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The interaction of phosphonium ions with *Acinetobacter calcoaceticus*: evidence for the operation of an efflux system

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Experiments with washed cell suspensions of *Acinetobacter calcoaceticus* NCIB 8250 indicated that this organism possesses an energy-linked efflux system for phosphonium ions and related compounds. Evidence is presented to show that the apparent impermeability to low concentrations of methyltriphenylphosphonium ion is a result of the combination of the outer membrane acting as a partial permeability barrier and the activity of the efflux system.

Introduction

The distribution of phosphonium ions has been widely used in bioenergetic studies to quantitate the membrane potential ($\Delta\psi$) across many biological membranes [1,2]. A number of investigators have pointed out some of the limitations concerning the use of such probes [3–5]. One major assumption when using phosphonium ions is that they distribute across the membrane in question by a non-mediated process. With one strain of yeast, this assumption has been shown to be invalid and the entry of methyltriphenylphosphonium ion (MTP^+) was observed to occur via the thiamine transport system [4].

We have recently presented evidence for the presence of an efflux system for phosphonium ions in *Arthrobacter globiformis* [6]. The operation of this system was detected by an apparent stimulation of uptake of one substrate by a second substrate of the same system, due to an inhibition of efflux. In addition, the efflux system was partially deactivated by starvation of the cells [6].

Our studies with *Acinetobacter calcoaceticus* reported here indicate the presence of a mediated, energy-linked efflux system in this bacterium. Our results are consistent with the outer membrane of the organism acting as a significant diffusion barrier to phosphonium ions. This factor, combined with the operation of the efflux system we have demonstrated, results in an apparent impermeability of the organism to compounds such as MTP^+.

Materials and Methods

Cultivation and harvesting. The organism used was *A. calcoaceticus* NCIB 8250. It was grown in batch culture on the defined medium previously described [7], using 30 mM sodium succinate as the carbon source. Cells were harvested in the early stationary phase and washed once in ice-cold 67 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0). Washed cell suspensions were stored on ice.

Ethidium uptake. This was measured fluorimetrically as previously described [8,9].

EDTA treatment. This was based on the procedure of Leive [10]. Cells (1 mg dry wt./ml) were

Abbreviations: MTP^+, methyltriphenylphosphonium ion; TPP^+, tetraphenylphosphonium ion; TPA^+, tetraphenylarsonium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

suspended in 0.12 M Tris chloride (pH 7.0) containing 1 mM EDTA and shaken on ice for 30 min. For further use, the cells were sedimented and resuspended in sodium phosphate buffer.

Uptake of phosphonium ions. Experiments were conducted at 30°C using sodium phosphate buffer. The cell density was 1 mg dry wt./ml and the concentration of sodium succinate, when present, was 10 mM. Uptake was measured using a rapid filtration technique [11]. Data presented have been corrected for filter and extracellular binding obtained with controls that were incubated on ice with labelled phosphonium ions for less than 20 s.

Materials. ^{3}H-labelled phosphonium ions (MTP^+ and TPP^+) were purchased from Amersham International. Other reagents were obtained from Sigma and Aldrich.

Results

Studies on MTP^+ accumulation

When washed cell suspensions of *A. calcoaceticus* were incubated in the presence of succinate with 10 μM MTP^+, there was no detectable MTP^+ uptake by the cells (Fig. 1). However, in the presence of structurally related compounds, there was a marked stimulation of uptake (Fig. 1) which was concentration-dependent (Fig. 2). Compounds that were tested, at a concentration of 20 μM, and found not to stimulate MTP^+ uptake when assayed under the conditions described in Fig. 1, were ethidium bromide, dibenzyldimethylammonium chloride, safranine, quinaldine red, euflavine, auramine O, thiamine, trimethylphenylammonium iodide, malachite green, rosaniline hydrochloride and 2-(dimethylaminostyryl)-1-ethylpyridinium.

The extent of stimulation of MTP^+ uptake was dependent on the concentration of MTP^+, decreasing at higher concentration (Fig. 3). As shown, high concentrations of MTP^+ (100 μM) allowed demonstration of MTP^+ accumulation in the absence of a stimulatory analogue. The uptake of MTP^+ promoted by the addition of 20 μM TPP^+ was inhibited by 25 μM CCCP (data not shown).

With some batches of the organism, when cells were treated with EDTA as described in Materials and Methods, then MTP^+ uptake, measured at 10 μM MTP^+, was detectable but uptake was further stimulated by the addition of TPP^+ (Fig. 4). However, despite the fact that no known variable was introduced into the growth and EDTA treatment procedures, some batches of cells did not show MTP^+ uptake (measured at 10 μM) under these conditions. Experiments measuring ethidium

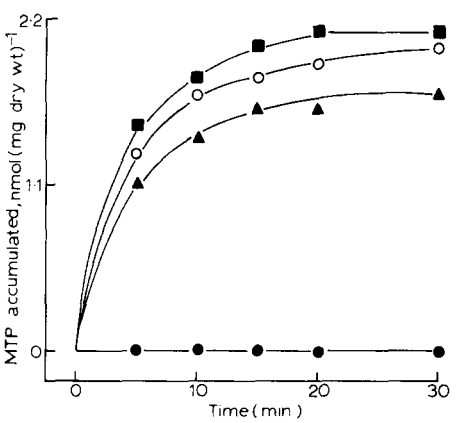

Fig. 1. Uptake of MTP^+ by *A. calcoaceticus*. MTP^+ uptake was assayed using 10 μM MTP^+ (spec. act. 12.5 μCi/μmol). Cell suspensions were preincubated for 10 min with 10 mM sodium succinate and the indicated analogue prior to the addition of MTP^+. ●, No analogue added; ▲, 20 μM crystal violet; ○, 20 μM TPP^+; ■, 20 μM TPA^+.

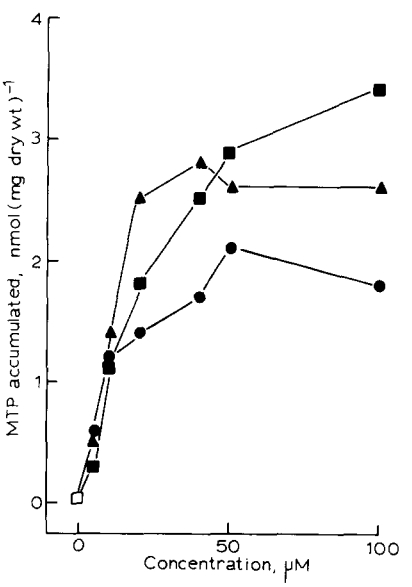

Fig. 2. Stimulation of MTP^+ uptake by varying concentrations of analogues. The experiment was performed as described in the legend to Fig. 1. Samples were removed for assay of the steady-state accumulation of MTP^+ between 15 and 25 min. ●, TPP^+; ▲, TPA^+; ■, crystal violet; □, MTP^+ alone.

penetration using a fluorescence assay based on the interaction of ethidium with intracellular nucleic acids indicated a marked increase in permeability in response to EDTA treatment. Those batches of cells that did not take up MTP^+ after EDTA treatment, did so in the presence of TPP^+. This behaviour was consistent with some batches of cells having sufficient efflux activity to maintain an apparent impermeability to MTP^+, even when the permeability of the outer membrane was increased.

Experiments with 100 μM MTP^+ showed an uptake of MTP^+ in the absence of succinate (Fig. 5). The addition of succinate, which stimulated the rate of respiration 2-fold, produced a diminished accumulation and an efflux of accumulated MTP^+ (Fig. 5). The absolute value of the succinate-dependent rate of respiration was 60 μmol O_2/min per g dry wt. MTP^+ uptake in the absence of succinate was inhibited by the addition of 25 μM CCCP (Fig. 5).

Studies on TPP^+ accumulation

TPP^+ uptake, using TPP^+ in the concentration range 10 nM–100 μM, was readily detectable with

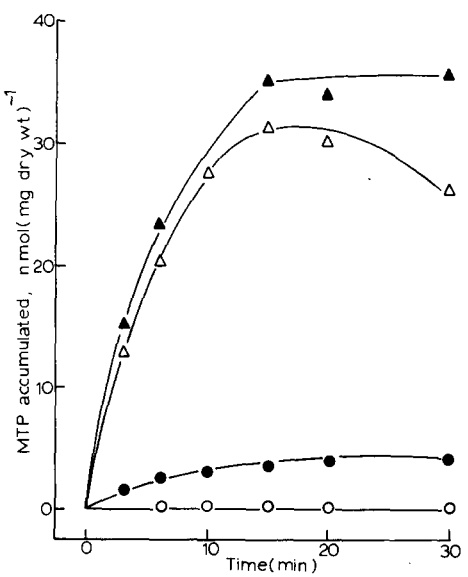

Fig. 3. Interaction of TPP^+ with MTP^+ uptake measured at different concentrations of MTP^+. MTP^+ uptake was assayed as described in the legend to Fig. 1, except that the MTP^+ concentration was 100 μM (△, ▲) or 10 μM (○, ●) in the presence (▲, ●) or absence (△, ○) of 50 μM TPP^+.

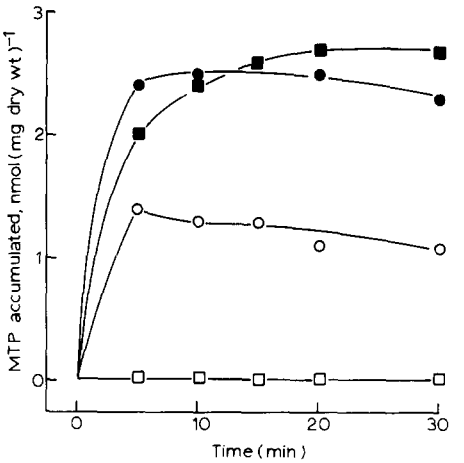

Fig. 4. Effect of EDTA treatment on MTP^+ uptake by *A. calcoaceticus*. MTP^+ uptake was assayed as described in the legend to Fig. 1 using EDTA-treated (○, ●) or untreated cells (□, ■) assayed in the presence (□, ●) or absence (□, ○) of 20 μM TPP^+.

untreated cells and uptake was inhibited by 25 μM CCCP (data not shown). TPP^+ accumulation was markedly stimulated by TPA^+ and crystal violet, and weakly stimulated by MTP^+, the exent of stimulation varying with the concentrations of TPP^+ and the stimulant (Table I). The extent of stimulation was also modified by treatment of the cells with EDTA. Using the same batch of cells,

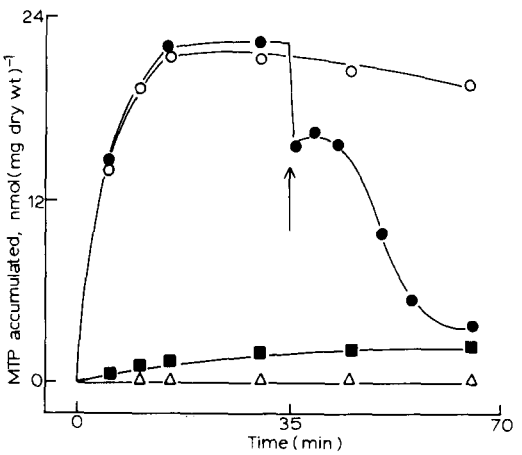

Fig. 5. Succinate-activated efflux of MTP^+ from *A. calcoaceticus*. The experiments were carried out as described in the legend to Fig. 1 with the following variations: ○, 100 μM MTP^+; ●, 100 μM MTP^+ plus 10 mM succinate added at time indicated by the arrow; ■, 100 μM MTP^+ plus 10 mM succinate throughout; △, 100 μM MTP^+ plus 25 μM CCCP throughout.

TABLE I

INTERACTION OF TPP^+ WITH *A. CALCOACETICUS*: EFFECT OF ANALOGUES

All incubations were carried out in the presence of 10 mM succinate. In experiments A and B, the TPP^+ concentration was 1 μM (spec. act. 125 μCi/μmol) and 40 μM (spec. act. 3.1 μCi/μmol), respectively. The values recorded are the steady-state values between 30 and 60 min (experiment A) or 15 and 30 min (experiment B). n.p., not performed.

Addition	Concn. (μM)	TPP^+ accumulated (nmol/mg dry wt.)	
		expt. A	expt. B
None	–	0.017	12.75
MTP^+	100	0.05	13.9
TPA^+	40	0.40	15.8
	100	0.50	n.p.
Crystal violet	50	0.45	16.6
	100	0.48	n.p.

the stimulation of TPP^+ uptake by 40 μM TPA^+ (measured with 1 μM TPP^+ in the presence of succinate) was 23-fold, and 1.4-fold in untreated and EDTA-treated cells, respectively. The untreated and treated cells, in the absence of TPA^+, accumulated 0.013 and 0.067 nmol TPP^+/mg dry wt., respectively.

A diminished accumulation of TPP^+ in response to succinate addition was observed in untreated cells incubated with 1 μM TPP^+; cells in the absence or presence of succinate accumulated 0.026 or 0.009 nmol TPP/mg dry wt., respectively.

Attempts were made to minimise the activity of the efflux system by employing low external concentrations of TPP^+ so that the internal concentration would also be low. However, the apparent $\Delta\psi$ values (calculated assuming a value of 2 ml intracellular water/g dry wt.) were observed to be 65, 70, 52, 110, 140, 148 and 143 mV at 0.01, 0.1, 1, 10, 20, 40 and 100 μM TPP^+, respectively. Thus, attempts to work at intracellular concentrations too low for efflux activity to be significant were probably offset by the lower rate of influx at the lower external concentrations, so that even a diminished efflux activity significantly depressed the steady-state intracellular concentration of TPP^+.

Discussion

The stimulation of MTP^+ uptake by TPP^+ could be due to a hyperpolarisation of the cell membrane, as observed with yeast treated with certain lipophilic cations [13]. However, we have been unable to demonstrate any marked changes in K^+ fluxes under conditions where TPP^+ stimulated influx (unpublished observations).

Our observations concerning the interaction of phosphonium ions with *A. calcoaceticus* are consistent with a scheme in which these compounds enter the organism by a non-mediated process in response to their electrochemical gradients. Efflux from the organism will also be non-mediated, but, in addition, a mediated (stereospecific), energy-linked process must also play a role. This mediated efflux system will presumably show saturation effects with regard to substrate. As a consequence of this behaviour, the proportion of the total efflux that occurs via this system will vary with internal substrate concentration and have a limiting value. In the case of MTP^+, with organisms possessing an intact outer membrane, the activity of the efflux system appears to be sufficient to lower the intracellular concentration of MTP^+ to the extent that an apparent impermeability at low concentrations of MTP^+ results (Fig. 1). As the concentration of MTP^+ increases, the rate of unidirectional influx will also increase and eventually allow measurable accumulation as observed at 100 μM. The small degree of stimulation of MTP^+ uptake by TPP^+ under these conditions (Fig. 3) is consistent with non-mediated fluxes of MTP^+ maintaining the steady-state accumulation, whereas at lower MTP^+ concentrations, the influx is mainly balanced by the mediated efflux. The stimulatory effect of EDTA is consistent with the outer membrane of the organism acting as a diffusion barrier for MTP^+ so that once this is modified, presumably by release of lipopolysaccharide, the increased influx of MTP^+ allows the system to move to a new steady state and, with some batches of cells, net accumulation can then be demonstrated (Fig. 4).

The uptake of TPP^+ by untreated cells may be explicable on the basis of a higher permeability constant, as indicated by studies on phosphonium ion permeability with membrane vesicles derived

from *Halobacterium halobium* [12], or on the basis of a different affinity for the efflux system. The method we have used to investigate the specificity of the efflux system (stimulation of influx at the steady state) requires the establishment of an intracellular concentration of a putative substrate that is significant with regard to the K_m (K_i) of the system for that substrate. This characteristic can only be used in a positive manner, since compounds not stimulating uptake may not reach a sufficiently high intracellular concentration. This may be the reason for the weak stimulation of TPP^+ uptake by MTP^+ when TPP^+ uptake was assayed at 1 μM (Table I). Clearly, a full explanation of the interactions we have observed will require detailed knowledge of the kinetic characteristics of the efflux system.

Our observations indicate that in the case of phosphonium ions, and probably TPA^+ and crystal violet, the apparent permeability of *A. calcoaceticus* is a function of both the outer membrane permeability and the activity of an efflux system. These observations may have more widespread implications for the penetration of other compounds into bacteria.

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