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Transport of Vitamin B_{12} in *Escherichia coli*: Energy Dependence

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This paper presents some evidence that the osmotic shock-sensitive, energy-dependent transfer of vitamin B_{12} from outer membrane receptor sites into the interior of cells of *Escherichia coli* requires an energized inner membrane, without obligatory intermediation of adenosine 5'-triphosphate (ATP). The experiments measured the effects of glucose, D-lactate, anaerobiosis, arsenate, cyanide, and 2,4-dinitrophenol upon the rates of B_{12} transport by starved cells of *E. coli* KBT001, which possesses a functional Ca^{2+}, Mg^{2+}-stimulated adenosine triphosphatase (Ca,MgATPase), and of *E. coli* AN120, which lacks this enzyme. Both strains were able to utilize glucose and D-lactate aerobically to potentiate B_{12} transport, indicating that the Ca,MgATPase was not essential for this process. When respiratory electron transport was blocked, either by cyanide or by anaerobic conditions, and the primary source of energy for the cells was presumably ATP from glucose fermentation, the rate of B_{12} transport was much reduced in *E. coli* AN120 but not in *E. coli* KBT001. These results support the view that the Ca,MgATPase can play a role in B_{12} transport but only when the energy for this process must be derived from ATP. The results of experiments with arsenate also supported the conclusion that the generation of phosphate bond energy was not absolutely required for B_{12} transport.

The availability of strains of *Escherichia coli* that are mutant in the Ca^{2+},Mg^{2+}-stimulated adenosine triphosphatase (Ca,MgATPase) has facilitated studies on the energy coupling of several active transport systems. This ATPase evidently participates in the phosphorylation of adenosine diphosphate (ADP) at the expense of the energized membrane state (or proton-motive force) generated by the oxidation-reduction reactions of the respiratory electron transport chain (1, 11, 19). In the reverse direction, this enzyme participates in the generation of the energized membrane state from adenosine 5'-triphosphate (ATP). The active transport systems of *E. coli* have been divided into two groups on the basis of energy coupling. One group is potentiated directly by the energized membrane state without the obligatory intermediation of ATP (2, 3, 13-16, 20, 22, 23). In the presence of a functional Ca,MgATPase, these transport processes can also derive energy from ATP. The second group of active transport processes has an obligatory requirement for ATP and requires the intermediation of the Ca,MgATPase for utilization of energy from the energized membrane state (2, 3, 6, 12, 22). An interesting correlation is that the members of this group of transport processes are sensitive to osmotic shock treatment of the cells and seem to require the participation of specific periplasmic binding proteins. The energized membrane-dependent transport processes, on the other hand, are relatively insensitive to osmotic shock and presumably do not require specific periplasmic proteins.

As shown previously, the energy-dependent phase of vitamin B_{12} transport in cells from *E. coli* is severely diminished by an osmotic shock, but it is not yet known whether the few molecules of a B_{12}-binding protein that are released by the shock treatment have an essential role in B_{12} transport (9, 17, 18, 21). In this paper we present some evidence that this shock-sensitive, active transport of vitamin B_{12} requires specifically an energized membrane, without the obligatory involvement of ATP.

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MATERIALS AND METHODS

Bacterial strains. The two strains used in this study were *E. coli* KBT001 (F⁻ *leu pro lysA trp purE metE str lac tonA*) (4, 9, 10, 21), and *E. coli* AN120 (F⁻ *argE3 thi-1 str uncA401*) (5). A culture of the latter strain was obtained from H. R. Kaback. Although these two *E. coli* K-12 strains are not an isogenic pair, we have found no differences in their transport of vitamin B_{12} that could not be explained most readily in terms of the function of the

Ca,MgATPase. Samples of liquid cultures, mixed with equal volumes of sterile 40% glycerol, were stored in liquid nitrogen until required.

Bacterial growth and starvation. Cells from both strains were grown aerobically at 37°C on the minimal medium of Davis and Mingioli (7), supplemented appropriately with amino acids (50 μg/ml), adenine (30 μg/ml), and glucose (1%). Growth was followed by measuring the optical density at 660 nm (OD_{660}).

The experiments all used cells that had been starved by the method described by Berger (2). Mid-log-phase cells (OD_{660} 0.6 to 0.7) were washed once with minimal phosphate [containing 34 mM NaH_2PO_4, 64 mM K_2HPO_4, 0.4 mM $MgSO_4$, and 0.02 mM $Ca(NO_3)_2$, adjusted to pH 7.0] and then suspended at a density of 1 g of cells (wet weight) per 200 ml of minimal phosphate containing 5 mM 2,4-dinitrophenol. This suspension was incubated in a water bath shaker at 300 rpm and 37°C for periods that were usually 1 to 2 h for *E. coli* AN120 and 2 to 12 h for *E. coli* KBT001. These periods of starvation were determined experimentally to give the maximum difference between the rates of B_{12} transport in the presence and absence of 20 mM glucose. After starvation, the cells were washed three times with minimal phosphate and finally stored in minimal phosphate (pH 6.6) at a concentration of 1 g of cells per 20 ml. The number of cells per milliliter was measured with a microscope, with the aid of a Petroff-Hausser slide. The preparation of cells for experiments with sodium arsenate as an inhibitor used minimal HEPES (pH 6.6), instead of minimal phosphate to wash and suspend the cells. Minimal HEPES contained 90 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 15 mM KCl in place of the phosphates of minimal phosphate.

Radiochemicals. Tritium-labeled cyanocobalamin ($[^3H]B_{12}$; specific activity, about 1.7 mCi/μmol) was obtained from Amersham/Searle, Arlington Heights, Ill.

Uptake of vitamin B_{12}. The reaction mixtures (10 ml) in 25-ml Erlenmeyer flasks contained (final concentrations) 0.1 M potassium phosphate (pH 6.6), 6.9 nM $[^3H]B_{12}$, approximately 6×10^8 cells per ml, and energy sources (20 to 40 mM) and inhibitors (<2 mM) where appropriate. The incubations were usually aerobic in a water bath shaker at 300 rpm and 37°C, with a 4-min preincubation under the same conditions before addition of the $[^3H]B_{12}$. Samples (1 ml) were removed at intervals, filtered through membrane filters (Millipore Corp., 0.45-μm pore size, 25 mm diameter), washed twice with 10 ml of 0.2 M lithium chloride, and dried in an oven. The dry filters, in vials with 10 ml of toluene containing 0.4% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP), were counted in a Beckman LS-100C liquid scintillation counter. The results are expressed as picomoles of B_{12} taken up per 10^9 cells.

RESULTS

Energy sources for B_{12} transport. Figure 1 shows the effects of glucose and of D-lactate upon the uptake of $[^3H]B_{12}$ by starved cells of *E. coli* KBT001. In the absence of an added energy source, there was rapid uptake of about 0.4 pmol of B_{12}, followed by a much slower rate of further B_{12} uptake. We showed previously that the initial rapid uptake consists of binding of B_{12} to the outer membrane B_{12} receptors and is not energy dependent (8, 21). The subsequent B_{12} uptake, shown here between 1 and 30 min, represents the rate of the energy-dependent transfer of B_{12} from the receptors into the interior of the cell. The slow secondary increase in the B_{12} content of the starved cells without an added energy source in Fig. 1 indicates that the endogenous sources of metabolic energy were not completely depleted during the starvation process. The secondary rate of B_{12} uptake was stimulated approximately fivefold by the inclusion of either 20 mM glucose or 20 mM D-lactate in the reaction mixtures. Glucose was approximately equally effective as an energy source under both aerobic and anaerobic conditions. Thus, these cells are capable of utilizing energy derived from glucose fermentation for the transport of vitamin B_{12}.

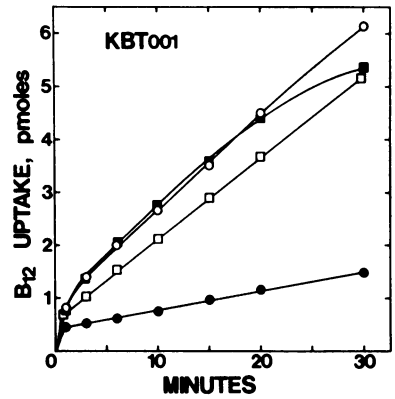

FIG. 1. *Potentiation of B_{12} transport by glucose and D-lactate in starved cells from E. coli KBT001. The reaction mixtures contained 0.1 M KPO_4 (pH 6.6), approximately 6×10^8 cells per ml, 6.9 nM $[^3H]B_{12}$, and other additions. The incubations were in a water bath shaker at 300 rpm and 37°C, with a 4-min preincubation period before addition of the $[^3H]B_{12}$. Samples (1 ml) were removed at intervals and treated as described in the text. The other additions and conditions were: no added energy source, gas-phase air (●); 20 mM glucose, gas-phase air (○); 20 mM glucose, gas-phase nitrogen (■); and 20 mM lithium D-lactate, gas-phase air (□). Results are expressed as picomoles of B_{12} taken up per 10^9 cells.*

Figure 2 shows the results of a similar experiment with starved cells of the Ca,MgATPase mutant strain, *E. coli* AN120. Aerobically, the uptake of $[^3H]B_{12}$, in the presence of either glucose or D-lactate, was essentially identical to that for strain KBT001. A crucial difference,

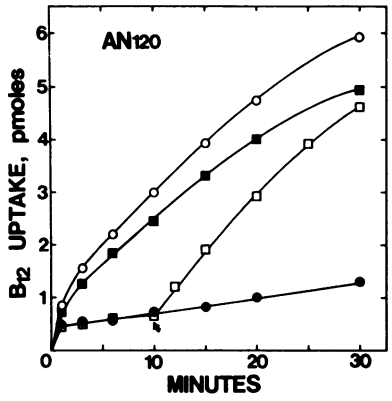

FIG. 2. *Potentiation of B_{12} transport by glucose and D-lactate in starved cells from E. coli AN120. The reaction mixtures and assay procedures were the same as those described for Fig. 1, except that the cells were from E. coli AN120. Other additions and conditions were: no added energy source, gas-phase air (●); 20 mM glucose, gas-phase air (○); 20 mM glucose, gas-phase nitrogen initially, but changed to air after 10 min as shown by the arrow (□); 20 mM lithium D-lactate, gas-phase air (■). Results are expressed as picomoles of B_{12} taken up per 10^9 cells.*

however, was that cells of *E. coli* AN120 were unable to use glucose anaerobically to potentiate B_{12} transport. The anaerobic energy-dependent rate of B_{12} uptake in the presence of glucose was no greater than that of aerobic cells without an added energy source. This indicates that the Ca,MgATPase is necessary for coupling the energy derived from glucose fermentation to the transport of vitamin B_{12}. When air replaced the nitrogen of the anaerobic reaction mixture (shown by the arrow in Fig. 2), the rate of B_{12} transport was immediately stimulated about fivefold.

One of our checks on the authenticity of the cell preparations from *E. coli* AN120 was to compare the rates of uptake of [^{14}C]glycine and [^{14}C]arginine by starved cells in the presence of 20 mM glucose with the rates obtained in the presence of 20 mM sodium ascorbate-140 μM phenazine methosulfate (PMS). As expected, the ascorbate-PMS was much more effective than glucose in potentiating glycine uptake, which is not dependent upon ATP, but was unable to potentiate arginine transport (an ATP-dependent system) (3). It would also be expected that ascorbate-PMS would be an effective energy source for B_{12} transport, but this was not the case. The usual combination of ascorbate (20 mM) and PMS (140 μM) inhibited B_{12} uptake by both strains of cells (data not shown). This seemed to be primarily inhibition of receptor B_{12} binding. A separate experiment, with ascorbate constant at 20 mM and varying PMS concentrations, showed that 50% inhibition of receptor binding of B_{12} was given by approximately 100 μM PMS.

Inhibitors of B_{12} transport. Figure 3 shows the effects of various concentrations of potassium cyanide and 2,4-dinitrophenol upon the rate of the energy-dependent phase of B_{12} transport by starved cells of *E. coli* strains KBT001 and AN120. Each reaction mixture also contained 20 mM glucose. The time course of B_{12} uptake was determined for each inhibitor concentration by taking several samples between 1 and 20 min. In each case, the amount of B_{12} taken up between 1 and 10 min was used as a measure of the rate of the energy-dependent phase of B_{12} transport. In Fig. 3 these rates, expressed as percentages of the rate obtained in the absence of inhibitor, are plotted against the inhibitor concentrations. Dinitrophenol was an equally effective inhibitor of B_{12} transport in cells from both strains, giving 50% inhibition at 0.5 to 0.6 mM. An important difference in the pattern of inhibition in the two strains was obtained with cyanide. At the concentrations used (<2 mM), this compound strongly inhibited B_{12} transport only in cells of *E. coli* AN120 with 50% inhibition at about 0.25 mM. This again shows that, when respiratory elec-

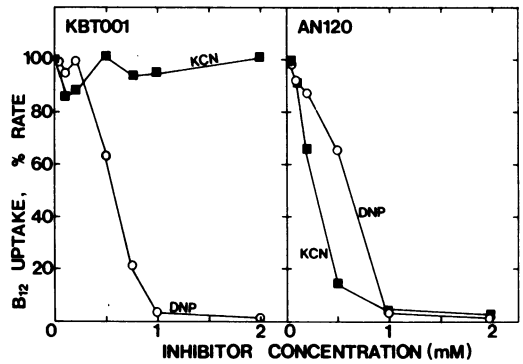

FIG. 3. *Effects of potassium cyanide and of 2,4-dinitrophenol upon the rates of B_{12} transport by cells of E. coli KBT001 and E. coli AN120. The reaction mixtures were as described for Fig. 1, with 20 mM glucose, various inhibitor concentrations, and starved cells from either E. coli KBT001 (left) or E. coli AN120 (right). At each inhibitor concentration, a time course of [^{3}H]B_{12} uptake (similar to those shown in Fig. 1 and 2) was determined. The amount of [^{3}H]B_{12} taken up between 1 and 10 min was used as a measure of the rate of the energy-dependent phase of B_{12} transport. These rates, expressed as the percentages of the rate in the absence of the inhibitors, are plotted versus the inhibitor concentrations. The inhibitors used were: 2,4-dinitrophenol (○) and potassium cyanide (■).*

tron transport is blocked, the cells need a functional Ca,MgATPase to couple nonrespiratory energy to B_{12} transport.

The effects of arsenate upon the rate of B_{12} transport are shown in Fig. 4. The reaction mixtures contained minimal HEPES (pH 6.6) in place of potassium phosphate. Aerobically, with glucose as the energy source, arsenate gave either slight (<20%) inhibition or slight stimulation of B_{12} transport in cells from *E. coli* AN120. This is consistent with the view that the generation of phosphate bonds is not obligatory for this transport process. The slight stimulation given by the higher arsenate concentrations was not always seen. In some experiments these concentrations were slightly inhibitory (<20%). The same arsenate concentrations gave rather more inhibition (<60%) in *E. coli* KBT001, which may indicate an appreciable diversion of energy from the energized membrane state to the Ca,MgATPase in an attempt to maintain normal cellular levels of ATP. In other experiments (data not shown), the maximum arsenate inhibition of aerobic, glucose-dependent B_{12} transport in this strain was not more than about 30%. This variability is probably a reflection of the residual phosphate concentrations in the cells. Arsenate would also be expected to inhibit glucose utilization in cells from both strains and give some indirect inhibition of B_{12} transport in this way. This possibility is supported by the results given in Fig. 4, showing that D-lactate-dependent B_{12} transport in strain KBT001 was less sensitive to arsenate than glucose-dependent transport.

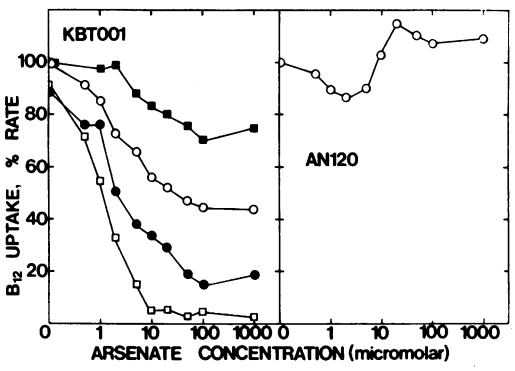

FIG. 4. *Effects of sodium arsenate upon the rates of B_{12} transport by cells of E. coli KBT001 (left) and E. coli AN120 (right). The experimental design was the same as that for Fig. 3. After starvation the cells were washed three times and suspended in minimal HEPES (pH 6.6), and the reaction mixtures also contained minimal HEPES (pH 6.6) in place of potassium phosphate. The rates of the energy-dependent phase of B_{12} transport are plotted against the arsenate concentrations in the reaction mixtures. The other reaction additions and conditions were: 40 mM glucose, gas-phase air (○); 40 mM glucose, gas-phase nitrogen (●); 20 mM lithium D-lactate, gas-phase air (■); 40 mM glucose, 2 mM potassium cyanide, gas-phase air (□).*

As shown above (Fig. 1 and 3), neither an anaerobic atmosphere nor 2 mM KCN gave appreciable inhibition of B_{12} transport in cells from *E. coli* KBT001, presumably because, in the presence of a functional Ca,MgATPase, these cells could generate an energized membrane state from the ATP produced by substrate level phosphorylations. It would be expected that, under these conditions where respiratory electron transport is blocked, the rate of B_{12} transport would be more sensitive to arsenate. Figure 4 shows that the arsenate inhibition of glucose-dependent B_{12} transport in *E. coli* KBT001 increased to about 85 and 95% in the presence of either anaerobic conditions or 2 mM KCN, respectively. This inhibition could be reversed by added phosphate.

None of the metabolic inhibitors used in Fig. 3 and 4 had any detectable effect upon receptor binding of vitamin B_{12}.

DISCUSSION

The data presented above are consistent with the view that the energy-dependent part of vitamin B_{12} transport in cells of *E. coli* requires an energized membrane without the obligatory intermediation of ATP. This conclusion rests most firmly on the results of experiments with cells of the Ca,MgATPase mutant strain, *E. coli* AN120, in which the reversible phosphorylation of ADP at the expense of the energized membrane state is prevented. This ATPase was evidently not required for B_{12} transport under conditions of active respiratory electron transport. Thus, glucose and D-lactate were about equally effective aerobically as energy sources for B_{12} transport in cells from both strain AN120 and strain KBT001. However, when electron transport was inhibited, by anaerobiosis or by cyanide, a functional Ca,MgATPase was clearly required for the glucose-dependent potentiation of B_{12} transport, since these conditions inhibited B_{12} transport only in cells of *E. coli* AN120. The primary source of energy under these conditions was presumably ATP from substrate level phosphorylations, and the Ca,MgATPase would then be essential for generation of an energized inner membrane. Consistent with this view are the observations that glucose-dependent B_{12} transport in *E. coli* KBT001 was much more sensitive to arsenate when the respiratory electron transport chain was blocked. The relative insensitivity of B_{12}

transport to arsenate in the presence of active electron transport is further evidence that phosphate bond energy is not an obligatory requirement. The potent inhibition of B_{12} transport by dinitrophenol is also consistent with the dependence of this system upon the energized membrane state. Dinitrophenol is believed to act as a proton conductor, preventing the generation of an energized membrane state of normal magnitude (11).

We indicated previously that B_{12} transport in *E. coli* was not catalyzed by a group translocation mechanism since we were unable to detect an essential chemical change in the transported molecule (8). The total intracellular B_{12} concentration often increases to about 5 μM, but most of this is apparently bound to soluble macromolecules with K_S values for binding in the micromolar range. We estimate that the free, intracellular concentration of B_{12} is frequently at least as high as 300 nM, which is about 50 times as large as the usual extracellular concentration used in the transport experiments. The uptake of vitamin B_{12} thus appears to be an active transport process dependent, as shown above, upon energy from the energized membrane state, without requiring ATP. B_{12} transport is unusual for an active transport system that depends directly upon the energized membrane state in that it is sensitive to an osmotic shock (9, 17, 18). It should be noted that B_{12} transport in cells of *E. coli* AN120 is fully as sensitive to osmotic shock as that process in *E. coli* KBT001. In both strains, an osmotic shock gives 85 to 95% reduction in the rate of the energy-dependent phase of B_{12} transport. As has been shown by others, active transport systems that are sensitive to an osmotic shock usually require ATP, whereas those that are shock resistant do not require ATP and are potentiated by an energized membrane directly (2, 3, 6, 12, 22). Shock sensitivity is usually interpreted as indicating the probable involvement of a periplasmic binding protein in the transport process. Small amounts (<10 molecules per cell) of a small-molecular-weight B_{12}-binding protein are released from cells of *E. coli* by an osmotic shock (9, 17, 18, 21), but there is at present no other evidence to support a role for this protein in B_{12} transport. All of our attempts so far to catalyze B_{12} transport in energized inner membrane vesicles, with or without added B_{12}-binding protein, have failed. Another important and unusual feature of B_{12} transport in *E. coli* is that the initial B_{12}-binding sites are known to be external to the inner membrane, and are tightly bound to the outer membrane of the cell envelope (21). Osmotic shock treatment might inhibit B_{12} transport by disrupting some essential direct association between the outer membrane receptor and the inner membrane, rather than by the removal of a periplasmic protein. It remains evident that a primary problem in understanding the transport of vitamin B_{12} in *E. coli* is the elucidation of the means of communication between the outer membrane receptors and the interior of the cell.

ACKNOWLEDGMENTS

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