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A SYNTHESIS OF ACYLPHOSPHONIC ACIDS AND OF 1-AMINOALKYLPHOSPHONIC ACIDS: THE ACTION OF PYRUVATE DEHYDROGENASE AND LACTATE DEHYDROGENASE ON ACETYLPHOSPHONIC ACID

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Acylphosphonic acids, R–CO–$PO(OH)_2$, have been synthesized by the steps R–CO–Cl $\xrightarrow{P(OMe)_3}$ R–CO–$PO(OMe)_2$ $\xrightarrow{I^-}$ R–CO–PO(OMe)–O $\xrightarrow{HCOOH}$ R–CO–PO(OH)–O^- of which the last is new and provides a mild method for de-esterifying acylphosphonic acids. Their reductive amination gives a simple way of making 1-aminoalkylphosphonic acids. Acetylphosphonic acid inhibited NAD^+ reduction by pyruvate with the pyruvate dehydrogenases from *Escherichia coli* and *Bacillus stearothermophilus*. The inhibition was competitive with pyruvate, with K_i of 6 μM for the *E. coli* enzyme (pyruvate K_m 0.5 mM) and one of 0.4 mM fo the *B. stearothermophilus* enzyme (pyruvate K_m 0.1 mM). Acetylphosphonate and its monomethyl ester are substates for pig heart lactate dehydrogenase, with K_m values of 15 mM and 10 mM respectively (pyruvate K_m 0.05 mM) and specificity constants one thousandth that for pyruvate.

KEY WORDS: Pyruvate dehydrogenase, lactate dehydrogenase, acetylphophonate, acylphosphonate, aminoalkylphosphonate.

INTRODUCTION

Acylphosphonic acids, R–CO–PO_3H_2, have many properties of 2-oxo carboxylic acids, and have biochemical interest as their analogues. Similarly, 1-aminoalkylphosphonic acids, R–$CH(-NH_2)$–PO_3H_2, are analogues of natural amino acids. This paper presents simplified syntheses of both types of compound, and some actions of enzymes on acetylphosphonic acid.

Acetylphosphonic acid, CH_3–CO–PO_3H_2, a pyruvate analogue, appeared likely to react with thiamin diphosphate of the pyruvate dehydrogenase complex (for reviews, see references 1 and 2). We therefore tested it with this enzyme and it proved to be an effective and reversible inhibitor. Preliminary results by Harrison, Perham & Slater

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were cited by Ambrose & Perham.[3] Kluger & Pike[4] reported that monomethyl acetylphosphonate, which bears only a single charge at neutral pH, is a better inhibitor, with a K_i of 0.05 μM. Acetylphosphinic acid, CH_3–CO–PO_2H_2, is also a powerful inhibitor of this enzyme.[5–7] Kluger & Pike[4] were uncertain whether acetylphosphonic acid itself inhibited the enzyme, so we now report confirmation of the preliminary observation[3] that it is an inhibitor, and the present study is with well-characterized, crystalline acetylphosphonate. We similarly show it to be a substrate, albeit a poor one, of pig heart lactate dehydogenase (EC 1.1.1.27).

MATERIALS AND METHODS

Syntheses

Sodium methyl acetylphosphonate, CH_3–CO–PO(OMe)–O^- Na^+ Following Kabachnik & Rossiĭskaya,[8] acetyl chloride (20 ml, 0.28 mol) was cooled to 0°C. Trimethyl phosphite (30 ml, 0.25 mol) was added slowly with stirring and cooling. The mixture was then heated to 70°C for 5 min to drive off the excess of acetyl chloride. It was then cooled to 20°C and diluted with 100 ml of acetone; 40 g of NaI (0.27 mol) was gradually added with stirring. The heat of reaction raised the temperature to 43°C and the product crystallized. After 15 min the mixture was heated to 50°C for a further 15 min and cooled in ice; the product was filtered off and washed with acetone. Yield, 37.7 g (90%).

Sodium hydrogen acetylphosphonate, CH_3–CO–PO(OH)–O^- Na^+. The sodium salt of the half ester (31.2 g) was dissolved in 200 ml of 99% formic acid and boiled under reflux for 2.5 h. Paper electrophoresis[9] showed that the starting material had been completely converted into a substance with greater mobility at pH 6.5, as judged by staining for either carbonyl or $-PO_2^-$ – groups. The product was evaporated to dryness, and this was repeated three times after addition of portions of toluene. The crystalline mass formed was ground with acetone, washed well with acetone, and dried (yield 20.6 g). It was dissolved in aqeous acetic acid (2 ml of water and 10 ml of acetic acid per g of crude product) and it crystallized on addition of diethyl ether as a solvate with acetic acid (yield, 0.9 g of crude product, 47%).

Characterization of acetylphosphonic acid. N.m.r. (100 MHz, ^2H_2O δ = 2.06 [3H, s, CH_3–COOH], 2.40 [3H, d, J 5 Hz, CH_3–CO–PO_3H^-]. Found: C, 22.5; H, 3.8 · $C_4H_8NaO_6P$ requires C, 23.3; H 3.9%. Paper electrophoresis[9] at pH 6.5 showed a single spot positive for carbonyl group and for the $-PO_2^-$ – group, which ran about 20% faster than orthophosphate or the monomethyl acetylphosphonate. The n.m.r. line at δ = 2.4 and the electrophoretic behaviour were identical with those of the material[2] Dr Kluger kindly gave us. Titration of the dilithium salt (precipitated from water by acetone) showed a pK of 5.2, whereas the isomer[10] CHO–CH_2–PO_3^{2-} $2Li^+$ gave a pK of 6.5. The dilithium salt was obtained from the free acid, after a solution of this had been made by ion exchange and freed from acetic acid by evaporation. Incubation of a sample of Ac–PO_3HNa · AcOH for 20 h at 100°C at a pressure of 2.6 kPa gave the loss of weight theoretical for the loss of acetic acid, and the product showed an i.r. spectrum identical with material made by the previous method[4] (B.J.

Wright, unpublished work). The evidence that most clearly demonstrates the nature of the product is its X-ray crystallography by Jones & Kennard.[11]

Other acylphosphonic acids Isovalerylphosphonic acid and phenylacetylphosphonic acids were similarly prepared. The half salt of the former crystallized as its solvate with acetic acid in a yield of 67%, and exhibited the following properties. Paper electrophoresis showed a single spot on staining for 'phosphate' and with dinitrophenylhydrazine. N.m.r. at 60 MHz gave δ (^2H_2O) 2.6 [2H, d, 6 Hz, CH–C$\underline{H}_2$–CO], 2.0 [3H, s, C$\underline{H}_3$–COOH], 0.8 [6H, d, 6 Hz, –CH(C$\underline{H}_3$)$_2$], with the multiply split signal of H–3 not apparent. Found: C, 32.6, H, 5.5 · $C_7H_{14}NaO_6P$ requires C, 33.9; H, 5.7%. The half salt of the phenylacetylphosphonic acid also crystallized as a solvate with acetic acid; n.m.r. showed the expected signals but acetic acid was present in a less than 1 : 1 ratio, suggesting loss from a solvate on washing or drying. On repeated dissolution in water and drying the n.m.r. signal for the methyl group of acetic acid disappeared, and there remained, at 60 MHz in ^2H_2O, δ 7.3 [5H, broad, $C_6\underline{H}_5$–CH$_2$–CO], 4.8 [2H, s, C_6H_5–C$\underline{H}_2$–CO]. Both were characterized further by their reductive amination.

Reductive amination Sodium hydrogen isovalerylphosphonate (0.87 g, 5 mmol), triethylamine (2.5 g, 25 mmol), ammonium acetate (3.85 g. 50 mmol) and dimethylamine–borane complex (0.3 g, 5 mmol) were dissolved in methanol (200 ml), and stirred overnight. Paper electrophoresis showed that reaction was complete. The mixture was evaporated to dryness, water was added, and concentrated hydrochloric acid (5 ml) was added slowly to decompose the excess of Me_2NH^+–BH_3^-. The mixture was passed through a column (13 cm × 3 cm diameter) of the acid form of a sulphonic resin, washed through with water, and the product was eluted with 1 M-ammonia solution and evaporated to dryness. It was precipitated from methanol by addition of diethyl ether (0.55 g, 65% yield). It was dissolved in water, formic acid was added to pH 4, and the solution evaporated to dryness. The product crystallized from aqueous methanol on addition of acetone (0.4 g, 47% yield). It gave a single spot by electrophoresis at pH 2 and pH 6.5, staining for $-PO_3^{2-}$ – and with ninhydrin. Found: C, 35.2, 34.8; H, 8.5, 8.3; N, 8.2, 8.2 · $C_5H_{14}NO_3P$ requires C, 35.9; H, 8.44; N, 8.38%. 1-Aminophenethylphosphonic acid was similarly prepared (yield 64%); it also gave a single spot by electrophoresis at ph 2 and pH 6.5. Found: C, 47.9; H, 6.16; N, 6.91 · $C_8H_{12}NO_3P$ requires C, 47.8; H, 6.0; N, 7.0%.

Pyruvate Dehydrogenase and its Assay

The pyruvate dehydrogenase complex from *E. coli.* was purified as described by Danson *et al.*,[12] a method based on that of Reed & Mukherjee.[13] The enzyme complex from *B. stearothermophilus* (strain NCA 1503) was purified similarly.[14] The pyruvate dehydrogenase complexes were pure by the criteria of sodium dodecyl sulphate – polyacrylamide gel electrophoresis and analytical ultracentrifugation (Bates *et al.*[15]).

The pyruvate dehydrogenase complexes were assayed at 30°C and pH 7.0 in the direction of NAD^+-reduction.[12] The dihydrolipoamide dehydrogenase activity was assayed by the method of Reed & Willms.[16] Values of K_m and K_i were determined from double reciprocal plots or from fitting the progress curves to hyperbolae by computer (Cleland[17]).

Lactate Dehydrogenase and its Assay

The pig heart enzyme[18] (10 μM active sites) was incubated with 0.2 mM NADH and the phosphonate in 50 mM H_3PO_4 adjusted to pH 6.0 with 5 M NaOH at 25°C. The rate of NADH oxidation was followed by the fall in absorbance at 340 nm.

RESULTS AND DISCUSSION

Acetylphosphonate and Pyruvate Dehydrogenase

The effect of acetylphosphonate was first tested on the oxidative decarboxylation of pyruvate by the pyruvate dehydrogenase multienzyme complex of *E. coli*. Double reciprocal plots showed that acetylphosphonate was acting as an inhibitor competitive with pyruvate. By carrying out the kinetic analysis at several different inhibitor concentrations, several estimates could be made of the value K_i. A mean value of 5.9 $\pm$ 1.4 μM was calculated. This may be compared with the K_m for pyruvate of 0.48 mM (Danson *et al.*[19]). The reversibility of the inhibition, implicit of its competitive nature, seen in double reciprocal plots, was confirmed by incubating the enzyme (0.2 mg/ml) at 0°C in the assay buffer from which pyruvate was omitted and to which acetylphosphonate (0.14 mM) was added, and then dialysing the mixture at 2°C against 50 mM-potassium phosphate buffer, pH 8.0. Samples of the dialysed enzyme were found to have activities identical with that of control samples not treated with acetylphosphonate.

Similar experiments were carried out with the pyruvate dehydrogenase multienzyme complex of *B. stearothermophilus*. Acetylphosphonate was again found to act reversibly as an inhibitor competitive with pyruvate, the value for K_i being estimated as 0.41 $\pm$ 0.11 mM. The value of K_m for pyruvate was found to be 0.12 $\pm$ 0.01 mM.

One anomaly we observed in the kinetics remains unexplained. Linear rates of product formation could be observed in the presence of acetylphosphonate only when the inhibitor and enzyme were preincubated in the assay mixture for about 5 min, the assay being started by addition of pyruvate or, in some cases, of CoA. This is how the kinetic analyses described above were performed. When the assay was initiated by adding enzyme to the assay mixture that already contained acetylphosphonate, the initial rates decreased over a period of a few minutes before it became constant. This effect was independent of the length of time the acetylphosphonate was incubated with the assay mixture, showing that it was not caused by a reaction of acetylphosphonate with a component of the assay. Rather it appears to be caused by some slow (but evidently fully reversible) interaction of actylphosphonate with the pyruvate dehydrogenase complex, the nature of which is yet to be explained. The same phenomenon was observed for the *E. coli* and *B. stearothermophilus* complexes, which implies that it is a general property of the enzymes and the inhibitor.

Acetylphosphonate had no effect on the dihydrolipoamide dehydrogenase activity of the *E. coli* pyruvate dehydrogenase complex when the activity was assayed separately.

Thus acetylphosphonate can act as a reversible inhibitor of pyruvate hydrogenase multienzyme complexes, competitive with pyruvate, in keeping with the structural similarity of substrate and inhibitor.

Acetylphosphonates and Lactate Dehydrogenase

Both acetylphosphonic acid and its monomethyl ester were poor substrates for the enzyme. From double reciprocal plots with the substrate concentration varied from 1 mM to 100 mM the K_m for acetylphosphonate was determined as 15 mM and for the monomethyl ester as 10 mM at pH 6.0. The value for pyruvate is 0.05 mM for this isoenzyme[20] up to close to pH 7. At 100 mM substrates there was a drop in rate, much more marked for the methyl ester than for the free acid. One equivalent of the monomethyl ester oxidized one equivalent of NADH and one equivalent of the acetylphosphonate oxidized at least 0.95 equivalents of NADH.

The catalytic constant for acetylphosphonate was $0.25\,s^{-1}$ and for the methyl ester $0.05\,s^{-1}$. These values are 1000 times lower than for pyruvate, although they are similar to those for 3-(2-nitrophenyl)pyruvate (Holbrook & Stinson[21]). One possible explanation for the K_m of 15 mM for the acetylphosphonate at pH 6 is that this is the value for the dianion, the predominant form at this pH. Alternatively, however, the enzyme may bind only the monoanion, CH_3–CO–PO_3H^-. since this represents about a sixth of the total acetylphosphonate at pH 6, its K_m would be 2.5 mM, somewhat lower than that of the methyl ester. Indeed, the binding of a dianion would be most unexpected since Parker & Holbrook[22] have suggested that there is strict conservation of neutral charge, summed over the coenzyme, substrate or inhibitor, and active site histidine (His-195) in active ternary complexes of this enzyme. Unfortunately, the narrow range of pH over which rates can be measured does not allow us to distinguish whether the catalytically active form of the substrate is the mono- or dianion; at lower pH values the enzyme and NADH become increasingly unstable and at more alkaline values the observable rates of NADH oxidation are too slow for analysis.

Synthetic Route

Kabachnik & Rossiĭskaya[8] synthesized esters of acylphosphonic acids by reactions between acyl chlorides and trialkly phosphites. The difficulty in synthesizing acylphosphonic acids lies in liberating them from their esters. The first methyl group of Ac–$PO(OMe)_2$ is easily removed with sodium iodide; other authors[4,25] used a similar method. The second is harder to remove, but this has been done in several ways,[4,24–29] and the reaction here reported of formic acid with the salt CH_3–CO–PO(OMe)–O^-–Na^+ is particularly simple. Although formic acid is much weaker than the phosphonic acid, it apparently occasionally hydronates the substrate and labilizes the methyl group to attack by formate ion. No strong acid is used or formed in the reaction, so the product remains as its salt, CH_3–CO–PO(–OH)–O^- Na^+. Alkylphosphonic acids are not released from their monoesters by this treatment.

Reductive Amination

1-Aminoalkylphosphonic acids have been used as analogues of the natural amino acids in a variety of studies. Thus, for example, the anhydride of such an amino acid with AMP was a transition-state analogue of aminoacyl-AMP and bound tightly to the appropriate amino-acid – tRNA ligase (aminoacyl-tRNA synthease).[30] They are similarly substrates for aspartate aminotransferase, at least in reversibly modifying the pyridoxal form of the enzyme (Khurs *et al.*);[31] they also participate in non-enzymic transamination.[32]

Three main types of method have been used for preparing these compounds. Chambers & Isbell[33] converted R-CH(-COOH)-PO_3H_2 into R-CH(-NH_2)-PO_3H_2 via the amide and a Curtius rearrangement. In the second route (reviewed by Engel[34]), of which there are many forms, tervalent phosphorus compounds react with imines. In the third type, imines of acylphosphonic acids or related compounds are reduced. Khomutov *et al.*[35] showed that free acylphosphonic acids may be reductively aminated, and the method of making them now presented makes the route particularly simple; we use dimethylamine–borane and ammonia.[36]

When 1-aminoalkylphosphonic acids were isolated by evaporation of a solution in ammonia and then crystallized, high analyses for nitrogen showed that they retained some ammonia. Solutions of the crude compound were adjusted to pH 4 with formic acid before crystallization, so the excess ammonia stayed in solution as ammonium formate; in this way good analytical values were obtained.

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