Utilization of 2-aminoethylarsonic acid in *Pseudomonas aeruginosa*

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This paper describes the metabolism, transport and growth inhibition effects of 2-aminoethylarsonic acid (AEA) and 3-aminopropylarsonic acid (APrA). The former compound supported growth of *Pseudomonas aeruginosa*, as sole nitrogen source. The two arsonates inhibited the growth of this bacterium when 2-aminoethylphosphonic acid (AEP) but not alanine or NH₄Cl, was supplied as the only other nitrogen source. The analogy between AEA and the natural compound AEP led us to examine the *in vitro* and *in vivo* interaction of AEA with the enzymes of AEP metabolism. The uptake system for AEP (K_m 6 μ M) was found to be competitively inhibited by AEA and APrA (K_i 18 μ M for each). AEP-aminotransferase was found to act on AEA with a K_m of 4 mM (3.85 mM for AEP). Alanine and 2-arsonoacetaldehyde were generated concomitantly, in a stoichiometric reaction. *In vivo*, AEA was catabolized by the AEP-aminotransferase since it was able to first induce this enzyme, then to be an efficient substrate. The lower growth observed may have been due to the slowness with which the permease and the aminotransferase were induced, and hence to a poor supply of alanine by transamination.

Introduction

The recently synthesized compound 2-aminoethylarsonic acid (NH₂-CH₂-CH₂-AsO₃H₂; AEA) (Geoghegan & Dixon, 1989) is an analogue of the naturally occurring 2-aminoethylphosphonic acid (NH₂-CH₂-CH₂-PO₃H₂; AEP). This compound is found in several aquatic animals and in some protozoa, either free or incorporated into lipids or glycoproteins (Neuzil & Cassaigne, 1980).

The degradation pathway of AEP in *Pseudomonas aeruginosa*, which can utilize this aminophosphonate as a phosphorus, nitrogen or carbon source is well-known; a two-step pathway is involved. A specific 2-aminoethylphosphonate:pyruvate aminotransferase (EC 2.6.1.37) first catalyses the formation of alanine and phosphonoacetaldehyde (Dumora *et al.*, 1983); the C–P bond of phosphonoacetaldehyde is then split into P_i and acetaldehyde by a phosphonoacetaldehyde hydrolase (EC 3.11.1.1) (phosphonatase) (Dumora *et al.*, 1989).

Abbreviations: AEA, 2-aminoethylarsonic acid; APrA, 3-aminopropylarsonic acid; AEP, 2-aminoethylphosphonic acid; APrP, 3-aminopropylphosphonic acid.

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This led us to suppose that AEA might be a substrate for AEP-aminotransferase and that arsonoacetaldehyde, the product of its transamination, could act as a substrate or as an inhibitor of the phosphonatase. In this paper, we investigate the interaction of AEA with the proteins responsible for the metabolism of AEP in *P. aeruginosa*: the transport system, aminotransferase and phosphonatase. We also show that *P. aeruginosa* can grow in the presence of AEA as sole nitrogen source. We have therefore examined whether the *in vivo* degradative pathway of AEA conforms to the assays carried out *in vitro*, and the extent to which 3-aminopropylarsonic acid

(APrA), the next higher homologue of AEA, can substitute for it.

Methods

Bacterial strain. *Pseudomonas aeruginosa* A237, obtained from the Institut Pasteur, Paris, was routinely maintained on nutrient agar at 15–18 °C.

Arsonic and phosphonic compounds

2-Aminoethylarsonic acid. This was prepared from 2-chloroethylarsonic acid by the method of Geoghegan & Dixon (1989).

3-Aminopropylarsonic acid. This was synthesized by the method of Gough & King (1928); dichloro(3-chloropropyl)arsine was obtained by treating 3-chloropropan-1-ol successively with alkaline arsenite, SO₂ (with a catalytic amount of iodine) and thionyl chloride. The arsine was oxidized to 3-chloropropylarsonic acid with hydrogen peroxide rather than with chlorine. The arsonic acid was treated with 2-aminoethanol and the resulting compound cleaved with periodate, following the procedure of Geoghegan & Dixon (1989) for 2-aminoethylarsonic acid. On paper electrophoresis at pH 3.5, APrA migrated towards the negative electrode as did AEA; their mobilities were, respectively, 0.7 and 0.35 that of arsenate, which migrated in the opposite direction. Paper electrophoresis was performed with white spirit as coolant, with the buffers of Ambler (1963). Arsonates were detected as described for phosphonates by Dixon & Sparkes (1974), by staining with FeCl₃ and sulphosalicylic acid (Wade & Morgan, 1953).

2,3-Dihydroxypropylarsonic acid. Our method of making this acid proved to be almost the same as that of Tsigvoulis *et al.* (1991), which was published while our work was in progress. We prepared only the dilithium salt, however, and this avoided the difficulty of isolating the free acid. Arsenic(III) oxide (20 g, 0.2 mol) and NaOH (32 g, 0.8 mol) were dissolved in water (80 ml); 3-chloropropane-1,2-diol (24 g, 0.22 mol) was added dropwise with stirring at about 40–50 °C. The solution was then heated with stirring to 50–60 °C for 2 h. Electrophoresis showed that most of the arsenite had reacted, giving an arsonic acid. The solution was diluted with water to 200 ml, acidified with conc. HCl to pH 2, and evaporated to dryness with a rotary evaporator. The residue was extracted twice with ethanol to eliminate most of the NaCl formed. The ethanolic solution was evaporated to dryness; the crude product was dissolved in water and passed through a column (10 × 2.5 cm) of Dowex 50W X8 (acid form) and washed out with water. The pH was adjusted to 10.5 with LiOH and the solution evaporated to a small volume. The dilithium salt crystallized on addition of acetone, yield 23.0 g (57%). Electrophoresis showed a single spot. In proton NMR at 60 MHz in ²H₂O it showed bands at δ 2.07 [2H, d, 6Hz, CH-CH₂-As], 3.7 [2H, broad, HO-CH₂-CH] and 4.2 [1H, broad, CH₂-CH(OH)-CH₂]. Elemental analysis: C, 17.14, 17.05%; H, 3.29, 3.19%; C₃H₇AsLi₂O₅ requires C, 17.00%; H, 3.33%.

2-Oxoethylarsonic acid (2-arsonoacetaldehyde). A sample of 10 ml 50 mM-dilithium 2,3-dihydroxypropylarsonate at its original pH (10.5), and samples adjusted to pH 7.5 and 3.5 with HCl were each treated with 0.13 g (0.61 mmol) NaIO₄. After 10 min, samples were taken for paper electrophoresis, which was carried out at pH 3.5. The arsonate-positive spot of relative mobility 0.14 compared to arsenate had been converted to one of mobility 0.26; characterization of this solution as one of arsonoacetaldehyde was indirect as follows, because all attempts to isolate it from solution as a salt led to its partial breakdown to arsenate. Addition before electrophoresis of an equal volume of 0.2 M-NaBH₄ converted this spot to one of mobility 0.16, co-running with 2-hydroxyethylarsonic acid, confirming that the new spot was arsonoacetaldehyde. This identification was further confirmed by treating a

Fig. 1. Chemical formation of 2-oxoethylarsonic acid. *m*, electrophoretic mobilities of the arsonates relative to that of arsenate (*m* = 1).

solution of dilithium 2,3-dihydroxypropylarsonate at pH 10.5 with NaIO₄ as above, and oxidizing the aldehyde to the carboxylic acid as Isbell *et al.* (1969) had done with phosphonoacetaldehyde. Aqueous 5% (w/v) KMnO₄ was added dropwise until its colour persisted. The solid MnO₂ was filtered off and 5% (w/v) aqueous NaHSO₃ was added dropwise until the solution was colourless. Electrophoresis at pH 3.5 showed conversion of the spot of relative mobility 0.26 into one of relative mobility 0.87, co-running with arsonoacetate, and some formation of arsenate (see Fig. 1 below).

2-Aminoethylphosphonic acid. This was prepared by phosphonation of *N*-(2-bromoethyl)phthalimide with triethyl phosphite according to the method of Kosolapoff (1947). [1-¹⁴C]AEP [29 mCi mmol⁻¹ (1.07 GBq mmol⁻¹)] was purchased from CEA, France.

Phosphonoacetaldehyde. This was prepared according to the method of Isbell *et al.* (1969).

Enzymes. 2-Aminoethylphosphonate:pyruvate aminotransferase (EC 2.6.1.37) and phosphonoacetaldehyde hydrolase (EC 3.11.1.1) were purified from cell-free extracts of *P. aeruginosa* grown in media containing AEP as sole nitrogen source for the aminotransferase (Dumora *et al.*, 1983) or as both phosphorus and carbon source for the hydrolase (Dumora *et al.*, 1989).

Enzyme assays

AEP-aminotransferase. This was assayed by incubating at 30 °C a mixture containing AEA, APrA or AEP (1–20 mM), pyruvate (20 mM), pyridoxal 5'-phosphate (100 μM) and the enzyme (0.37 μM) in 2-amino-2-methylpropane-1,3-diol/HCl (Ammediol) buffer (100 mM), pH 8.5. The reaction was stopped by adding a 20% (w/v) solution of sulphosalicylic acid (0.125 ml per ml of reaction mixture). The precipitate was removed by centrifugation. The amount of alanine formed was measured from a sample of the supernatant, diluted and adjusted to pH 2.2, with a high performance Amino Acid Analyzer (Beckman 6300 series). In order to quantify arsonoacetaldehyde produced concomitantly with alanine, the assay was carried out in a volatile buffer, triethylammonium bicarbonate (100 mM, pH 8.5) in the presence of AEA (10 mM). After incubation, the reaction mixture was divided into two samples, A and B. Sample B was treated with borohydride. Reactions A and B were stopped by adding formic acid to pH 3.5. The reaction mixtures were then freeze-dried and the residues dissolved in water. An aliquot of each was electrophoresed at pH 3.5 to determine arsonoacetaldehyde in sample A and 2-hydroxyethylarsonic acid in sample B. The amount of arsonoacetaldehyde was estimated by comparison with standards prepared from 2,3-dihydroxypropylarsonic acid as above.

Phosphonoacetaldehyde hydrolase. We could not determine directly whether this enzyme hydrolysed arsonoacetaldehyde (CHO-CH₂-AsO₃H₂), because this compound broke down when we tried to isolate

it. We therefore estimated its rate of formation from the rate of alanine formation in the presence of aminotransferase and AEA ($\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-AsO}_3\text{H}_2$), and estimated its spontaneous rate of hydrolysis by determining the rate of acetaldehyde formation (using the coupled assay with alcohol dehydrogenase and NADH). Provided that formation was faster than hydrolysis, the breakdown of arsonoacetaldehyde accumulated in solution should be accelerated by phosphonate if it is a substrate. To follow this process, both AEP-aminotransferase (0.9 μM) and yeast alcohol dehydrogenase (5 IU) were added to a solution containing AEA (10 mM), pyruvate (10 mM), MgCl_2 (10 mM) and NADH (0.16 mM) in Ammediol buffer (100 mM), pH 8.5, and the oxidation of NADH was measured as the decrease in absorbance at 340 nm and 25 °C (Perkin Elmer 550 S). This measurement was made both with and without addition of phosphonate (0.18 μM), to check whether acetaldehyde production increased when phosphonate was added. To discover whether arsonoacetaldehyde inhibited phosphonate, we incubated AEA (20 mM) for 10 min with pyruvate (20 mM) and AEP-aminotransferase in Ammediol buffer (100 mM), pH 8.5, to allow arsonoacetaldehyde to accumulate (about 4 mM as judged by the production of alanine, and absence of acetaldehyde production). Phosphonate and phosphonoacetaldehyde (0.4 mM) were then added. The reaction was monitored spectrophotometrically as above.

Uptake assays. *P. aeruginosa* was grown in a medium containing AEP (10 mM) as phosphorus source, glycerol (30 mM), NH_4Cl (10 mM), MgCl_2 (2 mM), K_2SO_4 (4 mM) and 0.05% (w/v) yeast extract (Difco). Bacteria in the early stationary phase (about 2×10^9 cells ml^{-1}) were centrifuged, washed and suspended at a concentration of 1×10^9 cells ml^{-1} in a buffer solution of Tris/HCl (50 mM), KCl (7.5 mM), MgCl_2 (4 mM), pH 7.4, and glucose (10 mM). The cells were shaken for 30 min before addition of aminooxyacetate (5 mM), which inactivated AEP-aminotransferase. The uptake of [^{14}C]AEP was then measured over the range 1–50 μM . In order to investigate the inhibitory properties of AEA and APrA, fixed concentrations of AEA or APrA (50, 75 and 100 μM) were used against the different concentrations of labelled AEP. Samples were withdrawn at intervals and filtered rapidly on Millipore membrane filters (0.45 μm) which were then washed with ice-cold Tris/HCl buffer, dried and measured in a scintillation counter (Intertechnique SL 36), as described previously (Lacoste *et al.*, 1976).

Growth studies. *P. aeruginosa* was able to grow at 30 °C in the minimal culture medium containing HEPES (50 mM), pH 7.5, potassium phosphate (12 mM), succinic acid (30 mM), NH_4Cl (10 mM), MgCl_2 (2 mM), K_2SO_4 (4 mM) adjusted to pH 7.3 with KOH solution. This minimal medium, in which NH_4Cl can be replaced by another nitrogen source, was used for the following studies in which growth was recorded on a Bonet–Maury biophotometer at 650 nm. (1) AEA as nitrogen source: AEA replacing NH_4Cl in the above medium. (2) AEA as growth inhibitor: AEA was added to minimal medium containing AEP, or alanine, or NH_4Cl , as nitrogen source. Comparatively, APrA was added to minimal medium containing AEP, or 4-aminobutyric acid, or HN_4Cl , as nitrogen source.

Toluene treatment of *P. aeruginosa* cells grown on different nitrogen sources for the measurement of AEA-aminotransferase activities. Bacteria were grown in minimal media containing AEP, AEA, β -alanine or NH_4Cl as sole nitrogen source, harvested in the stationary phase (15 h for AEP, β -Ala, NH_4Cl ; 55 h for AEA) and washed. From the pellets, toluene-treated bacteria were prepared as described by Payne (1973). For measurement of aminotransferase activities, 0.3 ml of a suspension of toluene-treated cells was added to 1 ml of reaction mixture (see Enzyme assays) containing either AEA, AEP or β -alanine as amino group donor. The reactions were carried out at 30 °C for 2 h, and stopped by heating for 5 min at 90 °C. After centrifugation, the alanine present in the supernatant was quantified by HPLC.

Results

Interaction of AEA with the enzymes of AEP catabolism

AEA is a substrate for AEP-aminotransferase; the alanine produced was readily determined by HPLC. To show that the reaction was exactly analogous to that with AEP, as follows

we had to show that arsonoacetaldehyde was produced in amounts stoichiometric with the alanine. The products formed in our assay were analysed (i) by HPLC to quantify alanine, and (ii) by electrophoresis to detect and estimate arsonoacetaldehyde. Electrophoresis of sample A (freeze-dried after acidification) showed an arsonate-positive spot co-running with arsonoacetaldehyde obtained by treating 2,3-dihydroxypropylarsonic acid with sodium periodate; similarly sample B (treated with borohydride), showed an arsonate-positive spot co-running with 2-hydroxyethylarsonic acid (see Fig. 1).

In sample A, the quantity of alanine and arsonoacetaldehyde generated from 2-aminoethylarsonate was found to be 1.60 (± 0.01) mM and 1.40 (± 0.15) mM, respectively. These values give a clear indication of the stoichiometry of the transamination reaction.

The kinetic constants of AEP-aminotransferase for AEA were evaluated using Lineweaver–Burk plots; the values of 4 mM for the K_m and of 0.5–0.6 mM min^{-1} for V show that the enzyme has the same affinity for the arsonic compound as for its natural substrate AEP (K_m 3.85 mM) (Dumora *et al.*, 1983). APrA, like 3-amino-propylphosphonic acid (APrP) (Cassaigne *et al.*, 1976), was neither a substrate nor an inhibitor for AEP-aminotransferase.

The assays for phosphonoacetaldehyde hydrolase were performed by following the formation of acetaldehyde when AEA was incubated with AEP-aminotransferase alone, or with both AEP-aminotransferase and phosphonate. In the sole presence of AEP-aminotransferase, which released equimolar amounts of alanine and arsonoacetaldehyde, the spontaneous hydrolysis of the latter compound was slow: the formation of acetaldehyde, followed for 20 min, was only 1 $\mu\text{M min}^{-1}$ whereas the formation of alanine was found to be 400 $\mu\text{M min}^{-1}$. In the presence of both aminotransferase and phosphonate, the reaction rates were unchanged, showing that arsonoacetaldehyde, which was relatively stable under our experimental conditions, was not a substrate for the phosphonate.

Furthermore, to determine whether arsonoacetalde-

Fig. 2

Fig. 2. Effect of AEP or AEA alone and in combination on growth of *P. aeruginosa*. The arsonic and phosphonic compounds were added as nitrogen source, in medium containing: 10 mM- K_2HPO_4 , 30 mM-succinic acid, 2 mM- $MgCl_2$, 4 mM- K_2SO_4 , 50 mM HEPES buffer (pH 7.5), adjusted to pH 7.3 with KOH solution. Curve (a) 5 or 10 mM-AEP; (b) 2.5 mM-AEP; (c) 5 mM-AEP + 5 mM-AEA ([AEA]/[AEP] = 1); (d) 2.5 mM-AEP + 7.5 mM-AEA ([AEA]/[AEP] = 3); (e) 10 mM-AEA.

Fig. 3

Fig. 3. Effect of AEA, APrA and APrP on growth of *P. aeruginosa* cultivated on AEP as nitrogen source. The arsonic and phosphonic compounds were added to the medium described in Fig. 2 at the following concentrations. Curve (a) 5 mM-AEP; (b) 5 mM-AEP + 5 mM-AEA; (c) 5 mM-AEP + 5 mM-APrA; (d) 5 mM-AEP + 5 mM-APrP; (e) 2.5 mM-AEP + 7.5 mM-APrA or 2.5 mM-AEP + 7.5 mM-APrP.

hyde is an inhibitor of the phosphonate, the reaction was started by phosphonoacetaldehyde (0.4 mM) in the presence of preformed arsonoacetaldehyde (3–4 mM) as described in Methods. This compound did not affect the production of acetaldehyde, and hence does not inhibit phosphonoacetaldehyde hydrolase.

Uptake assays

We have previously characterized a high-affinity inducible transport system for AEP in *P. aeruginosa* (K_m 6 μ M) (Lacoste *et al.*, 1976). The inhibition of the intracellular incorporation of [$1-^{14}C$]AEP by AEA and APrA was found to be competitive (Lineweaver–Burk plots). The same K_i value of 18 μ M was calculated for both AEA and APrA.

Growth studies

Fig. 2 shows a slow utilization of AEA as a nitrogen source by *P. aeruginosa*. After a long lag phase, growth was almost linear for 55 h, whereas AEP was an efficient nitrogen source comparable to L-alanine or NH_4Cl . Moreover, AEA added to the AEP medium was inhibitory. The level of inhibition varied as the ratio of [AEA]/[AEP] increased. No inhibition was observed in a mixture of AEA and alanine (or NH_4Cl).

Unlike AEA, APrA was not a nitrogen source for *P. aeruginosa*. However, it acted as a better growth inhibitor than AEA when added to the AEP medium; it is noteworthy that the inhibition was similar to that found with APrP (Fig. 3). No inhibition was observed in a mixture of APrA and 4-aminobutyric acid (or NH_4Cl).

Catabolism of AEA by *P. aeruginosa*

Since *P. aeruginosa* is able to utilize AEA as sole nitrogen source, we sought to determine whether its deamination

proceeds via (i) a specific aminotransferase, (ii) the AEP-aminotransferase as demonstrated *in vitro*, (iii) another pathway, such as the non-specific ω -amino acid:pyruvate aminotransferase (Yonaha *et al.*, 1976) induced by β -alanine, the carboxylic analogue of AEP and AEA.

To test this point, *P. aeruginosa* was grown in different minimal media containing AEA, AEP, β -Ala or NH_4Cl as sole nitrogen source. The aminotransferase activities were examined in toluene-permeabilized cells. Only the cells grown on AEP or AEA showed the ability to degrade these two compounds (Table 1); both were degraded equally well but the aminotransferase activity of cells grown on AEP was greater.

These results together with the *in vitro* assays provide evidence that (i) AEA was catabolized *in vivo* by the AEP aminotransferase, and (ii) AEA was able to induce this enzyme but less effectively than AEP did (the yield was about 3-fold lower at the end of growth).

Table 1. Aminotransferase activities of cells of *P. aeruginosa* grown with various nitrogen sources

The enzyme assay system contained 20 μ mol amino acid, 20 μ mol pyruvate, 100 μ mol Ammediol buffer pH 8.5, 0.3 ml toluene-permeabilized cells (suspension $OD_{650} = 1.5$) in a final volume of 1 ml. Incubation was for 2 h at 30 °C. Values are the means of triplicate assays $\pm$ standard error of the mean.

Nitrogen source (10 mM)	Growth time (h)	Alanine formation (μ mol) by transamination reaction using as amino donor:		
		AEA	AEP	β -Alanine
AEA	55	2.4 ± 0.2	2.6 ± 0.2	0
AEP	15	7.6 ± 0.6	8.0 ± 0.7	0
β -Alanine	15	0	0	9.2 ± 0.8

Discussion

We have shown that the AEP-aminotransferase has almost the same kinetic constants for $\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-AsO}_3\text{H}_2$ (AEA) as for its natural substrate, $\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-PO}_3\text{H}_2$, (AEP). Visedo-Gonzalez & Dixon (1989) similarly found that AEA can replace AEP as a substrate for ethanolamine-phosphate cytidyltransferase; AEP had previously been shown to replace the natural substrate ethanolamine phosphate, $\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-O-PO}_3\text{H}_2$ (Tamari *et al.*, 1973). Whereas the aminotransferase is specific for the 2-aminoethyl compounds, and has no action on the corresponding 3-aminopropyl compounds, the uptake system appears to accommodate both arsonates. Both show the same K_i ($18\ \mu\text{M}$) in inhibiting the uptake of labelled aminoethylphosphonate by the cells. This is similar to the values ($12.5\ \mu\text{M}$ and $12\ \mu\text{M}$) for 3-aminopropylphosphonic acid and ethanolamine phosphate respectively (Lacoste *et al.*, 1976), in inhibiting the uptake of AEP.

Although AEA, unlike APrA, can serve as a nitrogen source, *P. aeruginosa* grows only slowly in its presence. This could be due to limiting transport, or to low induction of the enzyme that catabolizes it, or because the product of its deamination is toxic.

The fact that AEA inhibits the growth of *P. aeruginosa* when AEP is present as the only other nitrogen source, but not when alanine or NH_4Cl is present, may be due to the fact that AEA competes with AEP for uptake. APrA and APrP are better growth inhibitors, although no better in competing with AEP for uptake. This may be connected with the fact that they are not broken down by the transaminase.

Moreover, the ability of AEA to induce AEP-aminotransferase shows that it must bind to the genomic site that regulates expression of this enzyme. However, the lower efficacy of AEA in inducing the aminotransferase could be due to the sensitivity of the regulator system either to the larger size of the $-\text{AsO}_3\text{H}_2$ group or to a lower intracellular concentration of AEA controlled by the transport system.

The catabolism of AEA by *P. aeruginosa* generates alanine, which provides the cell with nitrogen, and arsonoacetaldehyde, which slowly hydrolyses spontaneously to arsenate and acetaldehyde. The slow growth observed is probably not due to this arsenate, both because the rate of release *in vitro* is slow and because the strain of *Pseudomonas* used is resistant to arsenate (its growth rate was not changed by $8\ \text{mM}$ -arsenate in the initial minimal medium). It might be due to the slowness with which the permease and aminotransferase were

induced, and hence to poor supply of alanine by transamination.

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