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EXPERIMENTAL ARTICLES

Phosphonoacetate Biosynthesis: In vitro Detection of a Novel NADP$^+$-Dependent Phosphonoacetaldehyde-Oxidizing Activity in Cell-Extracts of a Marine *Roseobacter*[1]

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Abstract—A novel phosphonoacetaldehyde-oxidizing activity was detected in cell-extracts of the marine bacterium *Roseovarius nubinhibens ISM* grown on 2-aminoethylphosphonic acid (2-AEP; ciliatine). Extracts also contained 2-AEP transaminase and phosphonoacetate hydrolase activities. These findings indicate the existence of a biological route from 2-AEP via phosphonoacetaldehyde for the production of phosphonoacetate, which has not previously been shown to be a natural product. The three enzymes appear to constitute a previously-unreported pathway for the mineralization of 2-AEP which is a potentially important source of phosphorus in the nutrient-stressed marine environment.

Keywords: phosphonoacetaldehyde dehydrogenase; phosphonoacetate biosynthesis; 2-aminoethylphosphonate.

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INTRODUCTION

Phosphonic acids are a class of organophosphorus compound characterised by the presence of a direct carbon-phosphorus (C–P) bond. Biogenic phosphonates are widely distributed in the living world, especially in more primitive organisms such as marine invertebrates [1]. They have a variety of biological roles, most commonly acting in a structural capacity, as peptide, glycan and lipopolysaccharide membrane conjugates: 2-aminoethylphosphonic acid (2-AEP; ciliatine), the most predominant biogenic C–P compound, is oftentimes found as a membrane lipid headgroup analogous to phosphatidylethanolamine [2–4].

Of particular importance however are those phosphonates which are synthesised as secondary metabolites by a wide range of both bacteria and fungi. Due to the structural similarity of the phosphonate moiety to the carbon-oxygen-phosphorus motif many of these phosphonate natural products have application in the agricultural, industrial and medical sectors. Examples include the broad-spectrum, cell wall-active antibiotic fosfomycin and the glutamine synthetase-inhibiting herbicide phosphinothricin (bialaphos) [3]. Not surprisingly this biotechnological potential has resulted in the manufacture of synthetic phosphonates, the most extensively used of which is the herbicide glyphosate.

Numerous studies have addressed microbial organophosphonate biosynthesis [3]. These have for the most part identified phosphonoacetaldehyde as a key building block in the anabolism of biogenic organophosphonates: formation of phosphonoacetaldehyde occurs via rearrangement of phosphonenolpyruvate to phosphonopyruvate (catalysed by phosphonenolpyruvate phosphomutase; E.C. 5.4.2.9) and subsequent decarboxylation by phosphonopyruvate decarboxylase (E.C. 4.1.1.82.) to yield phosphonoacetaldehyde. Currently there are three known routes by which phosphonoacetaldehyde is further transformed: (1) transamination to yield 2AEP; (2) reduction to generate 2-hydroxyethylphosphonate; and (3) through an aldol reaction with oxaloacetate to generate 2-keto-4-hydroxy-5-phosphonopentanoic acid [3].

In addition to playing a central role in phosphonate anabolism, phosphonoacetaldehyde is also an intermediate in one of the three known routes of 2AEP catabolism whereby biodegradation proceeds via the pyruvate-dependent transamination of 2AEP to yield phosphonoacetaldehyde (catalysed by 2AEP : pyruvate aminotransaminase; EC 2.6.1.37) and its subsequent hydrolytic cleavage by phosphonoacetaldehyde hydrolase ("phosphonatase"; EC 3.11.1.1) [5] to

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release acetaldehyde and orthophosphate (Pi). Expression of this 2AEP degradative operon, commonly known as the phosphonatase pathway, in many bacteria is *pho* regulon-controlled i.e. P starvation inducible and hence Pi-repressible [6]. Under such conditions 2AEP can serve as an alternative P source during conditions of P limitation. In some instances however expression of this operon is independent of the Pi status of the cell thus facilitating complete mineralisation of 2AEP [7, 8].

While studying the diversity of 2AEP metabolism amongst marine microorganisms we detected a further route for phosphonoacetaldehyde transformation in cells of *Roseovarius nubinhibens* ISM, which involves its oxidation to phosphonoacetate via a novel enzyme activity "phosphonoacetaldehyde dehydrogenase".

This report represents the first in vitro detection of this hitherto unrecognised enzymatic activity and points for the first time to a biogenic source of phosphonoacetate (previously thought to be only of synthetic origin being produced industrially as an antiviral agent).

MATERIALS AND METHODS

Microorganism and culture conditions. Cells of *Roseovarius nubinhibens* ISM (DSM 15170; obtained from DSMZ, Braunschweig, Germany) were grown in batch cultures at 28°C on an orbital shaker at 100 rpm in 50% v/v minimal Artificial Sea Water [9]. The pH of the medium was adjusted to pH 7.0 using 50 mmol l^{-1} HEPES-KOH buffer. Acetate (10 mmol l^{-1}) was supplied as sole carbon and energy source, while 2-AEP or phosphate buffer (each at 1 mmol l^{-1}) served as P sources. All glassware was stripped of contaminating phosphorus by overnight soaking in 2% Decon (Decon Laboratories, Hove UK) and rinsed repeatedly with distilled water. Microbial growth was measured by the increase in optical density at 650 nm (OD650) using a ATI-Unicam 8265 UV/VIS spectrophotometer (Pye-Unicam Ltd., Cambridge, UK) and by measurement of the increase in total culture protein concentration [10]. Pi release into the culture supernatant was monitored via the method of Fiske and Subbarow [11].

Preparation of cell-extracts and enzyme assays. Cell-extracts of either 2AEP or Pi-grown *R. nubinhibens* ISM cells were prepared by sonication as previously described [12]. The resulting homogenate was centrifuged at 25 000 g for 30 min at 4°C and the ensuing supernatant or crude extract stored at −20°C until required. Protein concentration was determined by the method of Bradford [13] using bovine serum albumin as standard.

Phosphonoacetaldehyde hydrolase (EC 3.11.1.1) was assayed as described by Ternan and Quinn [8] while the activity of phosphonoacetate hydrolase (EC 3.11.1.2) was determined via the method of McGrath et al. [12]. All assays were carried out in triplicate with activities expressed as nanomoles of phosphate liberated min^{-1} mg cell extract $protein^{-1}$. Acetic acid was measured using the Boehringer Mannheim Acetic Acid Test Kit (Boehringer Mannheim) with a lower detection limit of 0.3 mg l^{-1}. Acetaldehyde was determined using the spectrophotometric method of Toyama et al., [14] based on its reaction with *o*-aminobenzaldehyde. 2AEP : pyruvate aminotransaminase (EC 2.6.1.37) was assayed according to Ternan and Quinn [8]; values are reported as nmoles alanine produced min^{-1} mg cell extract $protein^{-1}$ and are the mean of triplicates.

Phosphonoacetaldehyde dehydrogenase (this study) was assayed by modification of the fluoroacetaldehyde dehydrogenase method of Murphy et al. [15]. Briefly dehydrogenase activity was measured at 35°C by monitoring the formation of NADPH at 340 nm when cell-extracts prepared from 2AEP grown cells of *Roseovarius nubinhibens* ISM (containing 0.2–0.4 mg protein) were incubated with $NADP^+$ (1 mmol l^{-1}) and phosphonoacetaldehyde (2 mmol l^{-1}) in 100 mM *Tris*-HCl buffer pH 7.5, in a final volume of 1.0 ml. Values reported are the means of triplicate samples, each assayed in duplicate and expressed as nanomoles of $NADP^+$ reduced to NADPH min^{-1} mg cell extract $protein^{-1}$: Assays in which either cell-extract or substrate had been omitted were used to determine the background levels of $NADP^+$ reduction. In determining the substrate specificity of the enzyme a range of other aldehydes were also tested under the same conditions, each at a concentration of 2 mM. For all enzyme assays control experiments were carried out in the absence of substrate or cell-extract.

Effect of pH, temperature and NAD^+/$NADP^+$ on phosphonoacetaldehyde dehydrogenase. The phosphonoacetaldehyde dehydrogenase temperature optima was established by assay at different temperatures over the range 25 to 55°C: pH optimum was determined by using 50 mmol l^{-1} succinate/sodium-hydroxide buffer, 50 mmol l^{-1} *Tris*-HCl buffer and 50 mmol l^{-1} glycine/sodium-hydroxide buffer, for pH ranges 4.0 to 5.8, 7.0 to 9.0, and 9.0 to 10.6 respectively. A comparison of the requirement for either NAD^+ or $NADP^+$ (1 mmol l^{-1} respectively) was also investigated.

RESULTS

Growth of *Roseovarius nubinhibens* ISM on 1 mM 2AEP as a sole source of *P* was accompanied by the concomitant release of up to 39% organophosphonate-derived Pi (Fig. 1). No spontaneous Pi release was observed in either uninoculated control experiments or from cultures incubated in the absence of substrate.

Cell-extracts, prepared from mid/late log phase cells of *R. nubinhibens ISM* grown on 1 mmol l^{-1} 2-

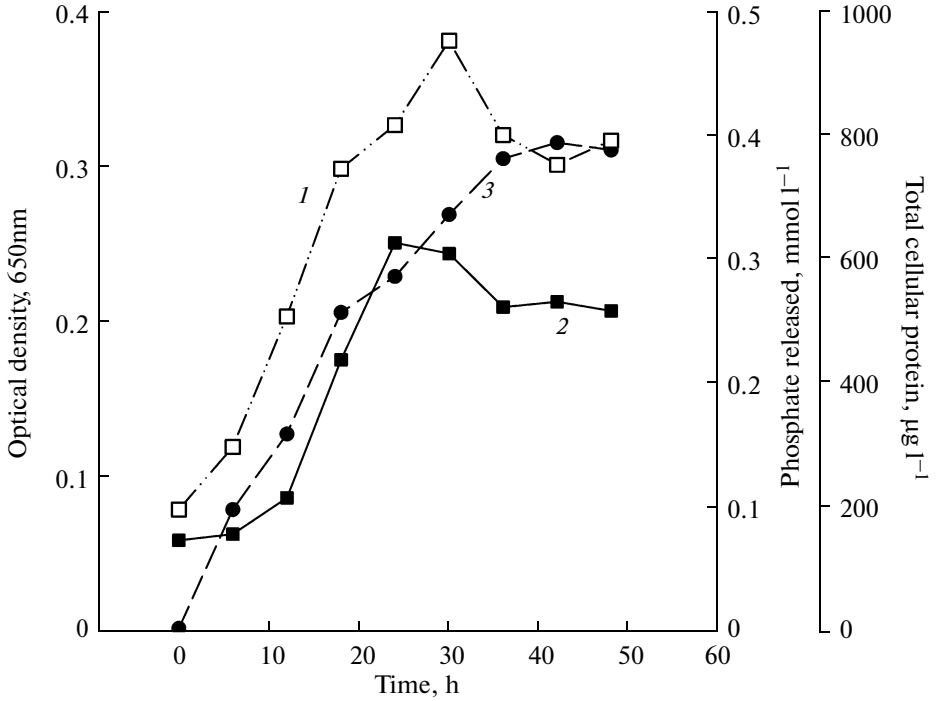

Fig. 1. Growth of *Roseovarius nubinhibens* ISM on 2-aminoethylphosphonic acid (1 mmol l^{-1}) as the sole phosphorus source. Cultures were incubated at 28°C on an orbital shaker at 100 rpm. Growth was measured both by the increase in optical density at 650 nm (*1*) and by the increase in total cellular protein (*2*). Phosphate release into the culture supernatant (*3*) was determined by the method of Fiske and SubbaRow [11].

AEP as a sole *P* source, were assayed for those enzyme activities associated with the phosphonatase pathway of 2AEP degradation i.e. 2AEP : pyruvate aminotransaminase and phosphonoacetaldehyde hydrolase. Although a 2AEP : pyruvate aminotransaminase activity of up to 70 nmoles alanine produced min^{-1} mg cell extract $protein^{-1}$ was routinely observed, no phosphonoacetaldehyde hydrolase activity could be detected; this correlated with a failure to detect acetaldehyde (the organic product of phosphonoacetaldehyde hydrolase mediated C–P bond cleavage) in phosphonoacetaldehyde (2 mmol l^{-1}) supplemented cell-free assays.

Cell-extracts from 2AEP grown *R. nubinhibens* ISM cells did however contain a putative soluble phosphonoacetaldehyde dehydrogenase activity (Fig. 2) which was dependent on the presence of both $NADP^+$ and phosphonoacetaldehyde. The rate of $NADP^+$ reduction was proportional to the amount of cell-extract protein present (Fig. 3) and to the assay time (Fig. 2). Highest activities were obtained when the assay was carried out at a temperature of 45°C and a pH of 8.5. In the course of three separate experiments phosphonoacetaldehyde dehydrogenase activities of up to 26.1 nanomoles $NADP^+$ reduced min^{-1} mg cell extract $protein^{-1}$ were found. Activity levels were stable in extracts stored at –20°C for periods of up to at least a week. When NAD^+ was supplied as an

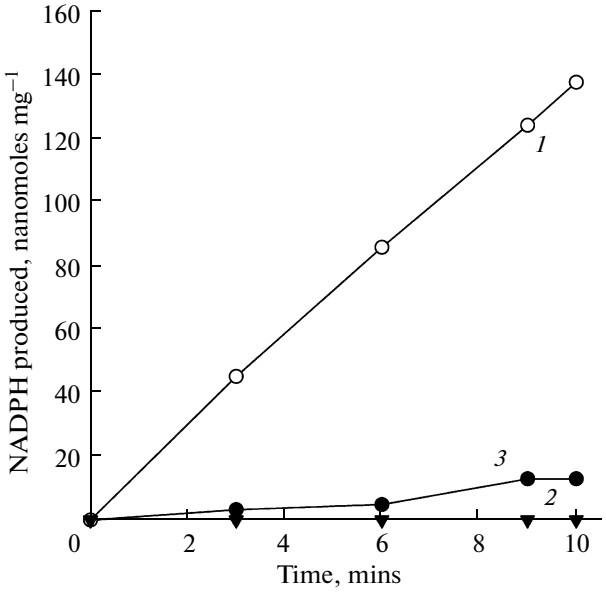

Fig. 2. Production of NADPH from phosphonoacetaldehyde (2 mmol l^{-1}) by the phosphonoacetaldehyde dehydrogenase activity of *Roseovarius nubinhibens* ISM with time (0–10 min) at 30°C (*1*). NADPH production from control assays incubated in the absence of cell-extract (*2*) and phosphonoacetaldehyde (*3*) are also shown.

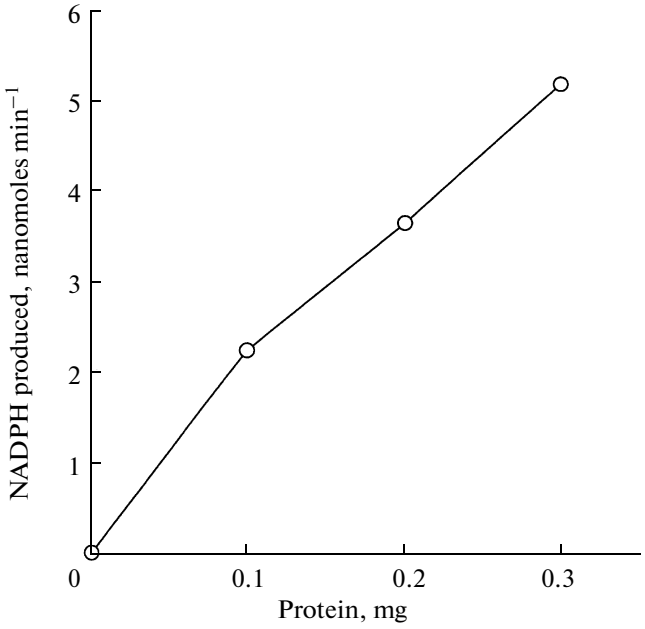

Fig. 3. Production of NADPH from phosphonoacetaldehyde (2 mmol l^{-1}) by the phosphonoacetaldehyde dehydrogenase activity of *Roseovarius nubinhibens* ISM in the presence of varying protein concentrations at 30°C for 40 minutes.

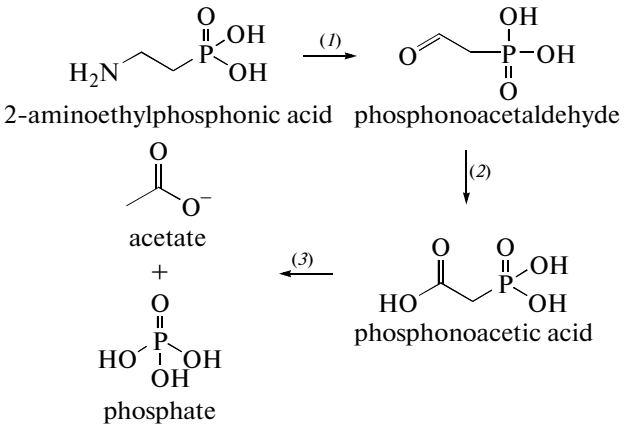

Fig. 4. Proposed novel route of 2AEP mineralisation. Transamination of 2AEP to phosphonoacetaldehyde (catalysed by 2AEP : pyruvate aminotransaminase (*1*); EC 2.6.1.37) is followed by oxidation of phosphonoacetaldehyde to phosphonoacetate by phosphonoacetaldehyde dehydrogenase (*2*: this study). Finally, cleavage of phosphonoacetate by phosphonoacetate hydrolase (*3*: EC 3.11.1.2) yields acetate and Pi.

alternative to $NADP^+$ in the assay the rate of coenzyme reduction fell by 71%. The dehydrogenase had a wide substrate spectrum; relative to phosphonoacetaldehyde, oxidation rates for sulphoacetaldehyde, formaldehyde, acetaldehyde, pyruvaldehyde and propionaldehyde were, respectively, 79, 59, 57, 44 and 37%.

In addition to 2AEP : pyruvate aminotransaminase and the putative phosphonoacetaldehyde dehydrogenase, phosphonoacetate hydrolase was also detected in cell-extracts of 2AEP grown *R. nubinhibens* ISM cells. Activity values of up to 68.9 nanomoles min^{-1} mg^{-1} cell-extract protein were observed with concomitant acetate and phosphate release occurring in the phosphonoacetate supplemented assays. Furthermore in vitro Pi release from 2AEP was also observed in assays supplemented with all the necessary components required for the operation of the 2AEP : pyruvate aminotransaminase, phosphonoacetaldehyde dehydrogenase and phosphonoacetate hydrolase activities: acetate was detected as the organic product.

DISCUSSION

Phosphonoacetaldehyde is a key intermediate in microbial organophosphonate metabolism undergoing either: transamination, reduction or an aldol reaction with oxaloacetate in phosphonate biosynthesis, or hydrolytic cleavage of the constituent C–P bond during catabolism of 2AEP via the phosphonatase pathway. Our identification of phosphonoacetaldehyde dehydrogenase, in cells of *Roseovarius nubinhibens* ISM grown on 2AEP as a sole source of P, provides evidence for a previously-undescribed oxidation route of phosphonoacetaldehyde transformation which leads to the formation of phosphonoacetate (Fig. 4). In *R. nubinhibens* ISM this dehydrogenase activity appears to form part of a novel route for 2AEP mineralisation which involves transamination of 2AEP to phosphonoacetaldehyde (catalysed by 2AEP : pyruvate aminotransaminase), oxidation of phosphonoacetaldehyde to phosphonoacetate by phosphonoacetaldehyde dehydrogenase (this study) and hydrolytic cleavage of phosphonoacetate by phosphonoacetate hydrolase to yield acetate and Pi (Fig. 4); each of these enzyme activities was detected in crude cell-extracts prepared from 2AEP grown *R. nubinhibens* ISM cells. Additionally both acetate and Pi were routinely detected in cell-free extracts supplemented with either 2AEP, phosphonoacetaldehyde, or phosphonoacetate as substrates. As growth of *R. nubinhibens* ISM on 2-AEP as a sole P source proceeds with the concomitant release of Pi into the growth medium (Fig. 1), this suggests that this 2AEP mineralization pathway is not under classical *pho* regulon control.

Our demonstration of a bacterial phosphonoacetaldehyde dehydrogenase-mediated pathway of 2-AEP catabolism by microorganisms indicates that degradation may proceed by a route that is similar to that of its sulfonate analogue taurine (2-aminoethylsulfonic acid). Inducible sulfoacetaldehyde dehydrogenase activity was detected by Denger et al. [16] and Krejcik et al. [17] in taurine-grown cells of *Rhodopseudomonas palustris*, and *Neptuniibacter caesariensis* MED92, although in both organisms the sulfoacetate produced was quantitatively excreted. However this may well be

the case for phosphonoacetate in microorganisms that possess 2AEP : pyruvate aminotransaminase and phosphonoacetaldehyde dehydrogenase but, unlike *Roseovarius nubinhibens* ISM, lack phosphonoacetate hydrolase. Such an explanation would account for the existence of a substrate-inducible pathway of phosphonoacetate degradation in some microorganisms e.g. *Pseudomonas fluorescens* 23F [18, 19].

Sulfoacetaldehyde dehydrogenase from *Neptuniibacter caesariensis* MED92 was purified and characterized [17]; the natural electron acceptor was NAD^+ and the enzyme showed no activity towards phosphonoacetaldehyde, or any other aldehyde tested. By contrast the phosphonoacetaldehyde dehydrogenase activity we report from *Roseovarius nubinhibens* ISM is $NADP^+$-linked and has a comparatively broad range of potential substrates. Its properties are consistent with those of similar NAD(P)-dependent aldehyde dehydrogenases identified in a wide range of bacterial sources [20–22] and often associated with metabolic detoxification.

The demonstration of a phosphonoacetaldehyde dehydrogenase activity also points for the first time to a biogenic source of phosphonoacetate, which was previously thought to exist only as a synthetic molecule (produced industrially in comparatively small quantities, for example as an antiviral agent). The lack of any identifiable biological source of phosphonoacetate has been puzzling [4], given the unique substrate specificity of phosphonoacetate hydrolase [12] and the fact that phosphonoacetate hydrolase (*phnA*) genes and transcripts have been shown to be widely distributed in both soil and marine environments [23–25]. Moreover the fact that *phnA* homologues are more abundant in marine metagenomic libraries than those of *phnX*, which encodes phosphonoacetaldehyde hydrolase [4] points to the likely ecological relevance of the novel pathway.

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