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In Vitro Characterization of a Phosphate Starvation-Independent Carbon-Phosphorus Bond Cleavage Activity in *Pseudomonas fluorescens* 23F

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A novel, metal-dependent, carbon-phosphorus bond cleavage activity, provisionally named phosphonoacetate hydrolase, was detected in crude extracts of *Pseudomonas fluorescens* 23F, an environmental isolate able to utilize phosphonoacetate as the sole carbon and phosphorus source. The activity showed unique specificity toward this substrate; its organic product, acetate, was apparently metabolized by the glyoxylate cycle enzymes of the host cell. Unlike phosphonatase, which was also detected in crude extracts of *P. fluorescens* 23F, phosphonoacetate hydrolase was inducible only in the presence of its sole substrate and did not require phosphate starvation.

While P_i is the preferred P source for microbial growth, many bacteria are able, under conditions of P_i limitation, to metabolize a wide range of organophosphorus compounds as the sole P source. These include both biogenic and xenobiotic members of the organophosphonates, a group of compounds containing the stable C-P bond.

Two bacterial C-P bond cleavage enzymes have been extensively studied to date. Phosphonoacetaldehyde hydrolase (phosphonatase; EC 3.11.1.1) has been purified and fully characterized (5, 12, 19, 20); it is specific to the activated C-P bond of phosphonoacetaldehyde, which in *Bacillus cereus* is cleaved via formation of a covalent imine intermediate between the substrate carbonyl group and the side chain of a lysine residue in the enzyme (11).

C-P lyase is the trivial name given to an enzyme (or a number of related enzymes [9]) which has a broad specificity toward both substituted and unsubstituted organophosphonates. It appears to catalyze a direct dephosphonylation of the substrate by a mechanism which may involve redox chemistry at the P center (1, 16). Efforts to obtain a fuller mechanistic understanding of the reaction catalyzed by C-P lyase have been repeatedly frustrated, however, by an inability to obtain in vitro activity of the enzyme (3, 7, 21, 24). This may indicate a fragile or membrane-associated enzyme complex, and evidence from *Escherichia coli* that indicates the probable involvement of nine genes in C-P bond cleavage would support this view (17, 26).

Virtually all studies to date have indicated that bacterial genes involved in the assimilation and metabolism of organophosphonates are tightly regulated by P_i and induced or derepressed only when growth becomes P_i limited. Our extensive knowledge of this phenomenon in *E. coli*, which possesses a C-P lyase of broad substrate specificity, has been reviewed elsewhere (26); recent data suggest that both phosphonatase and C-P lyase are under *pho* regulon control in *Enterobacter aerogenes* (13).

An apparent exception to the general rule has been our recent isolation of a strain of *Pseudomonas fluorescens* capable of the utilization of the antiviral antibiotic phosphonoacetate as the sole carbon and phosphorus source, with excretion of virtually equimolar quantities of P_i (14). A C-P bond cleavage activity detected in cell extracts of the organism catalyzed the production of acetate and P_i from phosphonoacetate (15). The enzyme involved, which we have provisionally designated phosphonoacetate hydrolase, appears to be distinct from phosphonatase, the only other C-P bond cleavage enzyme whose in vitro activity has been confirmed (12), since it showed no activity toward phosphonoacetaldehyde, the sole substrate of phosphonatase. Similarly, it was not active toward methylphosphonate or glyphosate, both typical C-P lyase substrates (9).

We now report the further characterization of this novel C-P bond cleavage activity and an investigation of its mode of regulation.

MATERIALS AND METHODS

Chemicals. Chemicals, all of highest available purity, were obtained from Sigma Chemical Co., Poole, United Kingdom, except for the following compounds: sulfonoacetate (Kodak Laboratory and Research Chemicals, Rochester, N.Y.); phenylphosphonate (Aldrich Chemical Co., Poole, United Kingdom); methyl- and ethylphosphonates (Ventron, Karlsruhe, Germany); and 2-phosphonopropionate, 2-phosphonobutyrate, and trimethylphosphonoacetate (MTM Research Chemicals Ltd., Morecambe, United Kingdom). Phosphonoacetaldehyde, phosphonopyruvate, and arsonoacetate (H. B. F. Dixon, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom), phosphonopyruvate (M. Seidel, Department of Chemistry, Harvard University, Cambridge, Mass.), glyphosate (Monsanto Agricultural Co., St. Louis, Mo.), alafosfalin (Roche Products, Welwyn, United Kingdom), and phosphinothricin (Hoechst AG, Frankfurt am Main, Germany) were all kind gifts.

Cultivation of organism. *P. fluorescens* 23F was isolated as described previously (14). The isolate was grown in a medium (pH 7.2) with the following composition (per liter): NH_4Cl, 5.0 g; Tris-HCl, 6.0 g; $CaCl_2 \cdot 2H_2O$, 0.08 g; $MgSO_4 \cdot 7H_2O$, 0.16 g; phosphate-free yeast extract, 0.05 g; and 1 ml each of vitamin solution and trace element solution (10). Phosphate-free yeast

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extract was prepared by magnesia treatment (18) and did not contain detectable levels of P_i. Phosphonoacetate (10 mM) routinely served as the sole carbon and phosphorus source, and the medium was filter sterilized. Where indicated, glucose (25 mM) and P_i (5 mM) were provided as alternative carbon and phosphorus sources. Flasks (2.5 liter) containing 500 ml of medium were incubated at 30°C on a rotary shaker at 100 rpm. All glassware was previously soaked in Decon (Decon Laboratories, Hove, United Kingdom) and rinsed repeatedly with distilled water.

Preparation of crude extracts. Cells were harvested in mid-log phase, washed once in 10 mM Tris-HCl buffer (pH 7.5), and then disrupted ultrasonically at 16 kHz on ice for 5 min (30 s of sonication followed by 2 min of cooling). The homogenate was centrifuged at 25,000 × *g* for 30 min at 3 to 5°C, and the supernatant was dialyzed overnight against 50 mM Tris-HCl (pH 7.5). It was stored at 3 to 5°C until needed.

Enzyme assays. (i) Phosphonatase. Phosphonatase activity was routinely assayed at 30°C in a reaction mixture (1 ml) containing the following: Tris-HCl (pH 7.5), 50 μmol; phosphonoacetaldehyde, 2 μmol; and crude extract, 0.25 to 0.75 mg of protein. The reaction was initiated by addition of crude extract and terminated by addition of 0.2 ml of 50% (wt/vol) trichloroacetic acid. After centrifugation, P_i release was assayed in the supernatant by the method of Fiske and Subba-Row (6), and acetaldehyde release was measured by using an enzyme-based acetaldehyde test kit (Boehringer Mannheim, Lewes, United Kingdom) with a lower detection limit of 5 μg ml^{-1}. Activity was expressed as nanomoles of P_i or acetaldehyde liberated per minute per milligram of protein.

(ii) Phosphonoacetate hydrolase. Routine assay of phosphonoacetate hydrolase activity was carried out at 30°C in a reaction mixture (1 ml) containing the following: Tris-HCl (pH 7.5), 50 μmol; phosphonoacetate, 5 μmol; and crude extract, 0.25 to 0.75 mg of protein. The reaction was initiated by addition of crude extract and terminated by addition of 0.2 ml of 50% (wt/vol) trichloroacetic acid. After centrifugation, the supernatant was assayed for P_i (6) and acetate release (15). Activity was expressed as nanomoles of P_i or acetate liberated per minute per milligram of protein.

(iii) Isocitrate lyase. The method described by Zarembinski et al. (27) was carried out at room temperature in a reaction mixture (1 ml) containing the following: Tris-HCl (pH 7.5), 10 μmol; phenylhydrazine hydrochloride, 4 μmol; $MgCl_2 \cdot 6H_2O$, 5 μmol; DL-isocitrate, 2 μmol; and cell extract (0.5 mg to 1.35 mg of protein). The reaction was initiated by addition of DL-isocitrate, and formation of glyoxylate phenylhydrazone was monitored at 324 nm for 10 min. A control containing no DL-isocitrate was run simultaneously with the assay. Enzyme activity was expressed as nanomoles of glyoxylate phenylhydrazone formed per minute per milligram of protein. The extinction coefficient of phenylhydrazone is 1.7×10^4 M^{-1} cm^{-1} (8).

Protein determination. The protein concentration in crude extracts was determined by the method of Bradford (2).

Glucose detection. Glucose was measured with a commercial detection kit (Sigma Diagnostics, Dorset, United Kingdom) by the method of Trinder (23).

All assays were carried out in duplicate.

RESULTS

Effects of carbon and/or phosphorus starvation on phosphonoacetate hydrolase activity in *P. fluorescens* 23F. *P. fluorescens* 23F was grown in mineral salts medium containing glucose and P_i. Phosphonoacetate hydrolase activity was detectable only in crude extracts when the medium was also supplemented with phosphonoacetate. Even if the isolate was starved of a carbon source and/or P_i, enzyme activity was again detectable only if the medium had also been supplemented with phosphonoacetate. Phosphonoacetate hydrolase activities of up to 25.9 nmol min^{-1} mg of $protein^{-1}$ were detected.

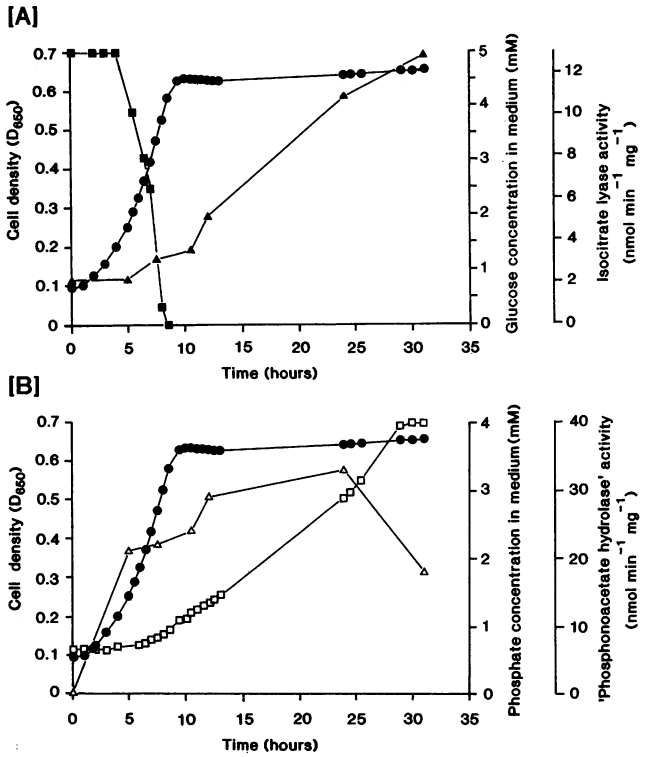

FIG. 1. Growth of *P. fluorescens* 23F on glucose (5 mM), phosphonoacetate (5 mM), and P_i (0.65 mM) as carbon and phosphorus sources. (A) D_{650} of culture (●), glucose concentration in the medium (■), and isocitrate lyase activity in cell extracts (▲). (B) D_{650} of culture (●), phosphate concentration in the medium (□), and phosphonoacetate hydrolase activity in cell extracts (△).

Induction of isocitrate lyase and phosphonoacetate hydrolase in *P. fluorescens* 23F. *P. fluorescens* 23F was grown on a medium containing glucose (5 mM), phosphonoacetate (5 mM), and P_i (0.65 mM) as sole carbon and phosphorus sources. Growth of the isolate was monitored, and the activities of isocitrate lyase and phosphonoacetate hydrolase were measured. Cell density at 650 nm (D_{650}) reached a value of 0.63 at 10 h, shortly after glucose exhaustion from the medium. Isocitrate lyase activity subsequently increased fourfold, rising from 3.0 to 12.6 nmol min^{-1} mg^{-1} (Fig. 1A). Later growth of *P. fluorescens* 23F, which was presumably at the expense of phosphonoacetate-derived acetate, as a result of the observed increase in isocitrate lyase activity, gave no significant increase in cell density (Fig. 1A). A control experiment indicated that isocitrate lyase activity did not increase after glucose exhaustion from a medium lacking phosphonoacetate.

In contrast to isocitrate lyase, phosphonoacetate hydrolase activity was found to increase rapidly after inoculation, reaching 21 nmol min^{-1} mg^{-1} after only 5 h (Fig. 1B). Subsequently phosphonoacetate hydrolase activity increased more gradually, reaching a peak of 33 nmol min^{-1} mg^{-1} after 24 h and then declining sharply. Release of phosphonate-derived P_i into the supernatant occurred gradually at first but then more rapidly once the concentration of glucose in the medium declined (Fig.

1B) (initial level of P_i supplied was 0.65 mM). Acetate was not detectable in the culture supernatant at any point.

Substrate specificity of phosphonoacetate hydrolase. Crude extract, prepared from *P. fluorescens* 23F grown on phosphonoacetate as the sole carbon and phosphorus source and known to contain phosphonoacetate hydrolase activity, was incubated in the presence of a wide range of compounds (final concentration, 5 mM). Enzyme activity, as measured by release of P_i and/or acetate production, as appropriate, was detected only in the presence of phosphonoacetate. Organophosphonates that failed to act as substrates included phosphonoacetaldehyde, phosphonopyruvate, phosphonoformate, 3-phosphonopropionate, hydroxymethylphosphonate, 2-phosphonopropionate, 2-phosphonobutyrate, 2-aminoethylphosphonate, 3-aminopropylphosphonate, 4-aminobutylphosphonate, 1-aminoethylphosphonate, 1-aminopropylphosphonate, 1-aminobutylphosphonate, glyphosate, aminomethylphosphonate, methylphosphonate, ethylphosphonate, phenylphosphonate, isopropylphosphonate, alafosfalin, phosphomycin, phosphinothricin, 2-amino-3-propylphosphonate, 2-amino-4-butylphosphonate, and trimethylphosphonoacetate. Other compounds that failed to act as substrates included arsonoacetate, sulfonoacetate, bromoacetate, chloroacetate, iodoacetate, iminodiacetate, malonate, glycolate, dimethylglycine, isethionate, phosphite, and PP_i.

Interaction of phosphonoacetate hydrolase with a range of nonsubstrate compounds. The effects of addition of various compounds (10 mM) on phosphonoacetate hydrolase activity in crude extracts, as monitored by release of both P_i and acetate from 5 mM phosphonoacetate, were examined. Phosphonocarboxylic acids, such as phosphonoformate and 3-phosphonopropionate, as well as the phosphonate herbicide glyphosate appeared to be moderate inhibitors of phosphonoacetate hydrolase activity (30, 58, and 38% enzyme activity remaining, respectively). The alkylphosphonic acids methyl- and ethylphosphonate had little or no effect on enzyme activity. Similarly, no inhibition of phosphonoacetate hydrolase was observed in the presence of the aminoalkylphosphonates 2-aminoethyl-, 3-aminopropyl-, and 4-aminobutylphosphonate. Unexpectedly, apparent enzyme activation was observed with several other aminoalkylphosphonates: 1-aminoethylphosphonate, 1-aminobutylphosphonate, and 2-amino-4-butylphosphonate (133, 104, and 132% enzyme activation, respectively). When a competition assay was carried out between an activator (2-amino-4-butylphosphonate, 10 mM) and an inhibitor (phosphonoformate, 10 mM), only inhibition of phosphonoacetate hydrolase was observed. Nonphosphonate analogs of phosphonoacetate, such as arsonoacetate, sulfonoacetate, and malonate, were poor inhibitors of phosphonoacetate hydrolase. Similarly, the presence of P_i, acetate, or the known phosphonatase inhibitor phosphite had little or no effect on enzyme activity. The reducing agents sodium sulfide and dithiothreitol partially inhibited the enzyme (60 and 17% enzyme activity remaining, respectively), and whereas the chelating agent iminodiacetate also partially inhibited enzyme activity (27% activity remaining), complete phosphonoacetate hydrolase inhibition was observed only when EDTA was added to the assay.

Effect of metals on phosphonoacetate hydrolase activity. The effects of various metal ions on phosphonoacetate hydrolase activity in dialyzed crude extract were examined (dialysis of cell extract was found to increase phosphonoacetate hydrolase activity 1.1-fold). Enzyme activity was initially tested under standard assay conditions with metal ions added to a final concentration of 1 mM. Zn^{2+}, Mn^{2+}, and Co^{2+} were found to increase phosphonoacetate hydrolase activity, whereas Fe^{3+}, Ca^{2+}, Mg^{2+}, and Cs^+ had no effect (Table 1). Addition of Zn^{2+}, Mn^{2+}, and Co^{2+} was also observed to allow recovery of EDTA-treated enzyme (Table 1). These results suggest pronounced metal dependency, with metal ions divided into two groups in terms of their effects on phosphonoacetate hydrolase.

TABLE 1. Effects of metal ions on phosphonoacetate hydrolase activity

Metal tested (final concn, 1 mM)	Phosphonoacetate hydrolase activity (%)[a]		
	A	B	C
None	100	7	270
Zn^{2+}	473	339	407
Mn^{2+}	373	277	377
Co^{2+}	252	144	315
Fe^{3+}	106	7	230
Ca^{2+}	94	11	246
Cs^+	102	7	270
Mg^{2+}	100	7	270

[a] The activity of phosphonoacetate hydrolase in dialyzed cell extract without any metal addition was 14.2 nmol min^{-1} mg^{-1} (100%). All other activities are compared with this. In treatment A, the metals were added directly to the enzyme assay, which contained dialyzed extract. In treatment B, the metals were added directly to the enzyme assay, which contained dialyzed extract treated with EDTA (1 mM). In treatment C, after treatment B, 1 mM $ZnCl_2 \cdot 7H_2O$ was added to each of the enzyme assays, containing the various metals, to determine whether further enzyme activity could be recovered.

Induction of phosphonoacetaldehyde hydrolase (phosphonatase) in *P. fluorescens* 23F. A second C-P bond cleavage activity was detectable in crude extracts of *P. fluorescens* 23F when the microorganism was starved of P_i or when it was grown on mineral salts medium supplemented with phosphonoacetaldehyde or 2-aminoethylphosphonate as the sole phosphorus source. The enzyme activity was capable of degrading phosphonoacetaldehyde to acetaldehyde and P_i but showed no activity against phosphonoacetate or ethylphosphonate (Table 2). If phosphite (10 mM) was present in the reaction mixture in addition to phosphonoacetaldehyde, the enzyme activity was inhibited by more than 95% compared with its activity in the absence of phosphite (Table 2). No enzyme activity was detectable in assays from which phosphonoacetaldehyde or crude extract had been omitted or boiled extract was used or in assays in which crude extract was prepared from cells of *P. fluorescens* 23F grown on mineral salts medium supplemented with glucose and P_i. The effect of various divalent metal ions on this C-P bond cleavage activity was investigated. Mg^{2+} supplementation led to a 1.5-fold increase in enzyme activity, whereas both Zn^{2+} and Ca^{2+} behaved as enzyme inhibitors. All of the foregoing characteristics are consistent with phosphonoacetaldehyde hydrolase (phosphonatase; E.C. 3.11.1.1) (12, 19).

TABLE 2. C-P bond cleavage activity in cell extracts of *P. fluorescens* 23F grown on 2-aminoethylphosphonate as the sole phosphorus source

Enzyme substrate (5 mM)	C-P bond cleavage activity (nmol of P_i liberated min^{-1} mg^{-1})
Phosphonoacetaldehyde	19.4
Phosphonoacetate	0.0
Ethylphosphonate	0.0
Phosphonoacetaldehyde + phosphite	0.8

DISCUSSION

Despite extensive research in recent years, our understanding of the enzymology of bacterial C-P bond cleavage remains limited. It is now clear, however, that several such enzymes exist and that more than one may be present in a single cell. Thus, in situ kinetic evidence for the existence in *Arthrobacter* sp. strain GLP-1 of two distinct C-P lyases has been provided (9). Similarly, the involvement of both a C-P lyase and phosphonatase in organophosphonate degradation by *Enterobacter aerogenes* has been demonstrated (13).

All of the foregoing enzymes share a common feature in that they are induced by P_i limitation of the host cell (22, 24, 26). Indeed, no mode of regulation has been described in which a specific phosphorus-containing compound is needed for induction of a gene required for its own utilization (25). In this report, however, we show that *P. fluorescens* 23F contains not only a phosphate starvation-inducible phosphonatase but also a hitherto undescribed C-P bond cleavage activity, detectable in dialyzed crude extracts, whose expression is independent of the phosphate status of the cell. Thus, it is not induced by phosphate starvation, and its induction is not repressed, and its activity is not inhibited, by the presence of high levels of P_i. Rather, its induction appears to require the presence of its sole substrate, phosphonoacetate (Fig. 1). We have provisionally designated this activity phosphonoacetate hydrolase in the belief that its cleavage of the C-P bond is hydrolytic rather than reductive; the fact that active extracts of phosphonoacetate-grown cells did not convert phosphite to P_i would support this view.

Through the action of phosphonoacetate hydrolase, the organism is enabled to use this xenobiotic organophosphonate as the sole carbon and energy source (14); it seems likely that the acetate produced is metabolized via the glyoxylate cycle. Thus, when *P. fluorescens* 23F was grown on a medium containing both glucose and phosphonoacetate (Fig. 1A), a fourfold increase in isocitrate lyase activity occurred, although only after depletion of glucose from the medium. Despite the fact that cells were found to contain high levels of phosphonoacetate hydrolase activity in the presence of high glucose concentrations, significant P_i release occurred only after glucose disappearance (Fig. 1B). This result suggests that while phosphonoacetate hydrolase is induced in the presence of its substrate, glucose availability may nevertheless play a regulatory role in phosphonoacetate metabolism, possibly through its control of phosphonoacetate uptake via catabolite inhibition.

In a preliminary report of the in vitro detection of phosphonoacetate hydrolase, we found that neither glyphosate, phosphonoacetaldehyde, phenylphosphonate, nor methylphosphonate was a suitable substrate for the activity (15). We have now investigated a wider range of 26 organophosphonate and 12 analogous nonphosphonate compounds, but again activity of the enzyme was observed only in the presence of phosphonoacetate.

Despite this strict substrate specificity, a number of compounds were nevertheless able to interact with phosphonoacetate hydrolase in crude extract as inhibitors or activators. The fact that only inhibition was observed if crude extract was incubated in the presence of both an inhibitor and an activator suggests the existence of different binding sites for these two classes of molecule and a higher affinity of the enzyme for the latter. The chelating agents EDTA, glyphosate, and iminodiacetate inhibited phosphonoacetate hydrolase activity to different degrees. The unexpected observation that sodium sulfide and dithiothreitol were also moderate inhibitors of activity probably results from their ability to interact with metals rather than their ability to cleave disulfide bridges and thus disrupt enzyme structure and function. As a result of the ability of various chelating agents to inhibit enzyme activity, the effects of metal ions on phosphonoacetate hydrolase were investigated by the method of De Frank and Cheng (4). Subsequently, we confirmed that the presence of certain metal ions was necessary for the stability of the enzyme and/or as a part of its catalytic mechanism (Table 1). If the latter, it is conceivable that these metals may serve either as Lewis acids or, through coordination, to stabilize a transition state for cleavage of the C-P bond.

An intriguing aspect of our findings is the fact that two enzymes of such closely related functions as phosphonoacetate hydrolase and phosphonoacetaldehyde hydrolase (phosphonatase) should exist within the same cell. Evidence has been presented (19) that phosphonatase has specifically evolved to catalyze the C-P bond cleavage reaction in phosphonoacetaldehyde; no homology between an apparent active-site peptide sequence and any other protein could be established by data bank searches. Phosphonoacetate hydrolase is clearly distinct from phosphonatase in its own unique substrate specificity, in its hydrolytic mechanism, and in its mode of regulation, although the two may share a common ancestry. Furthermore, it is clear that a third C-P bond cleavage system exists in *P. fluorescens* 23F, since several organophosphonates other than phosphonoacetate or 2-aminoethylphosphonic acid can be utilized as sole P sources (14).

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