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Evidence for two distinct phosphonate-degrading enzymes (C-P lyases) in *Arthrobacter* sp. GLP-1

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Abstract

Arthrobacter sp. GLP-1 can utilize a wide range of organophosphonates as its sole source of phosphorus. The in-situ formation of sarcosine and methane from glyphosate and methanephosphonic acid respectively was studied. These two processes are differentially induced during phosphorus-deprivation. Methanephosphonic acid strongly inhibits glyphosate degradation (I_{50} 10 μM), but glyphosate has very little effect on methane generation (I_{50} 150 mM). The pattern of inhibition by other organophosphonates and organophosphonate analogues is also very different for the two systems. Degradation of glyphosate and methanephosphonic acid therefore represent distinct processes.

Abbreviations: f.wt. – fresh weight, MP-lyase – methanephosphonate lyase

Introduction

Since the first isolation of a natural phosphonic acid derivative in 1959 (Horiguchi & Kandatsu 1959), a number of other members of this class of compounds have been discovered. Phosphonates are present in nature as phosphonolipids, as fosfomycin, phosphinothricin and other phosphonate antibiotics, and as 2-aminoethylphosphonic acid. In addition to the natural occurrence of these compounds, increasing amounts of man-made phosphonates are now entering the environment. These are important industrially as flame retardants, surfactants, plasticizers, chelating agents etc., and are also a vital part of modern agriculture in the form of the phosphonate or phosphinate insecticides and the broad-spectrum herbicides glyphosate and glufosinate (Hilderbrand 1983).

Since these compounds are ubiquitous in the environment, it might be expected that microorganisms may have evolved mechanisms for their degradation, and indeed, phosphonate breakdown has been observed in a number of different bacterial species (Cook et al. 1978; Moore et al. 1983; Shinabarger et al. 1984; Talbot et al. 1984; Balthazor & Hallas 1986; Wackett et al. 1987; Pipke et al. 1987a; Quinn et al. 1989). These range from enteric to soil bacteria, and include both Gram-positive and Gram-negative species. Mechanistically, two types of degradation are known, differing in the nature of the substrate used. For 2-aminoethylphosphonate, a specific enzyme system has been purified and characterized, which catalyzes the degradative process via transamination to phosphonoacetaldehyde and subsequent hydrolysis (LaNauze et al. 1970; LaNauze et al. 1977). For unacti-

vated phosphonates, however, this route is not available. Breakdown of the phosphonate seems to proceed by direct carbon-phosphorus cleavage (Cordeiro et al. 1986; Shinabarger & Braymer 1986; Avila et al. 1987; Kishore & Jacob 1987; Pipke et al. 1987a), but since this bond is chemically inert (C-P bonds are resistant to strong acid and base hydrolysis (Horiguchi 1984), the mechanism involved is unclear. From chemical model studies (Frost et al. 1987; Shames et al. 1987) it has been suggested that redox processes may play a role, and a radical-based mechanism has been proposed (Cordeiro et al. 1986).

A recurring problem in the study of the degradative mechanism is the lack of a reliable cell-free system. Several groups have reported studies aimed at isolating the 'C-P lyase' enzyme (Moore et al. 1983; Pipke et al. 1987a, b; Shames et al. 1987), but the only in vitro activity that has been obtained to date reported phosphate release in the presence of phosphonoacetate, and the fate of the organic moiety was not determined (Murata et al. 1988; Murata et al. 1989). Otherwise, in vivo systems have been employed, and phosphonate degradation has been monitored as cell growth in the absence of another phosphorus source, by gas chromatography of volatile products (Wackett et al. 1987; Cook et al. 1978), or by use of radiolabelled substrates (Pipke & Amrhein 1988b). Molecular biological methods have been used to identify and sequence the genes responsible for alkylphosphonate degradation in *Escherichia coli* (Chen et al. 1990; Wanner & Boline 1990). The seventeen genes found were under the apparent control of a Pho-box like promoter sequence, consistent with the observation that in several species phosphonate utilization is regulated by levels of phosphate (Pipke et al. 1987a, b; Wanner 1987).

However, though *E. coli* grows well on many alkyl phosphonates when induced, it cannot degrade the herbicide glyphosate (*N*-phosphonomethylglycine), even when supplied with the aromatic amino acids whose synthesis is inhibited by this compound. Of a number of glyphosate-degrading species that have been isolated by enrichment techniques, *Arthrobacter* sp. GLP-1 (Pipke et al. 1987a) has one of the broadest spectra of activity known to date (Pipke & Amrhein 1988a; Schowanek & Verstraete 1990). The only phosphonate yet found on which this species cannot grow is the sterically hindered *tert*-butylphosphonate (this paper). Uptake and breakdown of glyphosate by strain GLP-1 have previously been shown to be separate processes (Pipke & Amrhein 1987a); in this paper we demonstrate that *Arthrobacter* sp. GLP-1 contains distinct C-P lyases for methanephosphonate and glyphosate degradation.

Materials and methods

Chemicals and culture of bacteria

Glyphosate (free acid, 99.7% pure) was provided by Monsanto Agricultural Products Co., St Louis, USA. [3-^{14}C]Glyphosate (specific activity 2.1 GBq/mmol) was obtained from Amersham-Buchler, Braunschweig, Germany. *N*-methylglyphosate, *N*-phosphonoethylglycine, 4-phosphono-3-oxabutyrate, 4-phosphonobutyrate and *N*-sulfonomethylglycine were the gift of Monsanto Co., St Louis, Mo. Methanediphosphonic acid and benzenearsonic acid were obtained from Fluka (Buchs, Switzerland), and hydroxymethylphosphonic acid was from Ventron (Karlsruhe, Germany). All other phosphonates were purchased from Sigma (Munich, Germany), or Aldrich (Steinheim, Germany).

[3-^{14}C]-Glyphosate was purified by HPLC before use (column: MonoQ; elution buffer: 20 mM Tris/HCl pH 7.2, 0–200 mM linear KCl gradient). All other phosphonates were used as received, after checking that they did not contain any free phosphate (Lanzetta et al. 1979).

Arthrobacter sp. strain GLP-1 was isolated previously (Pipke et al. 1987a), and was routinely grown as described (Pipke et al. 1987b) on a synthetic medium based on that of Winkler and de Haan (1948). This contained 10 g l^{-1} glucose, 2 g l^{-1} NH_4Cl, 50 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 5 mg l^{-1} $FeSO_4 \cdot 7H_2O$, 0.2% (v/v) of a trace elements solution (Amrhein & Filner 1973), 0.1 M Tris/HCl, pH 7.2, and 1 mM glyphosate or methanephosphonic acid as the sole phosphorus source. All glassware, including headspace vials for gas chromatography,

was washed with 1 M HCl and thoroughly rinsed with glass-distilled, deionized water before use, to remove any contaminating phosphate.

Glyphosate uptake experiments

Uptake of labelled glyphosate by *Arthrobacter* sp. GLP-1 was measured by a modification of the method of Pipke & Amrhein (1987b). Cells from the logarithmic growth phase were harvested by centrifugation (7000 g, 10 min), washed once with phosphorus-free growth medium, and resuspended in the same medium. The cells were then starved of phosphorus (30° C, 220 rpm) for 3–4 hours, before being collected and washed once more, and resuspended in 50 mM Tris/HCl, pH 7.2 at a concentration of 250 mg f.wt.ml^{-1}.

The uptake assay (total volume 500 μl) contained 33 μM glyphosate (650 Bq), 50 mM Tris/HCl, pH 7.2 and the respective phosphonate test compound. The reaction was started by addition of 20 μl cell suspension. After incubation at 37° C for appropriate times, aliquots (70–240 μl) were removed, filtered by suction through membrane filters (0.45 μm pore size, cellulose mixed ester, Schleicher & Schuell, Switzerland) and washed rapidly with 5 ml of ice-cold 50 mM Tris/HCl, pH 7.2. The wet filters were transferred to 4 ml of scintillation fluid, and the radioactivity determined after one hour by scintillation counting.

Glyphosate degradation experiments

The rate of degradation of labelled glyphosate to sarcosine was determined by a method based on that of Pipke & Amrhein (1988b). The assay mixture (final volume 50 μl) contained 10 mM sodium acetate to inhibit sarcosine oxidase activity, 2% (v/v) dimethylsulfoxide, 33 μM glyphosate (400 Bq), 50 mM Tris/HCl, pH 7.2 and the respective test compound. The reaction was started by addition of 20 μl of the phosphorus-starved cell suspension described above, and incubation continued at 30° C for 30 minutes. After addition of acetone (50 μl) to terminate the reaction and centrifugation (10000 g, 5 min), the mixture was concentrated to ca. 10 μl and 5 μl aliquots were subjected to thin layer chromatography on cellulose plates (solvent: 7 : 2 : 1 methanol/water/acetic acid, saturated with EDTA). Radioactive glyphosate (R_f 0.45) and sarcosine (R_f 0.68) were quantified using a Berthold Tracemaster 20 radioscanner.

Methanephosphonic acid degradation experiments

Arthrobacter sp. GLP-1 was first adapted to growth in liquid medium on 1 mM methane-phosphonic acid as sole phosphorus source. These cells were then depleted of phosphorus as described above, and resuspended in 50 mM Tris/HCl, pH 7.2 at 250 mg f.wt.ml^{-1}. Samples (50–100 μl) of this suspension were then used to start the assay reaction (final volume 500 μl). This contained 70 mM Tris/HCl, pH 7.2, 2% (v/v) dimethylsulfoxide, 100 μM methanephosphonic acid and the respective test compound, in 1.5 ml screwcap headspace vials sealed with Teflon coated silicon seals (Alltech, Pully, Switzerland). The mixture was incubated at 30° C for 20 minutes, and 50 μl of the headspace gas was then removed with a gastight syringe, and methane determined by gas chromatography (column: Porapak R, 2mx1/8'; temp: 125° C; carrier gas: nitrogen; detector: FID).

Other analytical procedures

Protein was determined colorimetrically (Bradford 1976), using bovine serum albumin as standard. Inorganic phosphate was measured using the procedure of Lanzetta et al. (1979).

Results and discussion

As for several other organisms, attempts to demonstrate C-P lyase activity in cell-free extracts of *Arthrobacter* sp. GLP-1 have been unsuccessful in recent years. However, previous work with this strain considered only glyphosate degradation, and since the strain grows well on a broad range of

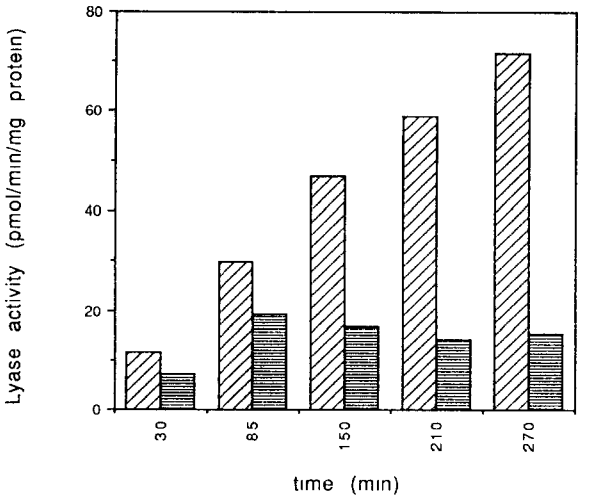

Fig. 1. C-P lyase activities in *Arthrobacter* sp. GLP-1 at various times after transfer from growth medium containing 1 mM glyphosate to medium without a source of phosphorus. Methane release from methanephosphonate (diagonal hatching); sarcosine production from glyphosate (horizontal hatching).

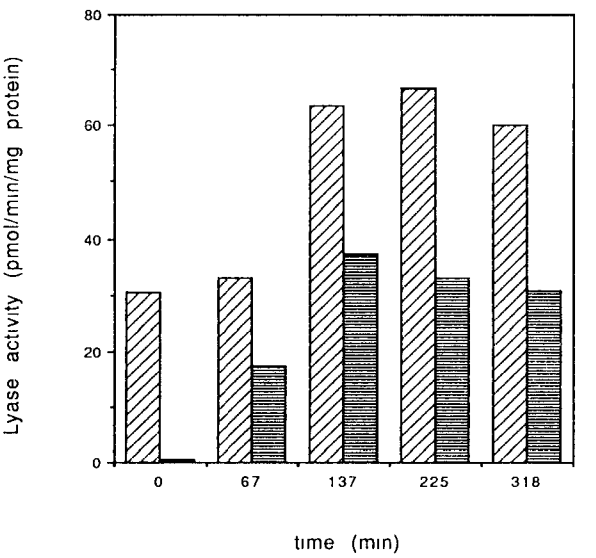

Fig. 2. C-P lyase activities in *Arthrobacter* sp. GLP-1 at various times after transfer from growth medium containing 1 mM methanephosphonate to medium without a source of phosphorus. Methane release from methanephosphonate (diagonal hatching); sarcosine production from glyphosate (horizontal hatching).

other phosphonates as sole phosphorus source, methane production from methanephosphonic acid was now also studied. *Arthrobacter* sp. GLP-1 grows strongly on this compound (generation times: phosphate 3.2 h, glyphosate 4.1 h, methanephosphonate 3.3 h), and the methane produced can be measured easily by headspace gas chromatography. The rate of methane production exhibits Michaelis-Menten kinetics, with an apparent K_m value of 25 μM and a V of 8.3 pmol $min^{-1}mg^{-1}$ f.wt. (cf. for glyphosate, app. K_m = 43 μM, V = 4.3 pmol $min^{-1}mg^{-1}$ f.wt.). As in other organisms (Wackett et al. 1987), the activity is inhibited by phosphate (data not shown), and is induced by phosphorus-deprivation (Fig. 1, Fig. 2).

A comparison of the induction kinetics of sarcosine production from glyphosate and methane production from methanephosphonate during phosphorus starvation shows significant differences between the two. The rate of methane production continues to increase for about four hours, while glyphosate degradation reaches a maximum after only one and a half to two hours, and subsequently begins to fall again. This pattern of induction is not dependent on the phosphonate supplied to cells during growth (the low level of sarcosine production immediately after transfer from medium containing methanephosphonic acid (Fig. 2) is probably due to strong inhibition of the glyphosate-degrading system by residual methanephosphonate, *vide infra*). The differential induction of methane release and sarcosine production seems, then, to indicate that two separate systems may be involved.

In order to investigate this further, we undertook a study of the inhibitory action of other phosphonates on both glyphosate and methanephosphonate degradation. A variety of differently substituted phosphonates were used, and their I_{50} values (concentration required for 50% inhibition of the respective lyase reaction) are presented in Table 1.

For glyphosate degradation, the strongest inhibitors were the alkyl phosphonates *1–4*. These showed 50% inhibition at levels below 10 μM, and an increase in inhibitory action with steric size from methyl to *tert*-butyl phosphonate. The latter was also the only phosphonate tested which the strain GLP-1 was unable to use as a sole source of phos-

phorus (data not shown). Aryl phosphonates (*5*, *6*) show a slightly reduced effect, as do both primary and secondary 1-amino-substituted phosphonates (*8–12*). However, alkyl organophosphonates bearing an amino group further along the alkyl chain (2-, 3-, and 4-amino substituted phosphonates *13–17*) are much less active in inhibiting glyphosate breakdown. Use of glyphosate analogues where the amino group is replaced by an oxa (*18*) or methylene (*19*) moiety leads to progressively reduced inhibition, suggesting that the positively charged amino group may be important for binding. However, phosphonoacetic acid (*20*) and methylene diphosphonic acid (*21*) both inhibit relatively well, even though they carry a negative charge at the pH used.

The actual binding site for the phosphonic acid group itself was probed by use of the phosphonate analogues sulfonate, carboxylate and arsonate (Table 2). The sulfonate analogue of glyphosate (*23*) inhibits well, though it is at present not clear whether or not the sulfonate group is also cleaved.

In contrast, the carboxyl analogue (*24*) does not inhibit glyphosate degradation at all. The arsonate analogues of several phosphonates inhibited the reaction an order of magnitude more strongly than the corresponding phosphonates. It seems, then, that the phosphonate-binding site requires a tetrahedral group, but that the charge may play a lesser role.

It has previously been reported (Pipke et al. 1987b) that uptake of glyphosate is inhibited by several other phosphonates (e.g. 50% inhibition with 500 μM methanephosphonic acid). This effect was now reinvestigated for representative organophosphonates, using the concentrations found to cause 50% inhibition of the lyase reaction. At these concentrations, the initial uptake rate was never reduced more than 15% (Table 3). It is also worth noting that the absolute rate of glyphosate uptake

Table 1. 'In situ' inhibition of glyphosate and methyl phosphonate degradation by other organophosphonates

Phosphonate	I_{50} value (μM)[a]	
	MP-lyase	glyphosate lyase
1. Methylphosphonate	(25)[b]	10
2. Ethylphosphonate	4.3	8
3. Propylphosphonate	3.6	5
4. *t*-Butylphosphonate	405	0.7
5. Phenylphosphonate	50	14
6. 4-Aminophenylphosphonate	n.d.[c]	11
7. Hydroxymethylphosphonate	190	22
8. Aminomethylphosphonate	9600	16
9. 1-Aminoethylphosphonate	11200	19
10. 1-Aminobutylphosphonate	23000	21
11. Glyphosate	53000	(43)[b]
12. *N*-Methyl glyphosate	20000	40
13. 2-Aminoethylphosphonate	41	137
14. 2-Amino-3-phosphonopropionate	n.d.	180
15. 2-Amino-4-phosphonobutyrate	1300	144
16. 4-Aminobutylphosphonate	44	17
17. *N*-Phosphonoethylglycine	5400	44
18. 4-Phosphono-3-oxa-butyrate	180	72
19. 4-Phosphonobutyrate	650	99
20. Phosphonoacetic acid	580	25
21. Methylenediphosphonate	n.d.	27

[a] I_{50} values were measured at a methylphosphonate concentration of 100 μM, and a glyphosate concentration of 33 μM, respectively.
[b] Apparent K_m values for the respective phosphonates.
[c] n.d. = not determined

Table 2. 'In situ' inhibition of glyphosate and methyl phosphonate degradation by organophosphonate analogues

Phosphonate analogue	I_{50} value (μM)[a]	
	MP-lyase	glyphosate lyase
23. *N*-sulfonomethylglycine		15
24. Iminodiacetic acid		> 100000
25. Methylenediarsonate		4
26. Phenylarsonate		5
27. 4-Aminophenylarsonate		5
28. Acetic acid	> 100000	
29. Aminomethylsulfonate	> 100000	
30. Methanesulfonate	> 100000	

I_{50} values were measured at a methylphosphonate concentration of 100 μM, and a glyphosate concentration of 33 μM, respectively.

measured (ca. 1 nmol $min^{-1}mg^{-1}$ f.wt.) is much greater than that of the C-P lyase reaction (V = 4.3 pmol $min^{-1}mg^{-1}$ f.wt.), so that the latter is presumably always rate-limiting. The inhibition of the C-P lyase reactions observed in Tables 1 and 2 is therefore clearly not due to reduced phosphonate uptake into the cell.

The pattern of inhibition for methanephosphonate degradation is quite different to that observed for glyphosate degradation (Table 1). The effect of the alkyl phosphonates is distinguished by much weaker inhibition by the sterically larger *tert*-butyl compound. The 1-amino-substituted phosphonates are extremely poor inhibitors, the weakest of all being glyphosate itself. In contrast, the oxa (*18*) and methylene (19) analogues of glyphosate inhibit much better, the reverse situation to that observed for glyphosate degradation. Since *N*-methylglyphosate (*12*), like glyphosate itself, is a very weak inhibitor, the reduced inhibitory effect may be due to charge factors, rather than steric ones.

Table 3. Rate of glyphosate uptake by *Arthrobacter* sp. GLP-1 in the presence of other organophosphonates

Phosphonate	conc. (μM)[a]	Rate of uptake (% control)
None (control)		100[b]
1. Methylphosphonate	10	93
4. *t*-Butylphosphonate	0.7	99
5. Phenylphosphonate	14	87
8. Aminomethylphosphonate	16	99
15. Methylenediphosphonate	27	88
21. 4-Aminophenylphosphonate	11	88
25. Methylenediarsonate	4	108

[a] Concentration causing 50% inhibition of the C-P lyase reaction (Tables 1 and 2).
[b] 100% = 1.2 nmol $min^{-1}mg^{-1}$ f.wt.

The MP-lyase also differs from the glyphosate-lyase in its specificity for binding of the phosphonic acid group. The sulfonate (*30*) and carboxylate (*28*) analogues of methanephosphonic acid do not inhibit the MP-lyase reaction at all, suggesting that the centre may be quite phosphonate-specific (Table 2).

Arthrobacter sp. GLP-1 therefore contains at least two distinct C-P lyase systems for degradation of glyphosate and methanephosphonic acid, which are differentially inhibited by other phosphonates and phosphonate analogues, and induced at different rates under phosphorus-deprivation conditions. Investigations into the mechanism of degradation of simple alkylphosphonates may hence not be directly transferable to aminophosphonates such as glyphosate. A severe limitation for further studies is the fact that neither of the two C-P lyases has yet been identified in cell-free extracts.

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