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H^+- OR K^+-DEPENDENT TRANSPORT SYSTEMS OF PHOSPHATE IN ALKALOPHILIC *BACILLUS*

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Membrane vesicles isolated from an alkalophilic *Bacillus* accumulated phosphate when a K^+ or H^+ gradient (extravesicular high) was imposed across the membranes. K^+- or H^+-dependent uptake of phosphate was not influenced by the reverse gradient of H^+ or K^+. Phosphate preloaded into the vesicles was released by an imposed H^+ or K^+ gradient (intravesicular high) and the release was inhibited by the reverse gradient of these ions. The optimum pH of the uptake lay between 9–10 in H^+ and K^+-dependent case. K^+-dependent uptake was stimulated by valinomycin beyond pH 9, indicating the transport was electrogenic in the pH. The kinetic profile of the activities indicates these two transport mechanisms are different systems.

Introduction

In a previous paper, we have reported that both alkaline pH and Na^+ are essential for the growth of an alkalophilic *Bacillus* sp. A-007 [1]. All the transport systems so far examined (for organic solutes and calcium) are driven only by an Na^+ gradient [2,3] and the optimum pH of all these systems is at alkaline pH (pH 9–10).

Phosphate is one of the physiologically important anions, and the mechanism of its transport has been studied in bacteria which grow at neutral pH [4–6]. Recently, the presence of two kinds of phosphate transport in *Escherichia coli*, one H^+-dependent and the other K^+-dependent, has been reported [7,8].

In the alkalophilic *Bacillus* sp. A-007, we have been specially interested in the kinds of ion coupled with phosphate in the phosphate transport of the *Bacillus*, because Na^+ was the only ion which was coupled with nutrients in the *Bacillus* so far studied.

In this study, we describe the particular characteristics of the phosphate-transport system in *Bacillus* sp. A-007.

Materials and Methods

Cultivation of bacterium and preparation of membrane vesicles. Bacillus sp. A-007 was grown aerobically and membrane vesicles were prepared according to the lysozyme-protoplast method as described earlier [3].

Protein determination. Protein concentration was determined by the method described by Lowry et al. [9] using bovine serum albumin as a standard.

Uptake of phosphate by membrane vesicles. Membrane vesicles were suspended in a medium (total 1 ml) comprising (final concentrations) 25 mM buffer (Tris-HCl (pH 7.4 or $NaHCO_3$/NaOH

Abbreviations: Hepes; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP; Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

(pH 10.0)), 2.5 mM $MgCl_2$, 0.4 M salt (KCl, NaCl, LiCl, RbCl or choline chloride) and 1 mM $Na_2H^{32}PO_4$ (37 GBq · mol^{-1}). Uptake was started by the addition of phosphate to the preincubated medium at 37°C. At suitable intervals, 100-μl samples were filtered through membrane filters (pore size, 0.45 μm). Membrane vesicles on the filters were washed four times with 2.5 ml of the medium without phosphate at 0°C, and dried. The radioactivity of the filter was measured in a liquid scintillation counter.

Efflux of phosphate from loaded vesicles. [^{32}P]Phosphate-loaded vesicles were obtained from the uptake experiment in which a K^+ gradient was used as the driving force and uptake was studied for 1 min. The phosphate-loaded vesicles were washed once with 25 mM buffer (Hepes/NaOH (pH 7.4), or Na_2HCO_3/NaOH (pH 10.0)) containing 0.4 M choline chloride and 2.5 mM $MgCl_2$ by centrifugation (30000 × *g*, 10 min), and resuspended in a small amount of the same buffer. The composition of the efflux medium (total 1 ml) was similar to that of the uptake medium, except that vesicles were replaced by the phosphate-loaded vesicles and [^{32}P]phosphate was omitted. Efflux at 37°C was started by the addition of loaded vesicles. At intervals, 100-μl samples were filtered through membrane filters and radioactivity was measured by a liquid scintillation counter.

Radioactive compound and chemicals. Carrier-free [^{32}P]phosphoric acid was purchased from New England Nuclear. Gramicidin and valinomycin were obtained from Sigma. FCCP was from Boehringer. Other chemicals were of the best grade commercially obtainable.

Results

Uptake of phosphate into membrane vesicles was examined in the vesicles upon which were imposed various cation gradients. Among the cations tested (K^+, Na^+, Li^+, Rb^+ and choline$^+$), the uptake was stimulated by H^+ and K^+ which had been added outside the vesicles (Fig. 1). In the experiment shown in the figure, the H^+ gradient across the membrane was imposed by the membranes loaded with pH 10 (pH 10 vesicles), being suspended in the medium of pH 7.4. When the vesicles loaded with pH 9.0 (pH 9 vesicles) were suspended in pH 6.5 medium, the activity was significantly lower than that measured with pH 10 vesicles. Therefore, we used pH 10 vesicles in our usual experiments. The activity in pH 10 vesicles was quite stable and only 25% of the activity was lost after 20 min of standing in an ice-bath. This is

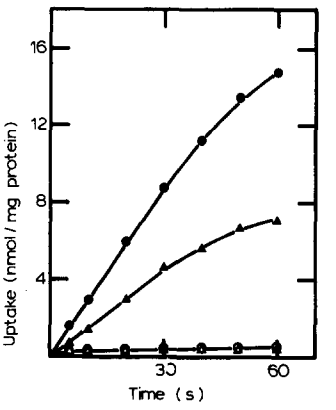

Fig. 1. Effect of H^+ and K^+ gradients on phosphate uptake in the membrane vesicles of alkalophilic *Bacillus*. Membrane vesicles were prepared in 25 mM $NaHCO_3$/NaOH buffer (pH 10.0) containing 0.4 M choline chloride and 2.5 mM $MgCl_2$. The vesicles were suspended in media containing 25 mM Tris-HCl (pH 7.4), 0.4 M choline chloride (▲, △) or 25 mM $NaHCO_3$/NaOH (pH 10.0), 0.4 M KCl (●, ○). All media also contained 2.5 mM $MgCl_2$ and $Na_2H^{32}PO_4$ (1 mM). Gramicidin (4 μg/ml) was added to some of the samples (open symbols).

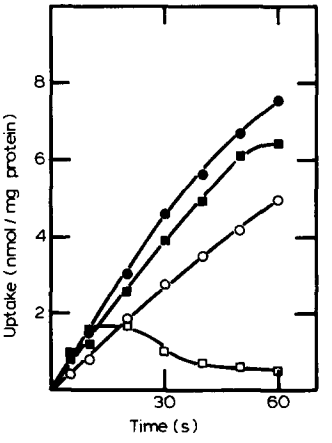

Fig. 2. Effect of pH on H^+-dependent uptake. Membrane vesicles were loaded with pH 11 (□), pH 10 (●), pH 9.0 (■) and pH 8.0 (○) buffers and the vesicles were suspended in the medium at pH 8.3, pH 7.4, pH 6.5 and pH 5.5, respectively. Buffers used were: $NaHCO_3$/NaOH (pH 11–10), Tris-HCl (pH 9.0–7.4) and HOAc/NaOAc (pH 6.5–5.5).

one of the typical characteristics of the membrane of *Bacillus* sp. A-007.

Then, we formed four kinds of vesicle which were loaded with pH 11, pH 10, pH 9.0 and pH 8.0, and H^+-gradient-driven phosphate uptake was assayed. In this experiment, the pH gradient across the membrane was fixed at about pH 2.5 difference, so these vesicles were suspended in media of pH 8.3, pH 7.4, pH 6.5 and pH 5.5, respectively. In all cases, phosphate was accumulated in the vesicles, but the initial rate of transport was highest in the case of the pH 10 vesicles (Fig. 2). These results may indicate that the pH optimum of the H^+-dependent system is above pH 8.3. At pH 11, the membrane become somewhat transparent at 37°C (but not below 25°C), and the transport continued only for a few seconds, decreasing thereafter.

We have examined the effect of a reverse gradient (intravesicular high) of H^+ or K^+ on the K^+- or H^+-gradient-driven phosphate uptake. In the presence of a reverse K^+ gradient, the rate of H^+-driven-phosphate uptake was almost the same as in the case of the absence of a K^+ gradient during first 10 s but was decreased thereafter, as shown in Fig. 3. The decrease after 10 s may indicate that the phosphate accumulated by the

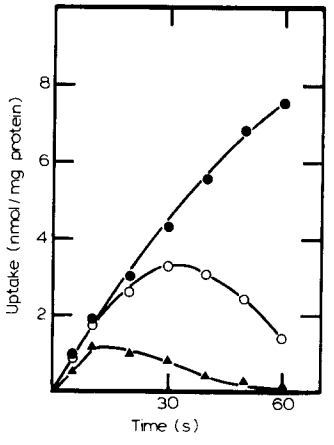

Fig. 3. Effect of a reverse K^+ gradient on the H^+-dependent phosphate uptake. Membrane vesicles were prepared in 25 mM $NaHCO_3$/NaOH buffer (pH 10) containing 0.4 M KCl and 2.5 mM $MgCl_2$. The vesicles were suspended in the medium containing 25 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 1 mM $Na_2H^{32}PO_4$ and 0.4 M choline chloride (○, ▲) or KCl (●). FCCP (4 μg/ml) was added to one sample (▲).

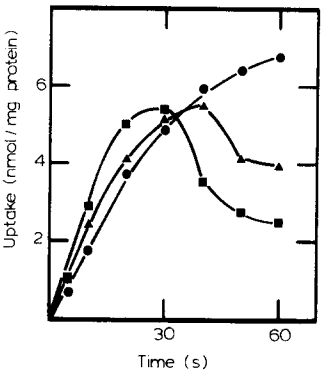

Fig. 4. Effect of a reverse H^+ gradient of K^+-dependent phosphate uptake. Mmebrane vesicles were prepared in 25 mM Hepes/NaOH buffer, pH 7.4 (●, ■) or pH 6.4 (▲), containing 0.4 M choline chloride and 2.5 mM $MgCl_2$. Membranes were suspended in 25 mM Hepes/NaOH (pH 7.4) (●) and 25 mM $NaHCO_3$/NaOH, pH 9 (▲) or pH 10 (■). These media contained 0.4 M KCl, 2.5 mM $MgCl_2$ and $Na_2H^{32}PO_4$.

H^+ gradient was extruded by the reverse K^+ gradient. In the absence of a K^+ gradient, phosphate accumulated constantly for about 45 s (Fig. 3). H^+-dependent uptake was inhibited by FCCP (Fig. 3), indicating the direct coupling of H^+ and phosphate in the transport.

On the other hand, phosphate uptake driven by a K^+ gradient was stimulated in the initial 10 s in the presence of reverse H^+ gradient and decreased thereafter (Fig. 4). The decreased rate after 10 s may also be indicative of the accumulated phosphate being extruded by the reverse H^+ gradient. The initial stimulation was also observed when pH 6.5 vesicles were suspended in a pH 9.0 medium, though the stimulation was much higher in the case of pH 10 vesicles. These stimulations might, however, be only a reflection of the pH-dependency of the transport activity. We therefore examined the pH-dependency of K^+-dependent phosphate uptake. In this experiment, the pH of the vesicles was fixed to be the same as in the reaction medium and K^+ was added only at the outside of the vesicles. The results presented in Fig. 5 show that the pH optimum of the K^+-dependent transport lay between 9 and 10.

K^+-dependent phosphate uptake was stimulated by valinomycin when the pH of the reaction (intravesicular = extravesicular) was beyond 9.0 (Fig. 5). Stimulation was not found under pH 8.0.

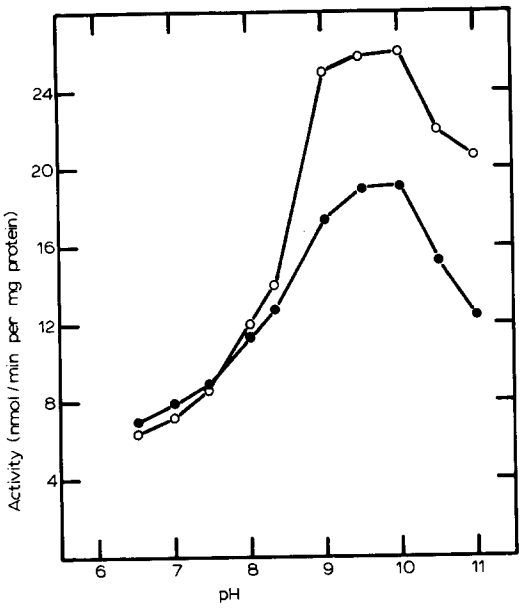

Fig. 5. Effect of pH on K^+-dependent phosphate uptake. Membrane vesicles were prepared in 25 mM buffers of pH 6.5, 7.0, 7.4, 8.0, 8.3, 9.0, 9.6, 10.0, 10.6 and 11.0 and these contained 0.4 M choline chloride and 2.5 mM $MgCl_2$. Transport was started by suspending these vesicles in a medium having the same pH as the vesicles and containing $Na_2G^{32}PO_4$, 0.4 M KCl (●) and valinomycin (○). Buffers used were HOAc/NaOAc (pH 6.5), Tris-HCl (pH 7.0–9.0) and $NaHCO_3$/NaOH (pH 9.5–11.0).

TABLE I

EFFECT OF CATION GRADIENT (IN < OUT) ON THE RELEASE OF PHOSPHATE FROM VESICLES

Phosphate was transported into membrane vesicles by a K^+-gradient at pH 10.0. The vesicles were washed once by centrifugation and resuspended in 25 mM $NaHCO_3$/NaOH (pH 10.0) containing 0.4 M choline chloride and 2.5 mM $MgCl_2$. Efflux media contained 25 mM Hepes/NaOH (pH 7.4) or $NaHCO_3$/NaOH (pH 10.0), 0.4 M cation, 2.5 mM $MgCl_2$ and loaded vesicles (0.5 mg protein) in total 1 ml. Release rate was calculated from release curves (within 30 s) obtained as shown in Fig. 7.

Extravesicular pH	Cation gradient	Release rate (nmol/mg protein per min)
10.0	choline $^+$	6.7
	K^+	3.7
	Rb^+	3.1
	Li^+	6.7
	Na^+	5.8
7.4	choline$^+$	1.4
	K^+	0.6

These results may indicate that the transport system is electrogenic above pH 9.0 but not under pH 9.0.

Kinetic studies of the uptake activity show the H^+- and K^+-dependent systems to be independent, one of the other. The apparent K_m of the K^+ system for phosphate ($3.3 \cdot 10^{-4}$ M) was about 6-fold larger than that of H^+-system ($6.0 \cdot 10^{-5}$ M).

The sensitivity of these transport systems to *N*-ethylmaleimide supported the above result. H^+-dependent activity was inhibited by $2 \cdot 10^{-2}$ M *N*-ethylmaleimide, while K^+-dependent activity was not affected by the drug (Fig. 6).

Release of phosphate from the vesicles were studied next. Efflux of the loaded phosphate in the vesicles was stimulated by an H^+ gradient (intravesicular high) and the H^+-stimulated efflux was inhibited by the reverse K^+ gradient (extravesicular high). Furthermore, as shown in Table I, H^+ and K^+ (Rb^+) gradient (extravesicular high) decreased phosphate efflux additively.

Discussion

The results of the present study indicate that two kinds of phosphate transport system, one H^+-dependent and the other K^+-dependent, are present in the alkalophilic *Bacillus* sp. A-007. An H^+-dependent phosphate uptake system is found in *Paracoccus dinitrificans* [4] and mitochondria [6,9]. On the other hand, an ATP-dependent phosphate uptake system has been reported in *Streptococcus faecalis* [10] and *Escherichia coli* [11]. Recently, a K^+- and H^+-linked phosphate transport system was found in *E. coli* [7,8].

Determination of the pH optimum of an H^+-dependent system is rather difficult. One cannot determine the exact optimum pH value. We have examined the pH dependency of the H^+-dependent system by shifting the pH under conditions in which the pH difference between intra- and extravesicular sides was fixed about 2.5. From the results shown in Fig. 2, it could be deduced that the optimum pH of the activity lies above pH 8.3.

On the other hand, the pH optimum of K^+-dependent system lay between pH 9 and 10, as shown in Fig. 6.

In the K^+-dependent system, it was significant

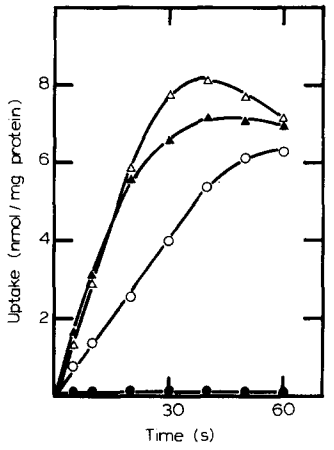

Fig. 6. Effect of *N*-ethylmaleimide on the phosphate uptake activity. Membrane vesicles were prepared in 25 mM $NaHCO_3$/NaOH (pH 10.0) containing 0.4 M choline chloride and 2.5 mM $MgCl_2$. Membranes were suspended in 25 mM Tris-HCl (ph 7.4), 0.4 M chloline chloride (○, ●), or 25 mM $NaHCO_3$/NaOH (pH 10.0) and 0.4 M KCl (△, ▲). These buffers also contained 2.5 mM $MgCl_2$ and 1 mM $Na_2H^{32}PO_4$, and *N*-ethylmaleimide was added to one portion of the samples (closed symbols).

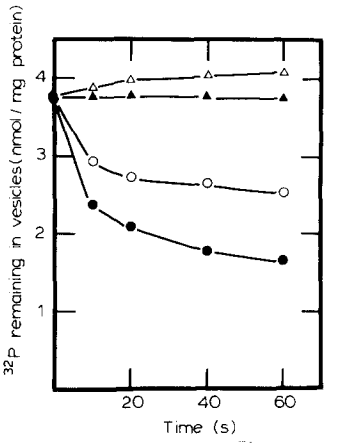

Fig. 7. Effect of H^+ and K^+ gradients on the phosphate release from preloaded vesicles. Phosphate was loaded in the vesicles by K^+ gradient at pH 7.4 (intra = extravesicular). The loaded vesicles were washed once with buffer at 4°C and suspended in a small volume (100 μl) of 25 mM Tris-HCl buffer (pH 7.4) containing 0.4 M choline chloride and 2.5 M $MgCl_2$ in an ice-bath. The release of phosphate from the vesicles was initiated by suspending 25 μl of vesicles in 1 ml of medium at 27°C. The media were as follows: (1) 25 mM Tris-HCl (pH 7.4) containing 0.4 M KCl (△) or choline chloride (▲); (2) 25 mM $NaHCO_3$/NaOH (pH 10.0) containing 0.4 M KCl (○) or choline chloride (●). All these media contained $Na_2H^{32}PO_4$ and $MgCl_2$.

that the system was stimulated by valinomycin at the pH 9.0, or more alkaline, side (Fig. 6). At neutral pH (under pH 8), phosphate ions are in the monovalent form but at pH 9–12, the phosphate anion is divalent. If 1 mol of cation were coupled with 1 mol of phosphate in the transport, one can assume that the transport should result in the net transfer of negative charge across the membrane in the reaction at pH 9–12. In the presence of valinomycin, the K^+-dependent system should induce an interior-positive membrane potential; therefore, the transport should be stimulated in the reaction at pH 9–12. As shown in Fig. 5, K^+-dependent uptake of phosphate was stimulated by valinomycin when the pH of the reaction was above 9.0. It could be deduced, therefore, that the carrier protein binds 1 mol of phosphate and K^+ in the K^+-dependent reaction in alkalophilic *Bacillus* sp. A-007.

Phosphate uptake activity of *Bacillus* sp. A-007 was dependent on the pH of the reaction. Both H^+- and K^+-dependent activities were activated at alkaline pH (above pH 8). When one estimates the initial rate of phosphate uptake in all the experiments in the present study, it can be easily seen that the rates were dependent on the pH of the substrate-binding side (extravesicular in Fig. 1–6).

Activation of the K^+-dependent system by a reverse H^+ gradient, as shown in Fig. 4, can be explained, therefore, by pH-dependency of the activity.

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