsenic (III) oxide was filtered off on a sintered glass funnel and the filtrate was desalted by passing through the hydrogen form of a sulphonic (Dowex 50XW8) resin. The reaction mixture was then applied to a column (25 $\times$ 3 cm) of Amberlite CG-400 resin in the acetate form. The column was washed successively with 200 ml each of 2.5% acetic acid, 5% acetic acid, 7.5% acetic acid, 10% acetic acid, 5% formic acid, 7.5% formic acid and finally 10% formic acid. The fractions were concentrated separately by rotary evaporation and analysed by electrophoresis. The compound with mobility 0.18 eluted in 7.5% acetic acid and its titration with NaOH revealed the absence of an arsono group and the presence of one or more carboxy groups. This may explain why the spot came up only slowly in the Fe^{3+}-binding test; many chelating agents without phosphono or arsono groups (e.g. oxalate) can give this test. No further attempts were made to characterize it. The presumed 3-arsono-2-(hydroxymethyl)propionic acid (electrophoretic mobility 0.33) eluted in 7.5% formic acid. This fraction was evaporated to dryness several times with additions of water to remove traces of formic acid as an azeotrope. The product was dissolved in water, adjusted to pH 7.0 with cyclohexylamine, and evaporated to dryness. It was taken up in ethanol and the addition of diethyl ether caused the product to crystallize as its salt with two molecules of cyclohexylamine. Elemental analysis gave C, 44.9; H, 8.4; N, 6.4; $C_{16}H_{35}AsN_2O_6$ requires C, 45.1; H, 8.3; N, 6.6%. N.m.r. spectroscopy (100.6 MHz ^{13}C) of the free acid in D_2O gave: δ 36.0 (t, CH_2As), 47.1 (d, CH). 66.3 (t, CH_2HOH), 18.0 (s, $COOH$).

Enolase Assay

Enolase (EC 4.2.1.11) from bakers yeast was purchased from Sigma Chemical Company as a freeze dried powder. All assays were carried out according to the method of Westhead,[13] following the absorbance at 240 nm in the broad absorption band of acrylate ions including PEP. The assay mixture, 1 ml, contained Tris/acetate buffer pH 7.8, 0.05 M in acetate, 1 mM magnesium acetate, 0.01 mM EDTA and variable concentrations of 2-phosphoglycerate or the analogue. The analogue was used as its disodium salt in case cyclohexylamine might interfere with the assay. This salt was made as above, but using NaOH instead of cyclohexylamine. The disodium salt was found to be the dihydrate from elemental analysis; unlike the cyclohexylamine salt it was highly hygroscopic. Analogue concentrdtions of 5 mM to 400 mM were used in the assays. The reaction was initiated by the addition of 56.6 μg of yeast enolase. The rate of dehydration was measured by following the increase in absorbance at 240 nm with a Pye Unicam PU 8800 UV/vis spectrophotometer. To monitor the enolase

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reaction with the analogue, it was assumed that the absorption coefficient of the presumed dehydration product, 2-(arsonomethyl)acrylic acid, at 240 nm is identical to that of PEP. Michaelis constants (K_m) for the enzyme were determined from initial velocity data using double reciprocal plots (Lineweaver-Burk plots).

RESULTS AND DISCUSSION

Synthetic Route

We have shown that (*RS*)-3-arsono-2-(hydroxymethyl)propionic acid can be synthesized (Scheme 1, step 3) by the action of alkali on 3-bromo-2-(bromomethyl)propionic acid, $(Br\text{-}CH_2\text{-})_2CH\text{-}COOH$, followed by careful addition of an equimolar amount of sodium arsenite. We believe that the synthesis proceeds via successive eliminations of HBr and additions of water and arsenite, with the addition of water, but not that of arsenite, being reversible. This would explain earlier observations made in this laboratory that addition of the $(Br\text{-}CH_2\text{-})_2CH\text{-}COOH$ to an excess of alkaline arsenite gave only 3-arsono-2-(arsonomethyl)propionic acid, $(H_2O_3As\text{-}CH_2\text{-})_2CH\text{-}COOH$, (M.J. Sparkes, unpublished results), as a result of two additions of arsenite.

Interaction of the Analogue with Enolase

Stubbe and Kenyon[11] found that replacing the $\text{-O-}PO_3H_2$ group of phosphoenolpyruvate with $\text{-}CH_2\text{-}PO_3H_2$ made it a worse substrate for enolase, raising its Michaelis constant from 0.09 mM to 0.25 mM, and diminished its catalytic constant 60-fold. Similarly we find that, in the reverse direction, replacing the $\text{-O-}PO_3H_2$ group of 2-phosphoglycerate by $\text{-}CH_2\text{-}AsO_3H_2$ raised its Michaelis constant from 0.08 mM to 6.5 mM, and lowered its catalytic constant 230-fold, from 35 s^{-1} to 0.15 s^{-1}. We used the racemate, so the true K_m for the active enantiomer, if the other binds negligibly to the enzyme, is 3.25 mM.

These results are simlilar to those of Adams *et al.*,[1] who found that the arsonomethyl analogue of 3-phosphoglycerate was a markedly worse substrate for phosphoglycerate kinase than the phosphonomethyl analogue. Engel[6] and Yount[5] compared bond lengths and angles for C-P-O and O-P-O; the C-P bond length (0.18 nm) is larger than the O-P bond length (0.15-0.16 nm), and this could contribute to the poor fit of the phosphonomethyl analogue. The C-As bond length is longer still (0.19 nm).[8] Phosphates, phosphonates and arsonates also differ in their acidities. The upper pK for phosphonic acids is in the range 7.0 to 8.2, about 0.5 to 1.0 higher than for the corresponding phosphate esters; this pK for arsonic acids is about 9.0. Hence the K_m could be raised by 10-100-fold by the need for the enzyme to bind the $\text{-}AsO_3^{2-}$ when, at pH 7.8, the $\text{-}AsO_3H^-$ form predominates in solution.

Acknowledgements

We thank Dr T.A. Moore for running the n.m.r. spectrum and for much advice on it. We also thank several colleagues, especially Mr M.J. Sparkes, for discussion. S. Chawla was supported by a Dharam Hinduja ODA Scholarship, for which we are grateful.

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