Na^+-DEPENDENT AMINO ACID TRANSPORT IN ISOLATED MEMBRANE VESICLES OF A MARINE PSEUDOMONAD ENERGIZED BY ELECTRON DONORS

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SUMMARY: Transport of L-alanine and AIB into isolated membrane vesicles of marine pseudomonad B-16 (ATCC 19855) was stimulated by NADH and ascorbate-TPD. Other electron donors tested and ATP had no stimulatory effect. The uptake of both amino acids required Na^+ specifically. Neither Li^+, K^+ nor Rb^+ could replace Na^+ for transport. Using L-alanine as the test amino acid, it was found that both endogenous transport and NADH stimulated transport was inhibited by anaerobiosis, HOQNO and CN^-. Transport in the presence of ascorbate-TPD was inhibited by anaerobiosis and by CN^- but not by HOQNO. PEA induced the loss of L-alanine from membrane vesicles which had been allowed to accumulate the amino acid, suggesting that L-alanine had been concentrated inside the vesicles against a gradient. Thus, the Na^+-dependent transport process is an active one and is coupled to the oxidation of electron donors. The site of energy coupling appears to lie below the quinone in the respiratory chain.

INTRODUCTION

Most marine bacteria require Na^+ specifically for growth and metabolism (1). In the case of a marine pseudomonad (2) and *Photobacterium fischeri* (3) this requirement for Na^+ for growth has been shown to reflect a requirement for Na^+ to transport metabolites into the cells. Na^+ has also been shown to be required for the transport of glutamate into *Escherichia coli* (4) and *Bacillus licheniformis* (MacLeod, Thurman and Rogers, unpublished observations) and for the transport of thiomethyl β-D-galactopyranoside into *Salmonella typhimurium* (5).

The mechanism of Na^+-dependent transport of AIB into intact cells of marine pseudomonad B-16 has been examined in some detail. Na^+ has been shown to decrease the K_m for AIB transport into the marine pseudomonad (6), as it does for Na^+-dependent solute transport into animal cells (7), suggesting

Abbreviations: AIB, α-aminoisobutyric acid; TPD, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride; HOQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; PEA, phenethylalcohol; PMS, phenazine methosulfate.

that Na^+ increases the affinity of a binding protein or carrier for the compound being transported. Na^+ has also been shown to permit the penetration of the cytoplasmic membrane by AIB in an energy uncoupled system. These observations have led to the conclusion that Na^+ is required for the facilitated diffusion of AIB across the membrane while the accumulation against a gradient requires the expenditure of energy and the presence of intracellular K^+ (8).

In Na^+-dependent transport in animal cells the mechanism of energy coupling is generally believed to be mediated through the ATP dependent maintenance of Na^+ and K^+ gradients in the cell (7). Kaback and co-workers (9, 10) and others (11, 12) using isolated bacterial membrane vesicles have shown that the transport of amino acids, sugars and other metabolites can be coupled to the oxidation of various electron donors in a system which depends on electron transport but not oxidative phosphorylation. Since procedures have been developed for the isolation of cytoplasmic membranes of marine pseudomonad B-16 which are completely free of cell wall material (13), Na^+-dependent transport by isolated membrane vesicles of this organism has been examined.

MATERIALS and METHODS

Cells were grown in a complex medium to mid log phase by methods described previously (13). Protoplasts were broken with the aid of a French pressure cell operating at 15,000 - 16,000 psi. The membrane fragments were treated with DNA'ase (50 µg/ml), RNA'ase (50 µg/ml) and lysozyme (150 µg/ml) in a solution referred to as complete-salts Tris and comprised of NaCl, 0.2 M; KCl, 0.01 M; $MgSO_4$, 0.05 M; and Trizma base, 0.05 M adjusted to pH 8.5 with HCl. Material sedimentable within 15 minutes at 4,080 x g was discarded. The supernatant was centrifuged at 75,000 x g for 35 minutes and the membranes which sedimented were washed twice with complete-salts Tris. The final suspension was adjusted turbidimetrically to 10 mg/ml with the aid of a previously calibrated curve. The membrane preparations were judged free of intact cells and protoplasts as determined by phase contrast and electron microscopy (13), and shown by plate count to contain insufficient numbers of viable cells to account for membrane transport activity.

The procedures used in the transport studies were a modification of those described by Kaback (9). The membrane suspension was diluted to 5 mg dry wt. of membranes per ml with complete-salts Tris solution, unless otherwise indicated, and 0.1 ml of the suspension was added to each of a series of serological tubes. The electron donor, dissolved in complete-salts

Tris solution, was added followed immediately by the ^{14}C-amino acid dissolved in complete-salts Tris solution. The final volume of the reaction mixture was 0.17 ml. After incubation for specified times on a rotary shaker at 25°C, the reaction mixture was diluted with 0.4 ml of complete-salts Tris solution. The suspension was filtered using a 0.8 μ Millipore filter overlaid with a Whatman #3 prefilter, and washed with 4.0 ml of complete-salts Tris solution. The filters were dried and then counted in a Nuclear Chicago Isocap/300 liquid scintillation spectrometer using 5.0 ml scintillation fluid (5 g 2,5-diphenyloxazole/1 toluene). Transport activity is reported as cpm/mg dry wt. of membranes. All results recorded represent the average of determinations performed in duplicate.

The ascorbate solution used was prepared from an ascorbic acid solution adjusted to pH 8.5 with Trizma base. Where necessary, pH adjustments of all other solutions were made with Trizma base. ^{14}C-L-alanine (156 mCi/m mole, uniformly labeled, Amersham Searle, Corp.) was used at 1.8×10^{-6} M and ^{14}C-AIB (8.6 mCi/m mole, carboxyl-labeled, New England Nuclear) at 5.5×10^{-5} M final concentration in the reaction mixture.

To determine the level of residual Na^+ in membrane preparations prepared without Na^+, samples were digested with HNO_3 and $HClO_4$ (14) and analyzed flame photometrically.

RESULTS and DISCUSSION

Compounds shown to be active as electron donors in other bacterial systems (9, 10, 11, 12) were tested for their capacity to stimulate L-alanine and AIB transport into isolated membrane vesicles of marine pseudomonad B-16. The results, Table 1, show that two of these, NADH and ascorbate-TPD, increased the uptake of both amino acids. NADH was equally as effective as ascorbate-TPD for the uptake of AIB but somewhat less active for alanine. It is of interest that ascorbate-PMS which is active as an artificial electron donor in the other systems tested (11, 12) was inhibitory or ineffective here. As can be seen, this was due to inhibition produced by PMS. ATP was also somewhat inhibitory at the level tested.

A highly specific requirement for Na^+ for the transport of L-alanine into isolated membrane vesicles could be demonstrated both in the presence and absence of an added electron donor, Figure 1. Neither Li^+, K^+ nor Rb^+ could replace Na^+ in this system. The uptake of AIB could also be shown to respond to Na^+ but less dramatically. Since a higher concentration of AIB than alanine was used in an effort to compensate for the lower specific activity of the AIB (see Materials and Methods), the higher non-Na^+-dependent

TABLE 1

Effect of potential electron donors and ATP on the uptake of L-alanine and AIB by isolated membrane vesicles of marine pseudomonad B-16

Compound tested	L-alanine		AIB	
	Uptake*	Ratio†	Uptake	Ratio
	cpm/mg		cpm/mg	
None	5,558	1.00	1,732	1.00
NADH	9,554	1.72	5,496	3.17
NADPH	5,798	1.04	2,045	1.18
Succinate	5,956	1.07	1,301	0.75
D(-)-Lactate	3,983	0.72	1,051	0.61
Ascorbate-TPD	15,100	2.72	5,667	3.27
Ascorbate-PMS	2,593	0.47	2,321	1.34
PMS	1,408	0.25	819	0.47
Ascorbate	4,836	0.87	1,279	0.74
ATP	4,241	0.76	932	0.54
D,L-α-Glycerophosphate	3,981	0.72	1,218	0.70

Compounds tested were at 20 mM with the following exceptions: NADH, 25 mM; NADPH, 25 mM; TPD, 150 μM; PMS, 100 μM. The compound tested was added just prior to the ^{14}C-amino acid.
*Uptake by membrane vesicle preparation after 5 minutes incubation.
†Ratio of the uptake in the presence, to uptake in the absence, of an added electron donor.

uptake of AIB observed is probably due to non-specific binding of AIB to the membranes. The level of residual Na^+ in the incubation mixture was 1.1 mM.

The stimulation of L-alanine transport by ascorbate-TPD required the presence of O_2, Figure 2. Replacing air with N_2 markedly depressed transport both in the presence and absence of the artificial electron donor. Thus, as in other bacterial membrane vesicle systems so far examined (9, 10, 11, 12), amino acid transport would appear to be energized by a flow of electrons from the electron donor to O_2 as the terminal acceptor.

Kaback and co-workers have presented evidence indicating that in *E. coli* the site of energy coupling for lactose and amino acid transport is localized between D-lactic dehydrogenase and cytochrome b_1, in the respiratory chain of *E. coli* (9, 15). In the *E. coli* system, the quinone inhibitor, HOQNO, inhibited lactose uptake (9). In the case of marine pseudomonad B-16, HOQNO inhibited endogenous L-alanine transport and transport in the presence of NADH, but it had no effect on transport in the presence of ascorbate-TPD,

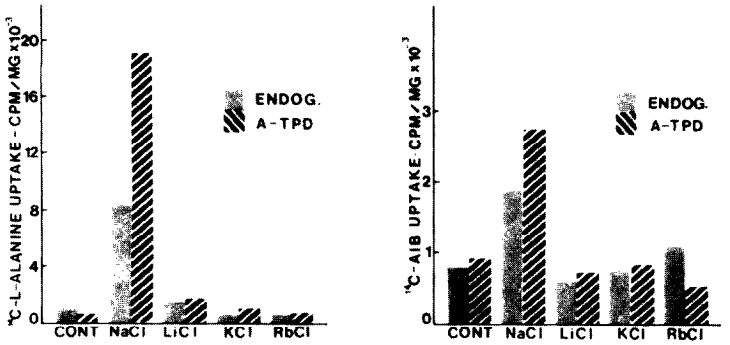

FIG. 1. Requirement for Na^+ for the uptake of L-alanine and AIB by isolated membrane vesicles of marine pseudomonad B-16. Left figure, L-alanine; right figure, AIB. Shaded bars, no added electron donor (ENDOG); hatched bars, ascorbate-TPD (A-TPD); control (CONT), no added salt. Membranes were prepared by substituting LiCl for NaCl in the complete-salts Tris solution during the final stages of isolation. The membranes were then collected by centrifugation and resuspended to 10 mg/ml in Mg-K-Tris solution ($MgSO_4$, 0.05 M; KCl, 0.01 M; Tris-HCl, 0.05 M; pH 8.5). Aliquots of this suspension were diluted to 5 mg/ml with Mg-K-Tris solution containing either no added salt (CONT) or sufficient NaCl, LiCl, KCl or RbCl to give 0.2 M in the final incubation mixture. For the transport studies the procedures described in Materials and Methods were used with the exceptions of adding the electron donor and the ^{14}C-alanine in Mg-K-Tris solution and of washing the membranes on the filter with complete-salts Tris solution containing LiCl in place of NaCl. Ascorbate, TPD and ^{14}C-alanine concentrations as in Table 1. Incubation time, 5 min.

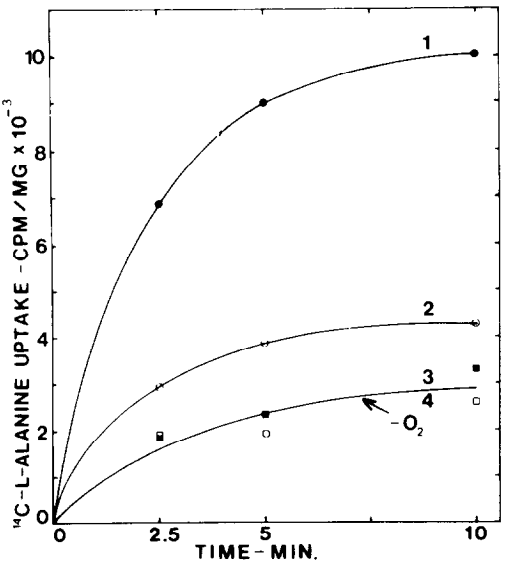

FIG. 2. Effect of aerobic and anaerobic conditions on L-alanine uptake by isolated membrane vesicles of marine pseudomonad B-16 in the presence and absence of ascorbate-TPD. The membrane suspensions contained in serological tubes were either left open to the atmosphere or sealed with serum caps and flushed with N_2 for 5 min. In the case of the sealed tubes, subsequent additions to the membrane suspension were made by injection through the cap. Curve 1, air, ascorbate-TPD; Curve 2, air, no added electron donor; Curves 3, 4, N_2, with or without ascorbate-TPD.

Table 2. Transport in the presence of both electron donors, as well as endogenous transport, was inhibited markedly by cyanide. These findings would suggest that in the case of marine pseudomonad B-16, the site of energy coupling for transport of L-alanine lies between the quinone and O_2 in the respiratory chain. Further support for this conclusion was obtained by irradiating the membranes with 360 nm light. This procedure is known to destroy the quinone component of the electron transport chain (16) and inhibits transport in membrane vesicles of *Mycobacterium phlei* (11) and *B. licheniformis* 6346 (MacLeod, Thurman and Rogers, unpublished observations). Irradiation inhibited endogenous transport by membrane vesicles of the marine pseudomonad and transport in the presence of NADH, but not transport coupled to ascorbate-TPD (data not shown).

It can be seen in Table 2 that there is a relatively high endogenous uptake of L-alanine into membrane vesicles of the marine pseudomonad. The fact that this uptake could be substantially reduced by inhibition of electron transport or anaerobiosis, Figure 2, suggests that there is an endogenous electron donor in the membranes capable of energizing transport. We have not yet identified this indigenous electron donor, but since HOQNO is

TABLE 2

Effect of inhibitors added in the presence and absence of electron donors on the capacity of isolated membrane vesicles of marine pseudomonad B-16 to take up L-alanine

Electron donor added	Inhibitor added	Uptake*
		cpm/mg
None	None	10,677
NADH	None	12,807
Ascorbate-TPD	None	46,607
None	HOQNO	3,009
NADH	HOQNO	3,363
Ascorbate-TPD	HOQNO	43,622
None	CN^-	2,034
NADH	CN^-	2,435
Ascorbate-TPD	CN^-	2,214

HOQNO and KCN were present at 2.0×10^{-5} M and 1.0×10^{-3} M, respectively. The inhibitors were added 15 minutes prior to the addition of exogenous donor and ^{14}C-L-alanine. Concentrations of added electron donor as in Table 1.
*as in Table 1.

able to inhibit endogenous transport activity, it would appear that the natural electron donor is introducing electrons into the respiratory chain at a level preceding the quinone.

Evidence that the uptake of L-alanine by the membrane vesicles is an active transport process was obtained using PEA. PEA is known to increase the leakage of solutes through the cytoplasmic membrane of this organism in a completely reversible manner (17). Membrane vesicles were allowed to accumulate ^{14}C-L-alanine to the maximum level. PEA was then added to the reaction mixture and the level of radioactivity in the membrane vesicles followed with time. In the presence of PEA, but not in its absence, radioactivity was quickly lost from the membrane vesicles, Figure 3. When the membranes exposed to PEA were resuspended in the absence of PEA they were found to be capable of taking up L-alanine again to levels prevailing before PEA treatment (data not shown). Ninety-eight per cent of the radioactivity in membrane vesicles preloaded with ^{14}C-L-alanine could be recovered by hot water extraction. Ninety per cent of this recovered activity remained as L-alanine. It is thus evident that L-alanine was being transported into the membrane vesicles against a gradient.

These results show that Na^+-dependent active transport of L-alanine and AIB into membrane vesicles of marine pseudomonad B-16 can be coupled to the

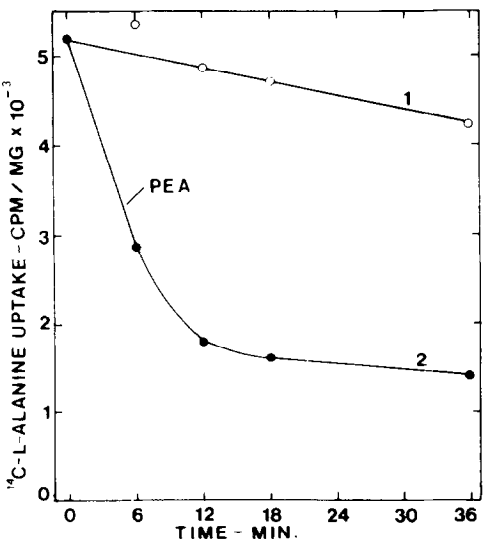

FIG. 3. PEA induced loss of ^{14}C-L-alanine from isolated membrane vesicles. Vesicles were allowed to accumulate L-alanine for 10 min in the absence of exogenous electron donors. At zero time either complete-salts Tris solution, curve 1, or PEA in complete-salts Tris solution, curve 2, was added. The final PEA concentration was 0.25%.

oxidation of electron donors. In consideration of the effects on transport of the donors and inhibitors used, and the fact that ATP failed to stimulate transport, oxidative phosphorylation and ATP are probably not directly involved in this process as was previously supposed (8). These findings provide further evidence that the mechanisms of Na^+-dependent transport in procaryotic and eucaryotic cells may be different.

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