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Characterization of the methylenediphosphonate transport system in *Arthrobacter* sp. GLP-1 using the novel tritium-labelled derivative

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The novel tritium-labelled derivative of methylenediphosphonate (MDP) was used in uptake studies of *Arthrobacter* sp. GLP-1 capable of utilizing a wide range of organophosphonates as its sole source of phosphorus. The MDP uptake was greatly stimulated upon phosphate deprivation. The uptake obeys Michaelis–Menten kinetics with respective K_m and V_{max} values of 33 μM and 0.3 nmol·min^{-1}·mg^{-1} fr.wt. Glyphosate and pyrophosphate were competitive inhibitors of MDP uptake. The effect of orthophosphate was more complex than a mere inhibition of MDP uptake since activation occurred at low concentrations. The uptake of MDP by *Arthrobacter* sp. strain GLP-1 appears to be mediated by a transport system different from the glyphosate uptake system operating in the same cells. The driving force for MDP uptake by *Arthrobacter* sp. GLP-1 may be a proton gradient across the cell membrane.

Methylenediphosphonate transport; Tritium-labelled methylenediphosphonate; *Arthrobacter* sp. GLP-1

1. INTRODUCTION

Methylenediphosphonate (MDP), a stable phosphonate analogue of inorganic pyrophosphate (PP_i) shows different kinds of cellular activity. MDP efficiently inhibits the growth of *Dictyostelium discoideum* amoebae presumably as a result of the in vivo formation of nonhydrolyzable methylene analogues of ATP and diadenosine tetraphosphate [1]. Despite of the stability of the C–P bond to chemical and to enzymatic hydrolysis there are at least two phosphonate-degrading microorganisms, namely *Pseudomonas* PG 2982 [2] and *Arthrobacter* sp. GLP-1 [3], capable of utilizing MDP as a sole source of phosphorus for their growth.

In order for MDP to manifest any kind of cellular activity, there must be MDP transport across the membrane to allow it to reach the target enzymes located inside the cell. Hence the study of the mechanism of MDP uptake and its regulation is of interest. This paper reports the application of the newly synthesized tritium-labelled methylenediphosphonate ([^{3}H]MDP) [4] for studying the transport of MDP in *Arthrobacter* sp. GLP-1. Previously, a glyphosate uptake system has been described for this strain [5]. Here we report that glyphosate and MDP appear to be taken up via two distinct transport systems which are differentially inhibited, as well as suppressed, by orthophosphate. We present the first data on the energetics of phosphonate uptake by bacterial cells.

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2. MATERIALS AND METHODS

The synthesis of [^{3}H]methylenediphosphonic acid trisodium salt (specific activity 30 mCi/mmol) was published elsewhere [4]. All other reagents were commercially available.

Arthrobacter sp. strain GLP-1 [6] was kindly provided by Prof. N. Amrhein and cultured as previously described [5]. Uptake of labelled MDP was measured by a modification of the method developed for glyphosate uptake experiments [7]. The uptake assay contained [^{3}H]MDP (1.11 kBq), 50 mM Tris-HCl, pH 7.2, and the respective test compound as indicated in the text. In the experiments with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP), valinomycin, nigericin, or *N*-ethylmaleimide (NEM), cells were pre-incubated with these compounds for 5 min at 37°C. Uptake studies were started by the addition of 33 μM MDP (1.11 kBq).

3. RESULTS AND DISCUSSION

Glyphosate uptake by *Arthrobacter* sp. GLP-1 was previously shown to be induced upon orthophosphate deprivation [5]. As is clear from Fig. 1, MDP uptake was considerably enhanced when cells were incubated for 5 h in phosphate-free medium prior to the uptake experiments. While the glyphosate transport system was completely suppressed in non-phosphate-starved cells [5], the slight entry of MDP still occurred in these cells (Fig. 1). However, this transport activity, presumably having a constitutive character, was too low to measure any kinetic parameters. All further experiments were carried out with phosphate-starved cells.

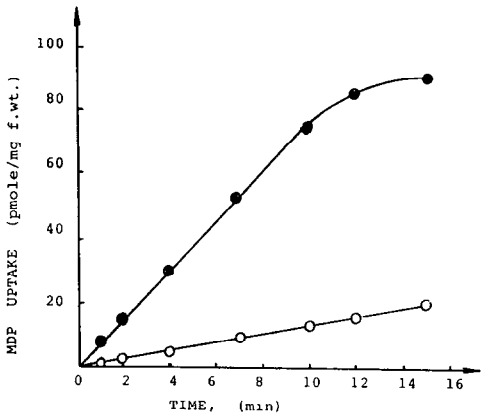

Fig. 1. Time course of MDP uptake in *Arthrobacter* sp. GLP-1. Cells were taken before (–○–) or after (–●–) phosphorus starvation.

The time course of MDP uptake is illustrated in Fig. 1. Uptake was linear during the 10 min incubation period, indicating that the reaction velocity was independent of time. Consequently, uptake during a 4-min incubation period may be taken as a measure of the reaction velocity (uptake rate per unit of time).

The rate of uptake was strongly affected by the MDP concentration in a hyperbolic fashion indicating site saturation. A Lineweaver–Burk presentation of such results yielded a value of about 33 μM for the K_m and $V_{max} = 0.3$ nmol·min^{-1}·mg^{-1} fr.wt (Fig. 2a). Thus the rate of MDP uptake by *Arthrobacter* sp. GLP-1 was about of 8 times lower than the rate of glyphosate uptake under the same conditions [7].

Glyphosate and PP_i were competitive inhibitors of MDP uptake with a K_i of 84 μM (Fig. 2a,b) and 100 μM (data not shown), correspondingly. The effect of inorganic phosphate on MDP transport in *Arthrobacter* sp. GLP-1 was more complex than a competitive inhibition demonstrated previously in the case of glyphosate uptake [5]. At low concentrations (5–10 μM) P_i appeared as an activator of MDP uptake, whereas at higher concentrations it exhibited inhibitory properties; moreover, this effect was dependent of the concentration of MDP, since the P_i activation occurred only at low concentrations of MDP (5–10 μM) (Fig. 3). Surprisingly, an inhibition of MDP uptake by P_i was even raised as the MDP concentration increased. This behaviour is rather unusual and cannot be interpreted in terms of known kinetic correlations for linear inhibition [8]. The physiological reason for such a complex regulation of MDP uptake by P_i is still unclear. The available data do not indicate how this regulatory pattern may be related to the phosphonate-utilizing phenotype.

Arsenate and methylphosphonate which bear a struc-

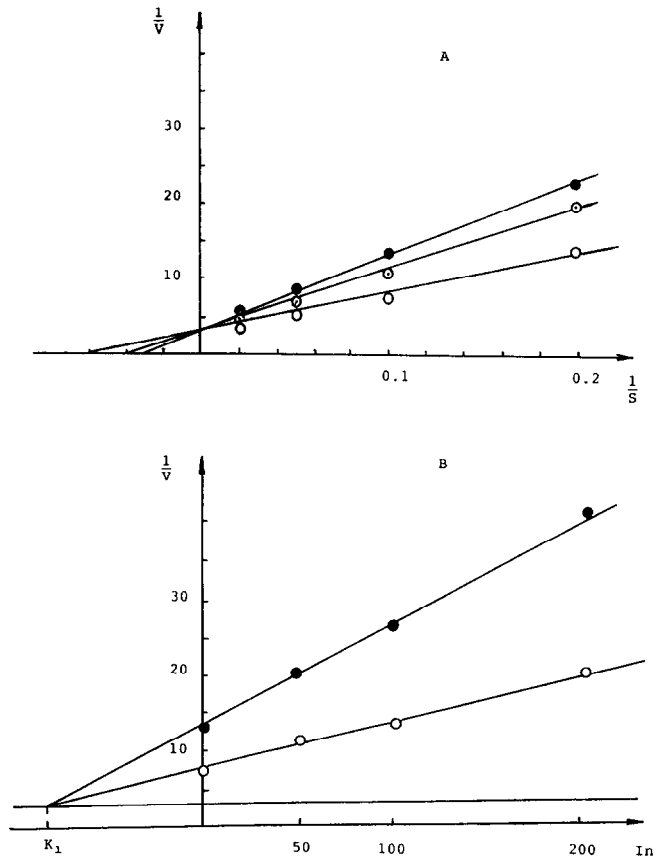

Fig. 2. Competitive inhibition of MDP uptake by glyphosate in *Arthrobacter* sp. GLP-1. (A) A double reciprocal (Lineweaver–Burk) plot. Control cells (–○–); Glyphosate concentrations: 50 μM (–○–) or 100 μM (–●–). (B) Dixon plot. In, nanomoles of glyphosate per milliliter. MDP concentrations: 5 μM (–●–) or 10 μM (–○–); the straight line corresponds to a saturated concentration of MDP.

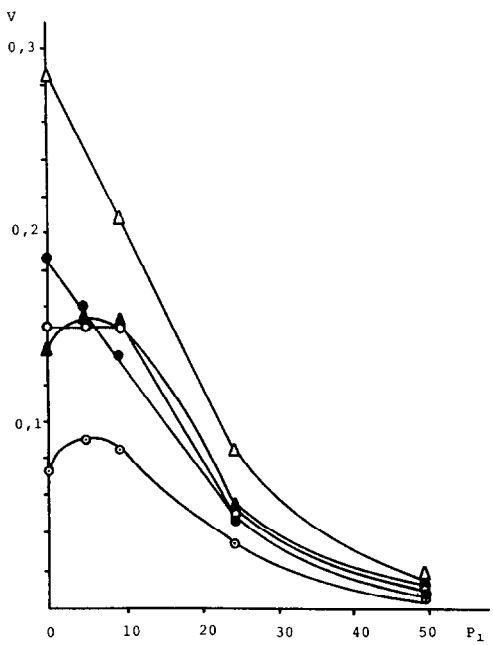

Fig. 3. Variation of MDP uptake as a function of orthophosphate concentration. P_i, Nanomoles of orthophosphate per milliliter. MDP concentrations: 5 μM (–○–); 10 μM (–▲–); 33 μM (–○–); 50 μM (–●–); 100 μM (–△–).

Table I

Inhibition of methylenediphosphonate uptake

Compound	Concentration (μM)	Flux (% of control)
None	–	100
CCCP	0.1	70.3
	0.5	10.5
	1.0	0
FCCP	5	23.2
	10	0
N-Ethylmaleimide	1,000	32.3

tural resemblance to P_i, effectively suppressed MDP transport. The I_{50} values of all these compounds are of the order of magnitude of 5–20 μM (data not shown). Since neither orthophosphate nor its structural analogues were able to compete with MDP in uptake assays, it seems unlikely that MDP is transported into *Arthrobacter* sp. GLP-1 by the phosphate transport system previously postulated for glyphosate uptake [5].

We are not aware of any previous studies of energetics of phosphonate uptake by bacterial or other cells. We studied MDP uptake in the presence of a number of compounds that might reasonably be expected to influence anion translocation. The protonophoric uncoupler CCCP produced a dramatic inhibition, an effect also observed with FCCP (Table I). Since CCCP acts by collapsing the proton gradient it can be supposed that the MDP transport system is energized by the protonmotive force similar to the Pit system in *E. coli* [9]. The idea of co-transport of MDP and protons is additionally supported by the observation that uptake of MDP was effectively inhibited by the sulfhydryl reagent NEM, which is the well-known selective inhibitor of the P_i/H^+ symporter [10]. Table II indicates that MDP uptake was stimulated by K^+ ions and inhibited by sodium. The potassium ionophore, valinomycin stimulated uptake, whereas nigericin, which exchanges potassium for protons, inhibited uptake (Table II). These results can be well interpreted in the framework of the modern concept of bacterial membrane transport [11]. According to this concept, the stimulation of MDP uptake by K^+ may be due to the fact that an electrophoretic K^+ influx (down electric gradient) results in a $\Delta\Psi$ being converted

Table II

Influence of univalent cations and ionophores upon methylenediphosphonate uptake[a]

Assay	Cations, 165 μM		Ionophores, 20 μM		Flux (% of control)
	K^+	Na^+	Valinomycin	Nigericin	
1	–	–	–	–	100
2	+	–	–	–	115.5
3	–	+	–	–	86.4
4	–	–	+	–	95.0
5	+	–	+	–	136.1
6	–	–	–	+	53.5
7	+	–	–	+	47.9
8	+	–	+	+	76.9

[a] + and –: presence or absence in assay mixture.

to ΔpH [12]. The thus formed proton gradient across the cell membrane appears then to be used as the driving force for MDP uptake by *Arthrobacter* sp. GLP-1.

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