Biochimica et Biophysica Acta, 508 (1978) 370—378

BBA 77978

PHOSPHATE TRANSPORT IN MEMBRANE VESICLES FROM *ESCHERICHIA COLI*

WIL N. KONINGS and HARRY ROSENBERG

Department of Biochemistry, The John Curtin School of Medical Research, Australian National University A.C.T., Canberra (Australia)

(Received September 7th, 1977)

Summary

Escherichia coli strain AN710 possesses only the PIT system for phosphate transport. Membrane vesicles from this strain, which contain phosphate internally, perform exchange and active transport of phosphate. The energy for active transport is supplied by the respiratory chain with ascorbate-phenazine methosulphate as electron donor. To a lesser extent also the oxidation of D-lactate energizes phosphate transport; the oxidation of succinate is only marginally effective. Phosphate transport is driven by the proton-motive force and in particular by the pH gradient across the membrane. This view is supported by the observation that phosphate transport is stimulated by valinomycin, inhibited by nigericin and abolished by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone. Neither inhibitor affects phosphate exchange. The phosphate analogue arsenate inhibits both the exchange reaction and active transport. Both processes are stimulated by K^+ and Mg^{2+}, the highest activities being observed with both ions present.

Membrane vesicles have also been isolated from *Escherichia coli* K10, a strain which possesses only a functional PST phosphate transport system. These vesicles perform neither exchange nor active transport of phosphate, although active transport of amino acids is observed in the presence of ascorbate-phenazine methosulphate or D-lactate.

Introduction

Phosphate transport has been studied in *Escherichia coli* by several investigators [1—10]. The presence of two major systems for transport of phosphate in *E. coli* was first demonstrated by Willsky et al. [4]. Recently Rosenberg et al. [9], using mutant strains of *E. coli* in which only one or the other system was operative reported that one system (PIT) was fully constitutive, required

no binding protein and operated in spheroplasts. The other system (PST) was repressed by phosphate concentrations above 1 mM, required the phosphate binding protein for full activity and did not operate in spheroplasts.

The energetics of these transport systems have been studied by H. Rosenberg, R.G. Gerdes and F. Harold (manuscript in preparation). Phosphate transport via the PIT system appears to be driven by a proton-motive force, generated by electron transfer in the respiratory chain or by anaerobic electron transfer systems; phosphate transport via the PST system appears to be driven by phosphate bond energy.

In view of these results it was of interest to study phosphate transport in membrane vesicles. Such studies can supply direct information about the mechanism of energization of phosphate transport via the two systems. These studies are also of interest for other reasons. Phosphate is one of the major components of the incubation mixture used with membrane vesicles. Translocation of phosphate could consume a considerable part of the energy supplied. Therefore, information about the translocation of phosphate and other ions is essential for studies on the stoichiometry of solute-proton translocation or for studies on the efficiency of energy utilization.

Materials and Methods

Strains and culture conditions. The *E. coli* strains, K10 and AN710, have been described elsewhere [9] as were the conditions for growth, except that potassium succinate (20 mM) was used as a carbon source.

Isolation of membrane vesicles. Cells were harvested at the late exponential stage of growth (A_{660nm} = 0.9) and spheroplasts were prepared as described previously [11,12]. Membrane vesicles, which contained phosphate internally, were prepared as described [11,12] by osmotic lysis of the spheroplasts in 50 mM potassium phosphate, pH 6.6. The vesicles were harvested by centrifugation (1 h, 46 000 × *g*), washed twice with 50 mM imidazole · HCl (pH 6.6) in order to remove external phosphate and finally suspended in this buffer to a protein concentration of 5—10 mg per ml.

Membrane vesicles devoid of phosphate were prepared by lysing the spheroplasts in 50 mM imidazole · HCl (pH 6.6) instead of phosphate buffer. Membrane vesicles were stored in aliquots of 0.5 ml in liquid nitrogen.

Uptake experiments. Uptake of L-[U-^{14}C]proline and [^{32}P]phosphate by membrane vesicles were measured as described previously [11,12]. [^{32}P]Phosphate (Australian Atomic Energy Commission, Lucas Heights, N.S.W., Australia) (specific activity 50 Ci/mol) was added at a final concentration of 0.83 mM; L-[U-^{14}C]proline (The Radiochemical Centre, Amersham, England) (specific activity 230 Ci/mol) was added at a final concentration of 11.6 μM. Uptake experiments in the presence of binding protein were performed by adding phosphate binding protein at a final concentration of 1 mg per ml 5 min prior to [^{32}P]phosphate. The effects on phosphate uptake of the ionophores valinomycin (30 μg/ml) and nigericin (30 μg/ml), the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (40 μM), the phosphate analogue arsenate (1 mM) and the respiratory chain inhibitors 2-heptyl-4-hydroxyquinoline-*N*-

oxide (10 μM) (HQNO) and KCN (10 mM) were studied as described previously [11—14].

Phosphate binding protein was isolated as described [10].

Protein was determined by the method of Lowry et al. [15].

Oxygen utilization. The consumption of oxygen by the membrane vesicles in the presence of electron donors was measured polarographically as described [12]. All data were corrected for endogenous respiration which was about 25 natoms oxygen/min per mg membrane protein.

Results

Phosphate buffers are commonly used in the lysis of spheroplasts in the isolation of membrane vesicles of *E. coli* and in the subsequent steps of the procedure [11]. Since accurate measurement of the accumulation of [^{32}P]phosphate is carried out with low concentrations of solute, membrane vesicles were prepared initially in a phosphate-free medium (buffered with imidazole · HCl) but these membrane vesicles transport neither phosphate nor L-proline in the presence of an electron donor.

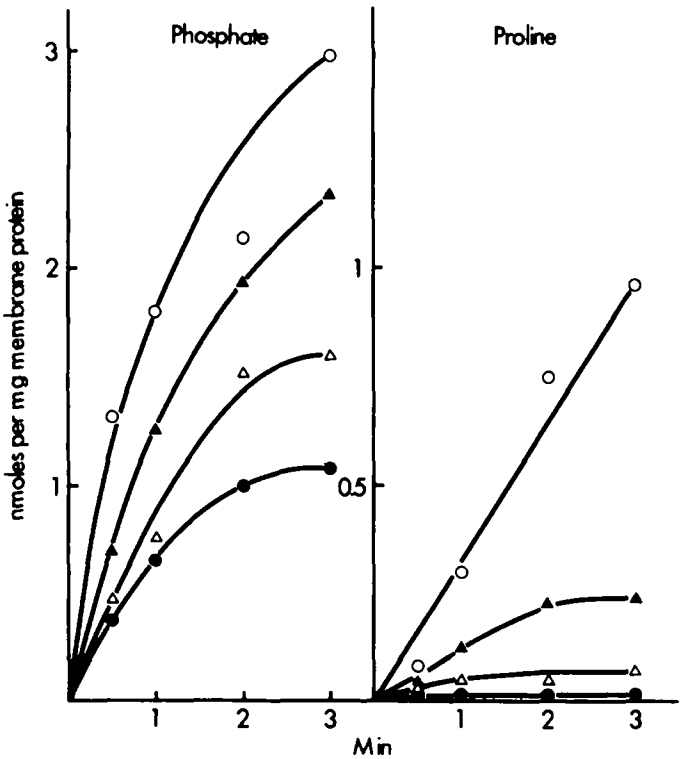

Fig. 1. Uptake of phosphate and L-proline by membrane vesicles from *E. coli* AN710. Phosphate uptake was performed in an incubation mixture consisting of 10-μl membrane vesicles (6 mg membrane/protein per ml) and 50 μl of 50 mM imidazole · HCl (pH 6.6) 20 mM KCl and 2.5 mM $MgCl_2$. For proline uptake experiments 10 μl of membrane vesicles were diluted with 50 μl of 50 mM potassium phosphate (pH 6.6) and 10 mM $MgCl_2$. Phosphate concentration: 0.83 mM. L-Proline concentration: $11.5 \cdot 10^{-6}$ M. ●, no electron donor added; △, 20 mM potassium succinate; ▲, 20 mM lithium D-lactate; ○, 20 mM potassium ascorbate (pH 6.6) and 200 μM phenazine methosulphate.

Membrane vesicles which actively transported both phosphate and L-proline were only obtained when the spheroplasts were lysed in phosphate buffers. This activity was retained after removal of external phosphate by washing and resuspension in imidazole · HCl buffers.

Fig. 1 shows the uptake of phosphate and proline by these membrane vesicles in the presence of various electron donors. Ascorbate (Asc) in the presence of phenazine methosulphate (PMS) was the most effective electron donor for the uptake of both solutes. A significant stimulation of phosphate and proline uptake was also observed with D-lactate while succinate was only marginally effective. NADH (10 mM) in the presence of phenazine methosulphate (200 μM) was as effective in energizing phosphate and proline uptake as ascorbate-phenazine methosulphate. The effect of NADH alone was similar to that of succinate (data not shown). In the absence of an electron donor the uptake of proline was negligible but a significant accumulation of phosphate label occurred. This observation is consistent with the finding that both whole cells and spheroplasts of strain AN710 carry out exchange of internal and external phosphate [9]. The vesicles used in this experiment contained internally 50 mM phosphate.

The electron donors NADH, D-lactate and succinate were oxidized by membrane vesicles from *E. coli* AN710 at rates of 160, 102 and 34 natom oxygen/min per mg membrane protein, respectively. Inhibitors of electron transfer

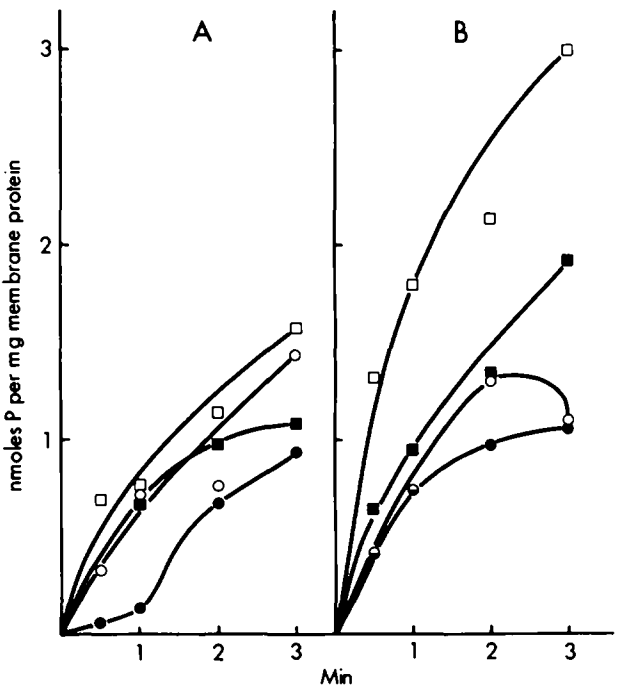

Fig. 2. Effect of cations on the accumulation of phosphate by membrane vesicles from *E. coli* AN710. (A) Without electron donor added. (B) In the presence of 20 mM imidazole-ascorbate (pH 6.6) and 200 μM phenazine methosulphate. Uptake experiments were performed in 50 mM imidazole · HCl (pH 6.6) as described in Materials and Methods and in the legend to Fig. 1. ●, no cations added; ■, with 40 mM KCl; ○, with 2.5 mM $MgCl_2$; □, with 2.5 mM $MgCl_2$ and 40 mM KCl.

(cyanide, HQNO) were found to inhibit the substrate-stimulated phosphate uptake (results not shown). It follows from these observations that the energy for active transport of phosphate by the PIT system is supplied by respiration.

Effect of cations

Phosphate uptake in whole cells of *E. coli* proceeds optimally in the presence of Mg^{2+} and K^+ [3]. We have studied the effects of these ions on phosphate exchange (Fig. 2A) and respiratory chain-linked phosphate transport (Fig. 2B) in membrane vesicles from *E. coli* AN710.

The exchange rates in the presence of K^+ and Mg^{2+} are higher than in the presence of either K^+ or Mg^{2+} alone and significantly higher than in the absence of these ions. These ions similarly affect ascorbate-phenazine methosulphate-driven phosphate uptake, the highest initial rates being observed with both K^+

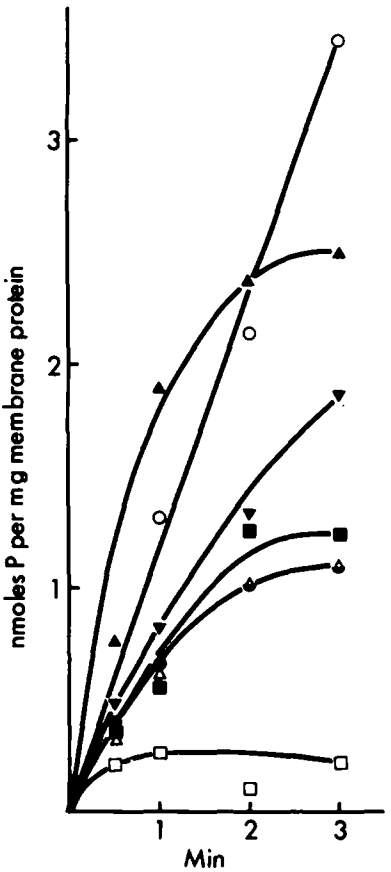

Fig. 3. Effect of ionophores and arsenate on respiration-driven phosphate uptake by membrane vesicles from *E. coli* AN710. Uptake experiments were performed in 50 mM imidazole · HCl (pH 6.6) as described in Materials and Methods and in the legend to Fig. 1 with membrane vesicles prepared in 50 mM potassium phosphate of pH 6.6. ●, no electron donor added; ○, with 20 mM potassium ascorbate and 200 μM phenazine methosulphate; ▲, with ascorbate-phenazine methosulphate and 30 μg/ml valinomycin; ▲, with ascorbate-phenazine methosulphate and 30 μg/ml nigericin; ■, with ascorbate-phenazine methosulphate and 30 μg/ml each of valinomycin and nigericin; △, with ascorbate-phenazine methosulphate and 40 μg/ml CCCP; □, with ascorbate-phenazine methosulphate and 1 mM potassium arsenate.

and Mg^{2+} present. The stimulatory effect of K^+ and Mg^{2+} on both the exchange and on active uptake of phosphate suggests that these ions are involved in the actual translocation step of phosphate across the membrane.

Effects of ionophores and of arsenate

In order to obtain further insight into the mechanism of energization of phosphate transport the influence of the ionophores valinomycin and nigericin was investigated. Valinomycin functions as a carrier of K^+ and thus collapses the membrane potential; this decrease of the membrane potential is partly compensated by an increase of the ΔpH [17,18]. Nigericin exchanges K^+ for H^+ and thus decreases the pH gradient across the membrane [16]. We have found (Fig. 3) that valinomycin stimulated and that nigericin inhibited the initial rate of phosphate transport. Almost complete inhibition (down to exchange levels) was observed in the presence of both ionophores or CCCP (Fig. 3).

These observations strongly implicate the proton-motive force as a driving force for active transport of phosphate. The results point to the ΔpH as the major driving force for phosphate accumulation. This conclusion is also supported by the result of studies on phosphate transport in valinomycin-treated vesicles at different external pH values. The highest initial rates of transport were observed between pH 5.5 and 6.0 (data not shown). Ramos and Kaback [17,18] demonstrated that this is the pH range at which the ΔpH has its highest value. Valinomycin, nigericin or CCCP have no effect on phosphate exchange. A proton-motive force therefore appears not to be involved in the phosphate exchange mechanism.

Both phosphate exchange and active phosphate uptake, however, are inhibited by the phosphate analogue arsenate (Fig. 3).

Membrane vesicles from E. coli K10

E. coli K10 lacks the PIT system for phosphate transport, consequently only the PST system is operative in this strain [4,9]. Membrane vesicles, prepared from *E. coli* K10 in the same way as described above for *E. coli* AN710, were used to study active transport activities of phosphate and proline (Fig. 4).

In the absence of an energy source membrane vesicles from *E. coli* K10 perform hardly any uptake of phosphate. In the presence of ascorbate-phenazine methosulphate a small stimulation of phosphate uptake is observed but the accumulation is significantly lower than that observed in *E. coli* AN710. The low rate of phosphate uptake was not due to an inefficient energy supply as these membrane vesicles accumulated L-proline in the presence of ascorbate-phenazine methosulphate at a rate and to a steady-state level comparable with those observed in membrane vesicles from *E. coli* AN710. Some stimulation of proline transport was also observed with D-lactate (Fig. 4). Furthermore, membrane vesicles from strain K10 oxidized the different electron donors at rates as high or higher than did membrane vesicles from *E. coli* strain AN710 (oxidation rate of NADH was 496, of D-lactate 98 and of succinate 40 natom oxygen/min per mg membrane protein).

The periplasmic phosphate binding protein is essential for activity of the PST system. Gerdes et al. [10] demonstrated recently that phosphate transport in spheroplasts via the PST system could be reconstituted with this binding pro-

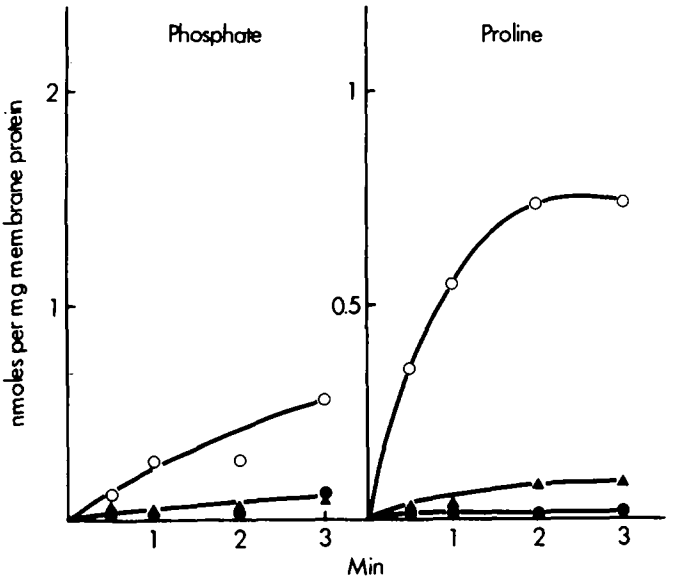

Fig. 4. Uptake of phosphate and L-proline by membrane vesicles from *E. coli* K10. The experiments were performed as described in Materials and Methods and in the legend to Fig. 1. ●, no electron donor added; ○, with 20 mM potassium ascorbate and 200 μM phenazine methosulphate; ▲, with 20 mM lithium D-lactate.

tein. We attempted to drive phosphate transport in membrane vesicles from *E. coli* K10 in the presence of the binding protein. However, no accumulation of phosphate could be observed with either ascorbate-phenazine methosulphate or with ATP.

Discussion

The results presented demonstrate that membrane vesicles from *E. coli* AN710 perform respiration-linked active transport of phosphate via the PIT system. Evidence is presented that the proton-motive force and in particular the ΔpH is the driving force for phosphate accumulation. Active transport of phosphate in *E. coli* via the PIT system therefore occurs by a mechanism which is very similar to the active transport mechanisms described for amino acids, β-galactosides, carboxylic acids and other solutes [19]. Respiration-linked transport of phosphate requires a specific membrane protein which is also involved in the exchange of internal phosphate with external phosphate, as indicated by the inhibitory effect of arsenate on both activities and by the absence of these activities from membrane vesicles of *E. coli* K10. The question arises whether the high rate of exchange observed in vesicles of AN710 takes place also under energized conditions, or whether all such uptake represents active transport. In view of the fact that both exchange and active transport appear to use the same carrier protein, the latter possibility seems to be the most likely.

Vesicles from strain K10 failed to take up phosphate in the presence of any of the electron donors tested, ATP, and high concentrations of the phosphate

binding protein. This, and the successful demonstration of such transport in spheroplasts of *E. coli* K10 supplemented with the binding protein, is consistent with the finding (Rosenberg, Gerdes, and Harold, manuscript in preparation) that phosphate transport in the PST system is energized by phosphate bond energy.

The PIT system is constitutive [9] and is thus present in membrane vesicles from wild type *E. coli*. Since such vesicles are usually prepared in phosphate buffers, they will, in the presence of an electron donor, accumulate not only any solute being tested but also phosphate and possibly other cations or anions. In studies involving the determination of the number of protons translocated during transport of a solute, the calculations are in most cases based on the direct measurements of pH changes during transport. These determinations are only meaningful when the movement of all other ions is either excluded or corrected for. Such corrections have occasionally been made with respect to the movement of K^+, but fluxes of phosphate have, with one exception [21], been largely ignored.

At any time, energy supplied by respiration is probably used to drive several translocation processes (active transport mechanisms and active extrusion mechanisms). The efficiency of utilization of respiratory energy for transport was claimed to be low in that the number of molecules of electron donor oxidized greatly exceed those of solute transported [20]. However, if in addition to the solute tested, the translocation of several inorganic ions of the medium is taken into account, then the energy appears to be expended much more efficiently.

Recently Rhoads and Epstein [22] reported that K^+ is transported in *E. coli* by three distinct transport systems. One of these systems appears to be dependent on a proton-motive force and is stimulated by phosphate. Rosenberg (unpublished data) observed that at pH 6.8, two potassium ions enter *E. coli* with each phosphate ion both in the PST and the PIT system. These results, taken together, suggest, that one of the systems for K^+ transport in *E. coli* may be involved in a co-transport with phosphate.

Acknowledgements

This study was supported by the Netherlands Organization for Advancement of Pure Scientific Research (ZWO). W.N.K. was on leave from the Department of Microbiology, University of Groningen, Kerklaan 30, Haren, The Netherlands.

References

1 Medveczky, N. and Rosenberg, H. (1970) Biochim. Biophys. Acta 211, 158—168
2 Bennett, R.L. and Malamy, M.H. (1970) Biochem. Biophys. Res. Commun. 40, 496—500
3 Medveczky, N. and Rosenberg, H. (1971) Biochim. Biophys. Acta 241, 494—506
4 Willsky, G.R., Bennett, R.L. and Malamy, M.H. (1973) J. Bacteriol. 113, 529—539
5 Willsky, G.R. and Malamy, M.H. (1974) Biochem. Biophys. Res. Commun. 60, 226—233
6 Rosenberg, H., Cox, G.B., Butlin, J.D. and Gutowsky, S.J. (1975) Biochem. J. 146, 417—423
7 Rae, A.S. and Strickland, K.P. (1976) Biochim. Biophys. Acta 433, 564—582
8 Rae, A.S., Strickland, K.P., Medveczky, N. and Rosenberg, H. (1976) Biochim. Biophys. Acta 433 555—560

9 Rosenberg, H., Gerdes, R.G. and Chegwidden, K. (1977) J. Bacteriol. 131, in the press
10 Gerdes, R.G., Strickland, K.P. and Rosenberg, H. (1977) J. Bacteriol. 131, in the press
11 Kaback, H.R. (1971) in Methods in Enzymology (Jakoby, W.B., ed.), Vol. 22, pp. 99—120, Academic Press, New York
12 Matin, A. and Konings, W.N. (1973) Eur. J. Biochem. 34, 58—67
13 Schuldiner, S. and Kaback, H.R. (1975) Biochemistry 14, 5451—5461
14 Boonstra, J. and Konings, W.N. (1977) Eur. J. Biochem., in the press
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
16 Harold, F.M. (1972) Bacteriol. Rev. 36, 172—230
17 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 848—854
18 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 854—858
19 Konings, W.N. (1977) in Advances in Microbial Physiology (Rose, A.H. and Tempest, D.W., eds.), Vol 15, pp. 175—250, Academic Press, London
20 Bisschop, A. and Konings, W.N. (1976) Eur. J. Biochem. 67, 357—365
21 Brand, M.D. and Lehninger, A.L. (1977) Proc. Natl. Acad. Sci. U.S. 74, 1955—1959
22 Rhoads, D.B. and Epstein, W. (1977) J. Biol. Chem. 252, 1394—1401