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## Amino Acid Transport by Membrane Vesicles of an Obligate Anaerobic Bacterium, *Clostridium acetobutylicum*

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Membrane vesicles were isolated from the obligate anaerobic bacterium *Clostridium acetobutylicum*. Beef heart mitochondrial cytochrome *c* oxidase was inserted in these membrane vesicles by membrane fusion by using the freeze-thaw sonication technique (A. J. M. Driessen, W. de Vrij, and W. N. Konings, Proc. Natl. Acad. Sci. USA 82:7555-7559, 1985) to accommodate them with a functional proton motive force-generating system. With ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine-cytochrome *c* as the electron donor, a proton motive force ( $\Delta p$ ) of -80 to -120 mV was generated in these fused membranes. This  $\Delta p$  drove the accumulation of leucine and lysine up to 40- and 100-fold, respectively. High transport activities were observed in fused membranes containing *Escherichia coli* lipids, whereas the transport activities in fused membranes containing mainly soybean lipids or phosphatidylcholine were low. It is suggested that branched-chain amino acids and lysine were taken up by separate systems. The effects of the ionophores nigericin and valinomycin indicated that lysine and leucine were translocated in symport with a proton.

Studies on the energetics of solute transport in aerobic heterotrophs and facultative anaerobes have been markedly stimulated since Kaback succeeded in isolating functional membrane vesicles (for a review, see reference 15). Similar investigations in anaerobic heterotrophs have been hampered by the difficulty of obtaining membrane vesicles that contain an accessible and functional proton pump. Since a cytochrome system is lacking, a proton motive force ( $\Delta p$ ) cannot be generated by respiration. Generation of a  $\Delta p$  in these organisms occurs mainly by  $H^+$  extrusion by the  $H^+$ -translocating ATPase upon hydrolysis of ATP obtained by substrate-level phosphorylation. The group of anaerobic heterotrophs includes lactic acid bacteria (*Streptococcus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Lactobacillus* spp.), several obligate anaerobic gram-positive cocci (*Sarcina* spp., *Peptococcus* spp., *Ruminococcus* spp., etc.), propionic acid bacteria, the spore-forming *Clostridium* spp., and many others (22). Whereas most lactic acid bacteria are oxygen tolerant, *Clostridium* spp. are usually rapidly killed by  $O_2$  unless they are in the spore form. This fact severely complicates bioenergetic studies. The industrial importance of these bacteria in the fermentation of sugars into the end products butanol, acetone, and ethanol (24), for instance, has provided the necessary impetus for research on sugar transport. Most sugars appear to be translocated in *Clostridium* spp. by phosphoenolpyruvate-dependent phosphotransferase systems (3, 4).

Sugars are not the only compounds that can be fermented by *Clostridium* spp. Certain *Clostridium* spp. can obtain energy from the fermentation of pyrimidine bases or amino acids, either singly or in pairs, via Stickland reactions (2, 18). In these processes, amino acids are translocated across the cytoplasmic membrane. For this and other reasons, it would be of particular interest to obtain information about the mechanisms and energetics in *Clostridium* spp. Such information has not yet been obtained. However, studies of these transport processes require an adequately defined model system in which the cytoplasmic enzymes are absent.

Recently, we developed a procedure for the incorporation

of proton pumps in bacterial membrane vesicles (8, 9). Membrane vesicles of the homofermentative lactic acid bacterium *Streptococcus cremoris* were fused with proteoliposomes containing beef heart mitochondrial cytochrome *c* oxidase, and the properties of a large number of amino acid transport systems were studied in detail with this system (7-13; A. J. M. Driessen, T. Zhen, G. In 't Veld, J. Op den Kamp, and W. N. Konings, Biochemistry, in press). In this article, we report on the insertion of cytochrome *c* oxidase into membrane vesicles of the obligate anaerobe *Clostridium acetobutylicum*. In these fused membranes, amino acid transport can be energized by the oxidation of reduced cytochrome *c*. Properties of leucine and lysine transport are discussed.

### MATERIALS AND METHODS

*C. acetobutylicum* NCIB 8052 (ATCC 824) was kindly provided by J. C. Gottschal (Department of Microbiology, University of Groningen). Cells were grown anaerobically on glucose (2% [wt/vol]) in complex medium (5) at 35°C in a stoppered bottle (1 liter of culture volume). Growth medium was freed of oxygen by a flow of  $N_2$ , which was passed over hot copper fillings. Cells were harvested during exponential growth (optical density at 660 nm, 0.9 to 1.1), when the medium pH had dropped from 6.5 to 5.5. Membrane vesicles were prepared by osmotic lysis essentially as described for *S. cremoris* (20) and stored in liquid nitrogen in 50 mM potassium phosphate (pH 7.0) supplemented with 5 mM  $MgSO_4$  to a protein concentration of 10 to 15 mg/ml. The membrane preparation was virtually devoid of intact cells as indicated by thin-section electron microscopy (data not shown).

Beef heart mitochondrial cytochrome *c* oxidase (23) was reconstituted into liposomes containing a mixture of acetone-ether-washed *Escherichia coli* phospholipid and egg yolk phosphatidylcholine (PC) (9:1 [wt/wt]) by dialysis, as described elsewhere (12). In a number of experiments, liposomes with different phospholipid compositions were used as indicated. *C. acetobutylicum* membrane vesicles (100  $\mu$ l and 1 mg of protein) and cytochrome *c* oxidase proteoliposomes (500  $\mu$ l, 10 mg of phospholipid, and 2.3

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nmol of haem a) were mixed and fused by freeze-thaw sonication (8, 9, 12).

Lipids were extracted from cells grown on sodium acetate-free complex medium containing 2.2  $\mu\text{M}$   $[\text{U-}^{14}\text{C}]$ acetic acid (4.7 TBq/mol). Lipids were separated by two-dimensional thin-layer chromatography on precoated silica gel plates (Kieselgel 60; Merck & Co., Inc.) by using the following solvent systems: (i) chloroform-methanol-ammonia-water (90:54:5.5:5 by volume) and (ii) chloroform-methanol-acetic acid-water (90:40:12:2 by volume). Soybean phospholipid was chromatographed in parallel as a reference compound. Chromatograms were stained with  $\text{I}_2$  vapor for lipids,  $\alpha$ -naphthol- $\text{H}_2\text{SO}_4$  for carbohydrates, and ninhydrin reagent to detect amino groups as described elsewhere (Driessen et al., in press). Phospholipid phosphorus was determined by the method of Ames and Dubin (1). The lipid composition of *C. acetobutylicum* membranes was quantitated by the amount of radioactivity incorporated in the acyl chains.

Amino acid transport driven by an imposed  $\Delta p$  or by cytochrome *c* oxidase activity was assayed as described previously (8–10, 12). Experiments were performed at 25°C. The  $\Delta\psi$  (interior negative) was determined from the distribution across the membrane of the lipophilic cation tetraphenylphosphonium ( $\text{Ph}_4\text{P}^+$ ) by using a  $\text{Ph}_4\text{P}^+$ -selective electrode (21) as described elsewhere (6, 9, 12). The  $\Delta\psi$  was calculated from the steady-state level of  $\text{Ph}_4\text{P}^+$  uptake and corrected for concentration-dependent binding of  $\text{Ph}_4\text{P}^+$  to the membrane (16, 23). Protein was determined by the method of Lowry et al. (17). The internal water space of fused membranes was estimated from the trapped amount of calcein (9, 19), yielding a value of 8.7  $\mu\text{l}/\text{mg}$  of protein.

An artificial  $\Delta p$  in membrane vesicles of *C. acetobutylicum* was generated by the simultaneous imposition of an outwardly directed valinomycin-mediated potassium and acetate diffusion gradient. Membrane vesicles were incubated in a solution containing 20 mM potassium phosphate (pH 6.0) and 100 mM potassium acetate and subsequently diluted in 20 mM sodium phosphate (pH 6.0) supplemented with 100 mM sodium piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES). By this procedure, both a  $\Delta\psi$ , inside negative, and a  $\Delta p\text{H}$ , inside alkaline, are generated.

## RESULTS AND DISCUSSION

The uptake of leucine by membrane vesicles driven by an artificially imposed  $\Delta p$  was studied. Under these conditions, rapid but transient uptake of L-leucine was observed (Fig. 1, inset). Only a low level of uptake was observed in control experiments in which no  $\Delta p$  was generated. In this control experiment, the membrane vesicles were diluted in 20 mM potassium phosphate (pH 6.0) supplemented with 100 mM potassium acetate (Fig. 1, inset). In membrane vesicles of *C. acetobutylicum* fused with proteoliposomes containing beef heart mitochondrial cytochrome *c* oxidase by the freeze-thaw sonication technique (8, 9, 12), leucine was rapidly accumulated in the presence of ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)-cytochrome *c* (Fig. 1). In contrast to imposed  $\Delta p$ -driven leucine transport, leucine uptake by the fused membrane continued for about 8 min and reached a steady state. Under these conditions, a constant  $\Delta\psi$  of about  $-100$  to  $-120$  mV was generated (data not shown). Very little uptake of leucine was observed when ascorbate and TMPD were omitted (Fig. 1). Cyanide strongly inhibited uptake, demonstrating the dependence on the activity of cytochrome *c* oxidase (Fig. 1).

To determine the optimal conditions for leucine uptake by fused membranes, the effect of the lipid composition of the

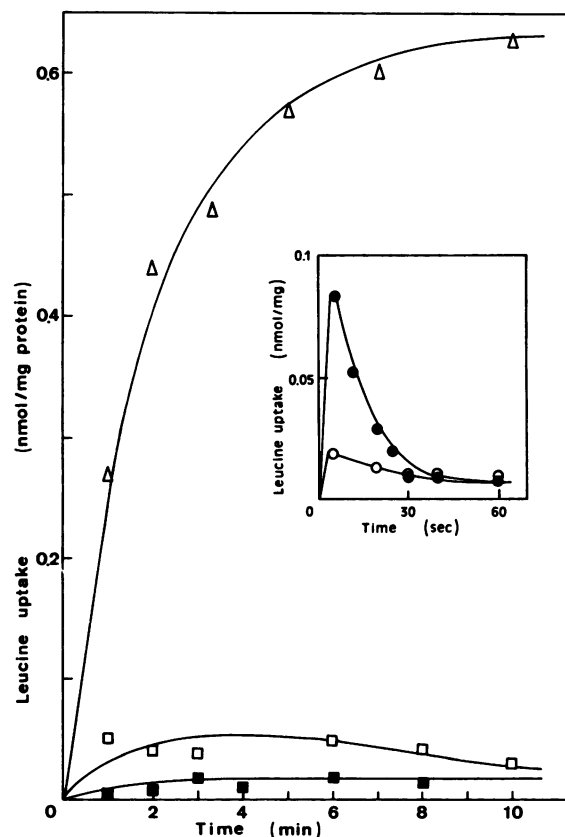


FIG. 1. Time course of leucine transport by *C. acetobutylicum* membrane vesicles fused with cytochrome *c* oxidase proteoliposomes containing *E. coli* phospholipid-egg yolk PC (3:1 [wt/wt]). Fused membranes (0.5 mg of protein per ml) were incubated in a medium containing 50 mM potassium phosphate (pH 6.0), 200  $\mu\text{M}$  TMPD, and 20  $\mu\text{M}$  cytochrome *c*. At 30 s after the addition of 10 mM potassium ascorbate (pH 6.0), 3  $\mu\text{M}$  L- $[\text{U-}^{14}\text{C}]$ leucine (12.4 TBq/mol) was added, and uptake in the presence ( $\Delta$ ) or absence ( $\blacksquare$ ) of ascorbate and TMPD and in the presence of ascorbate and 0.2 mM KCN ( $\square$ ) was assayed by filtration. Inset, Time course of leucine transport by *C. acetobutylicum* membrane vesicles upon imposition of a  $\Delta p$ . Membrane vesicles (20 mg of *C. acetobutylicum* membrane protein per ml) were incubated in 20 mM potassium phosphate (pH 6.0) supplemented with 100 mM potassium acetate and 2 nmol of valinomycin per mg of protein. Potassium-loaded membranes were diluted 50-fold into a solution containing 20 mM sodium phosphate (pH 6.0) supplemented with 100 mM sodium PIPES and 3  $\mu\text{M}$  L- $[\text{U-}^{14}\text{C}]$ leucine ( $\bullet$ ) ( $\Delta p$ , inside negative and alkaline) or into 20 mM potassium phosphate (pH 6.0) supplemented with 100 mM potassium acetate and 3  $\mu\text{M}$  L- $[\text{U-}^{14}\text{C}]$ leucine ( $\circ$ ) (no gradient).

proteoliposomes was investigated. Leucine uptake and  $\Delta\psi$  were simultaneously determined. When cytochrome *c* oxidase proteoliposomes containing only soybean phospholipid or only egg yolk PC were used, a low level of leucine transport activity was observed (Fig. 2), despite the presence of a  $\Delta\psi$  of up to  $-100$  mV (data not shown). With cytochrome *c* oxidase proteoliposomes containing *E. coli* phospholipid or mixtures of *E. coli* phospholipid with PC, considerably higher leucine uptake activities were observed (Fig. 2). The absolute value of  $\Delta\psi$  was higher when a small amount of PC was added to *E. coli* phospholipid, e.g., approximately  $-85$  mV for membranes fused with proteoliposomes containing *E. coli* phospholipid, compared with  $-105$  to  $-120$  mV for *E. coli* phospholipid-egg yolk PC (3:1

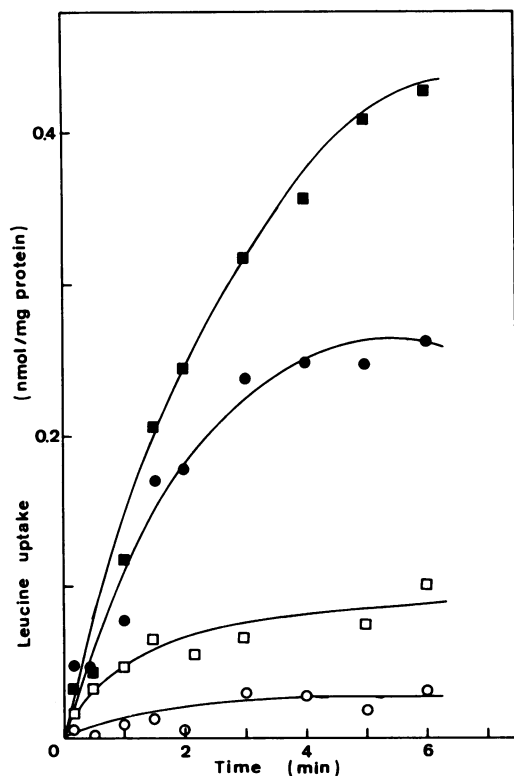


FIG. 2. Leucine uptake by *C. acetobutylicum* membrane vesicles fused with cytochrome *c* oxidase proteoliposomes with different lipid composition. *E. coli* phospholipid (●); *E. coli* phospholipid-egg yolk PC (3:1 [wt/wt]) (■), egg yolk PC (○), and soybean phospholipid (□). Experimental procedures are described in the legend to Fig. 1.

[wt/wt]). It should be stressed that fusion induced by freeze-thaw treatment does not demonstrate any preference for the lipid composition of the liposomes. An identical extent of the fusion efficiency has been observed for fusion between *S. cremoris* membrane vesicles and liposomes of various lipid composition (Driessen et al., in press), including those depicted in Fig. 2. *E. coli* phospholipid contains approximately 65% phosphatidylethanolamine, 30 to 35% phosphatidylglycerol, and 0 to 5% cardiolipin (Driessen et al., in press). The phospholipid composition of *C. acetobutylicum* membranes was estimated from the amount of label incorporated in the acyl chains during growth in the presence of [<sup>14</sup>C]acetate. The phospholipid composition was found to be very similar to that of *E. coli* phospholipid, i.e., approximately 60 to 65% phosphatidylethanolamine and mono-*N*-methylated phosphatidylethanolamine, 20% phosphatidylglycerol, and 15% cardiolipin (see also reference 14). All other experiments were performed with membrane vesicles of *C. acetobutylicum* fused with cytochrome *c* oxidase proteoliposomes composed of a mixture of *E. coli* phospholipid and egg yolk PC (3:1 [wt/wt]).

The following amino acids were accumulated severalfold by the fused membranes: glycine, L-lysine, L-leucine, L-isoleucine, L-valine, and L-glutamine (data not shown). No uptake or only a low level of uptake was observed with the following amino acids: L-proline, L-serine, and L-glutamate (data not shown).

The kinetics of L-leucine, L-isoleucine, and L-valine uptake were investigated. Initial rates of uptake were determined from the amount of label accumulated within 1 min.

TABLE 1. Kinetic constants of transport for branched-chain amino acids and lysine by membrane vesicles of *C. acetobutylicum* fused with cytochrome *c* oxidase proteoliposomes<sup>a</sup>

Transported substrate	$K_t$ ( $\mu\text{M}$ )	$V_{\text{max}}$ (nmol/min per mg of protein)
L-Leucine	11.9	0.34
L-Isoleucine	8.4	0.41
L-Valine	8.3	0.52
L-Lysine	2.9	0.68

<sup>a</sup> The initial rates of <sup>14</sup>C-labeled amino acid uptake were determined from the amount of label accumulated within 1 min. Branched-chain amino acids and lysine were used at concentrations of 0.75 to 20  $\mu\text{M}$  and 0.35 to 10  $\mu\text{M}$ , respectively.

Analysis of the data by Eadie-Hofstee plots revealed monophasic kinetics with an affinity constant ( $K_t$ ) for uptake of about 8 to 12  $\mu\text{M}$  (Table 1). The maximal rate of uptake ( $V_{\text{max}}$ ) varied between 0.3 and 0.6 nmol/min per mg of protein. Uptake of L-leucine was completely inhibited by a 70- to 80-fold excess of unlabeled L-leucine, L-valine, and L-isoleucine (data not shown). Similar results were obtained for L-isoleucine and L-valine uptake, indicating that branched-chain amino acids are transported by a common transport system. Monophasic kinetics were also observed for L-lysine uptake, yielding a  $K_t$  and  $V_{\text{max}}$  of 2.9  $\mu\text{M}$  and 0.7 nmol/min per mg of protein, respectively (Table 1). No mutual inhibition was observed between L-lysine and branched-chain amino acids, suggesting that separate systems are operational for these amino acids.

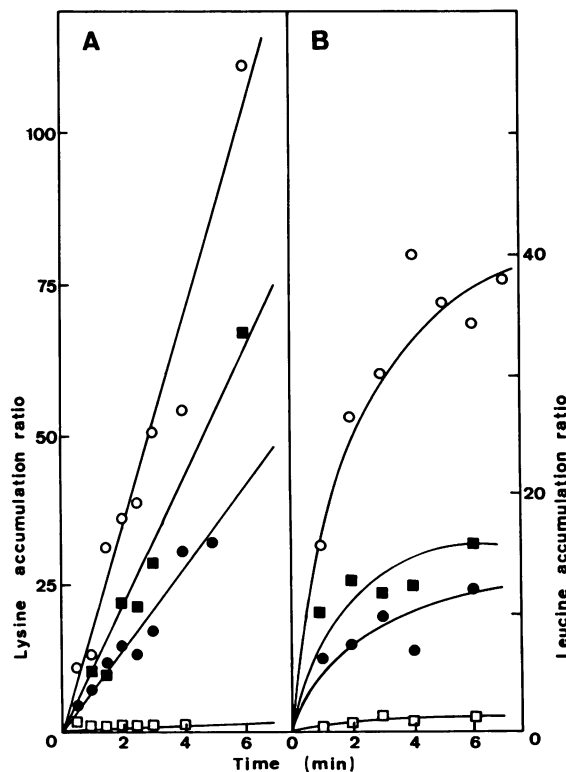


FIG. 3. Effect of ionophores on lysine (A) and leucine (B) uptake. Uptake in the absence of ionophores (○) or ascorbate (□) or in the presence of 10 nM nigericin (■) or 100 nM valinomycin (●). Experimental details are described in the legend to Fig. 1.

The effect of the ionophores valinomycin and nigericin on uptake was investigated. Addition of the ionophore valinomycin, which catalyzes electrogenic influx of  $K^+$ , results in a decrease of  $\Delta\psi$  with a noncompensating increase in  $\Delta pH$  (9, 12), whereas nigericin catalyzes an electroneutral exchange between  $H^+$  and  $K^+$ , thereby decreasing  $\Delta pH$  with a compensating increase in  $\Delta\psi$  (9, 12). Both ionophores caused inhibition of leucine and lysine transport (Fig. 3), indicating that the dipolar ionic amino acid leucine and the cationic amino acid lysine are most likely transported in symport with protons.

These results demonstrate that solute transport in obligate anaerobes such as *C. acetobutylicum* can be studied in membrane vesicles which have been fused with proteoliposomes containing a functional proton motive force-generating system. This system allows a much clearer definition of observed phenomena in solute transport than studies with intact cells do and may provide a valuable tool in studies on the energetics of amino acid uptake in these anaerobic heterotrophic bacteria.

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