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arcD, the First Gene of the *arc* Operon for Anaerobic Arginine Catabolism in *Pseudomonas aeruginosa*, Encodes an Arginine-Ornithine Exchanger

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In the absence of oxygen and nitrate, *Pseudomonas aeruginosa* metabolizes arginine via the arginine deiminase pathway, which allows slow growth on rich media. The conversion of arginine to ornithine, CO₂, and NH₃ is coupled to the production of ATP from ADP. The enzymes of the arginine deiminase pathway are organized in the *arcDABC* operon. The *arcD* gene encodes a hydrophobic polytopic membrane protein. Translocation of arginine and ornithine in membrane vesicles derived from an *Escherichia coli* strain harboring a recombinant plasmid carrying the *arcD* gene was studied. Arginine and ornithine uptake was coupled to the proton motive force with a bias toward the transmembrane electrical potential. Accumulated ornithine was readily exchangeable for external arginine or lysine. The exchange was several orders of magnitude faster than proton motive force-driven transport. The ArcD protein was reconstituted in proteoliposomes after detergent solubilization of membrane vesicles. These proteoliposomes mediate a stoichiometric exchange between arginine and ornithine. It is concluded that the ArcD protein is a transport system that catalyzes an electroneutral exchange between arginine and ornithine to allow high-efficiency energy conversion in the arginine deiminase pathway.

To survive anaerobiosis in the absence of nitrate as a terminal electron acceptor, *Pseudomonas aeruginosa* uses arginine as an energy source for viability, motility, and slow growth on rich media (31, 34). Arginine is metabolized in the arginine deiminase pathway (for a review, see reference 6). This pathway includes three cytoplasmic enzymes: (i) arginine deiminase, which catalyzes the conversion of arginine into citrulline and ammonia (in an essentially irreversible reaction); (ii) catabolic ornithine carbamoyl transferase, which catalyzes the reaction of citrullin with P_i to yield ornithine and carbamoyl phosphate; and (iii) carbamate kinase, which converts carbamoyl phosphate and ADP to ATP, CO₂, and ammonia. These enzymes are encoded by the *arcA*, *arcB*, and *arcC* genes, respectively (34). The *arcABC* genes have been cloned and sequenced (2, 3) and are organized in an operon (24, 30). The *arc* operon is under control of a positive regulatory protein, ANR, an FNR-like protein of *P. aeruginosa* (16, 18, 37), which is thought to activate transcription of the *arc* genes during oxygen limitation (26). The proximal part of the *arc* operon harbors the *arcD* gene (23). This *arcD* gene encodes a highly hydrophobic protein with a predicted molecular mass of 52 kDa. Hydropathy profile analysis (23) and topological studies (4) indicate that the ArcD protein is a cytoplasmic membrane protein that may traverse the membrane 13 times. Growth and complementation studies suggest that ArcD functions as a transport protein (26, 34). During anaerobic growth, arginine is stoichiometrically converted into ornithine (34), which is released into the growth medium. By analogy with the transport system of lactic acid bacteria (12), ArcD may specify an arginine-ornithine antiporter. The system of *Lac-*

tococcus lactis catalyzes an electroneutral one-to-one exchange between arginine and ornithine and requires no additional metabolic energy (12, 29).

In this paper we present an in vitro analysis of the activity of the *arcD* gene product of *P. aeruginosa* cloned in *Escherichia coli*. The presence of multiple transport systems for basic amino acids in *Pseudomonas* spp. (15) makes this organism less suitable as a host. The expression of the *arcD* gene product in an arginine-permease deficient *E. coli* strain (5) permits studies of its functions separated from regulatory or metabolism-related events. The ArcD protein mediates proton motive force (Δp)-driven uptake of arginine and ornithine and the stoichiometric exchange between arginine and ornithine. The ArcD protein was extracted from the cytoplasmic membrane with detergent and reconstituted into liposomes in a functional state.

MATERIALS AND METHODS

Materials. *E. coli* phospholipids and egg phosphatidylcholine were obtained from Sigma Chemical Co. (St. Louis, Mo.). *E. coli* phospholipids were washed with acetone-ether by a modification of the method of Kagawa et al. (21). Phospholipids were dissolved in chloroform-methanol (9:1, vol/vol) and stored under N₂ at -20°C. L-[U-¹⁴C]lysine (11 TBq/mol), L-[U-¹⁴C]arginine (11 TBq/mol), and L-[2,3-³H]ornithine (14.8 TBq/mmol) were purchased from New England Nuclear (Dreieich, Germany). L-[U-¹⁴C]ornithine (9.25 TBq/mol) was obtained from Amersham (Buckinghamshire, United Kingdom).

Bacterial strains, plasmids, growth conditions, and isolation of membrane vesicles. *E. coli* JC182-5 (5), which is deficient in arginine transport, was used as a host for the expression of the ArcD protein. The plasmids used in this study were pME3719 and pBluescript II KS(+) (Stratagene, La Jolla,

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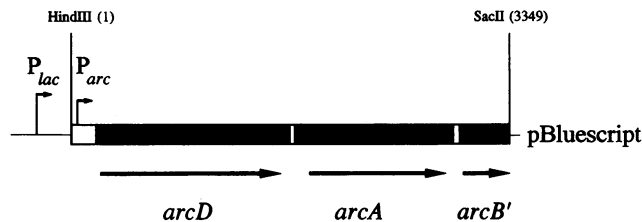


FIG. 1. *arcDA*⁺ recombinant plasmid pME3719. P_{lac} , promoter of the *E. coli lac* operon; P_{arc} , promoter of the *P. aeruginosa arc* operon. The numbers within parentheses denote the coordinates of the *arc* operon in base pairs (18).

Calif.) (32). Plasmid pME3719 is based on pBluescript KS(+) and contains the *P. aeruginosa* genes *arcD* and *arcA* and a part of the *arcB* gene located on a 3.3-kb DNA fragment (Fig. 1). Transcription of these genes can be induced by isopropyl- β -D-thiogalactopyranoside, and their expression can be monitored by measuring the arginine deiminase activity in toluene-treated cells (25). All strains were grown at pH 7.0 on minimal medium (7) supplemented with 1% (wt/vol) sodium succinate and 0.1% (wt/vol) yeast extract at 37°C. Maintenance of the recombinant plasmids was assured by ampicillin or carbenicillin at 100 $\mu\text{g} \cdot \text{ml}^{-1}$. Transcription of the *arcDA* genes was initiated during logarithmic growth (optical density at 660 nm of about 0.3 to 0.4) by the addition of 100 μM isopropyl- β -D-thiogalactopyranoside. Cells were harvested 1.5 to 2 h after induction.

Membrane vesicles were isolated by osmotic lysis by the method of Kaback (20). All buffers included 1 mM dithiothreitol as a stabilizer. Membrane vesicles were stored in liquid nitrogen until use.

Solubilization and reconstitution. Solubilization of membrane vesicles and the reconstitution of extracted proteins into proteoliposomes were performed as described by In 't Veld et al. (19). Membrane vesicles (1.5 mg of protein) were solubilized with 1.25% (wt/vol) *n*-octyl- β -glucopyranoside (27) in the presence of 45 mg of phospholipids (a mixture of *E. coli* phosphatidylethanolamine and egg phosphatidylcholine [3:1, wt/wt] in 4% [wt/vol] octylglucoside) and 20% (vol/vol) glycerol (1) in a final volume of 1.5 ml of 50 mM potassium phosphate (pH 7.0). The suspension was incubated for 30 min on ice and centrifuged for 1 h at 43,000 rpm (225,000 $\times g$) in a Beckman type SW 50.1 rotor at 5°C. The cleared supernatant (1.5 ml) was supplemented with 15 mg of phospholipids suspended in 4% (wt/vol) octylglucoside, and the octylglucoside concentration was adjusted to 1.5% (wt/vol) with 50 mM potassium phosphate (pH 7.0). The transparent solution of mixed micelles was kept on ice for 10 min and then injected into 50 mM potassium phosphate (pH 7.0), such that a 35-fold dilution was obtained. For the assay of arginine-ornithine exchange activity, the dilution buffer contained 500 μM ornithine. Proteoliposomes were collected by centrifugation for 2.5 h at 27,000 rpm (90,000 $\times g$) in a Beckman type 35 Ti rotor and stored in liquid nitrogen until use. Before the transport assays, proteoliposomes were thawed at room temperature and dispersed by ultrasonication with a probe sonicator (MSE Scientific Instruments, West Sussex, United Kingdom) for 8 s at an intensity of 4 μm (peak to peak) at 4°C.

Assay of transport. Δp -driven uptake of amino acids was assayed as follows. Membrane vesicles (0.12 to 0.24 mg of protein $\cdot \text{ml}^{-1}$) were suspended in 1.5 ml of 50 mM potassium phosphate, of the pH indicated, containing 5 mM

MgSO_4 . Membrane vesicles were energized by the addition of 20 mM glucose and the cofactor pyrroloquinoline-quinone (PQQ) at 2.2 μM (35). ^{14}C -labeled amino acids were added 60 s after the addition of glucose. At the indicated time intervals, samples of 100 μl were taken, diluted into 2 ml of ice-cold 0.1 M LiCl, and immediately filtered on 0.45- μm -pore-size cellulose-nitrate filters (Schleicher & Schuell).

For the assay of arginine-ornithine exchange, proteoliposomes were loaded with 500 μM ornithine as described previously (12). Samples were concentrated by centrifugation, and aliquots of 2 μl (approximately 2.5 to 3 mg of protein $\cdot \text{ml}^{-1}$) were diluted into 100 μl of 50 mM potassium phosphate (pH 7.0) containing L-[U- ^{14}C]arginine at the indicated concentrations. For the determination of the arginine-ornithine stoichiometry, proteoliposomes were loaded with L-[2,3- ^3H]ornithine by equilibrating ornithine-loaded proteoliposomes with a small aliquot of radiolabeled ornithine for 1 h at 23°C. Proteoliposomes were diluted 50-fold in an ornithine-free buffer containing 20 μM [^{14}C]arginine. Uptake was assayed as described above. For the analysis of the kinetic parameters of uptake, arginine and ornithine concentrations were varied between 1.5 and 152 μM and between 11.8 and 202 μM , respectively.

Other analytical procedures. The amount of protein in membrane vesicles was determined by the method of Lowry et al. (22). A Pierce micro BCA protein assay (33) was used for the determination of protein in the presence of lipids. Bovine serum albumin was used as the standard.

RESULTS

Δp -driven uptake of arginine and ornithine in *E. coli* membrane vesicles. A 3.3-kb *HindIII*-*SacI* fragment was taken from pME183, which contains the entire *arcDABC* operon (23, 24). The fragment contains *arcD*, *arcA*, and part of *arcB* (Fig. 1) and was inserted into a pBluescript KS(+) expression vector to yield the *arcDA*⁺ recombinant plasmid pME3719. *E. coli* JC182-5 (5) was used as a host because this strain is deficient in arginine transport. Membrane vesicles were prepared from *E. coli* JC182-5 harboring pME3719 (ArcD vesicles) or pBluescript KS(+) (control vesicles). Membrane vesicles were tested for their ability to accumulate lysine, arginine, and ornithine. In the absence of an energy source, none of these amino acids (except for arginine) was accumulated beyond the equilibrium level (Fig. 2). Uptake of arginine and ornithine was observed when ArcD membrane vesicles were energized by the electron donors glucose and PQQ in the presence of oxygen (Fig. 2). Hardly any uptake of these amino acids was detectable with control membrane vesicles. Lysine uptake did not require the ArcD protein, since it was accumulated by both membrane vesicle preparations (Fig. 2). These results demonstrate that the ArcD protein translocates arginine and ornithine across the cytoplasmic membrane.

Because both ArcD and ArcA proteins were expressed, we anticipated the possibility that arginine deiminase remaining in the membrane vesicles converts arginine to citrulline, thereby interfering with the uptake of arginine. Such metabolism may result in arginine uptake in the absence of an energy source (Fig. 2). Membrane vesicles derived from cells bearing pME3719 contained substantial arginine deiminase activity; ^{14}C -labeled arginine accumulated by the membrane vesicles was immediately degraded to citrulline (data not shown). For unknown reasons, deletion of the *arcA* gene had an adverse effect on the expression of the *arcD* gene (data not shown). Therefore, Δp -driven

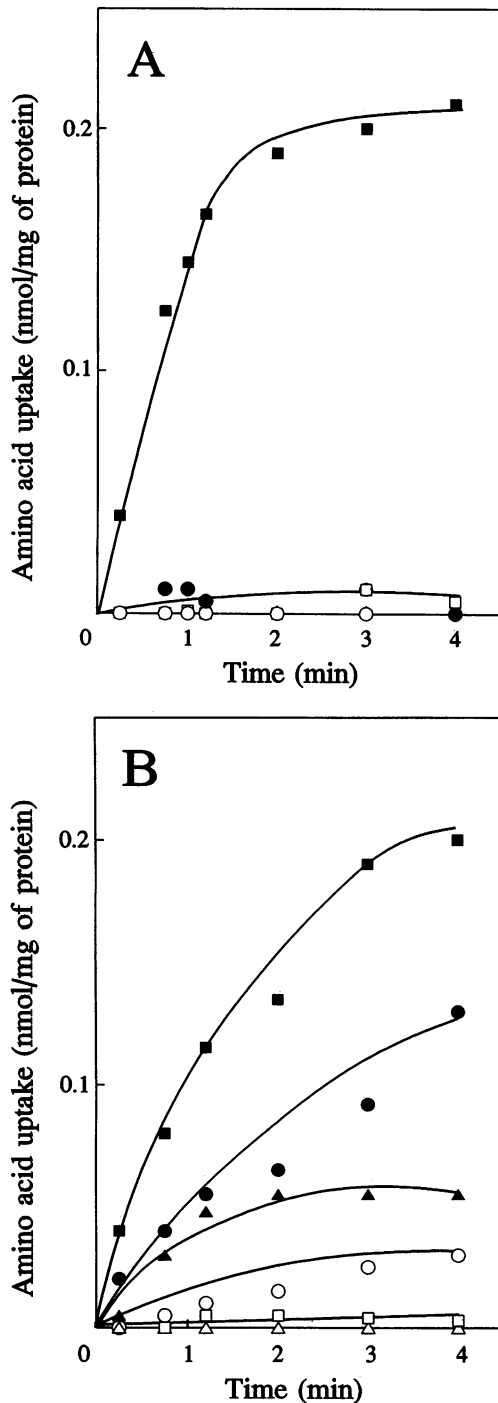


FIG. 2. Δp -driven uptake of lysine (■, □), arginine (●, ○), and ornithine (▲, △) by control (A) and ArcD (B) membrane vesicles. Uptake was performed aerobically in the presence (■, ●, ▲) and absence (□, ○, △) of glucose-PQQ.

uptake of ornithine was further studied because this amino acid is not metabolized.

The relation between Δp and ornithine transport by ArcD vesicles was studied by using the ionophores nigericin and valinomycin. Nigericin mediates the electroneutral exchange between K^+ and H^+ , thereby collapsing the transmembrane pH gradient (ΔpH) while the transmembrane electrical po-

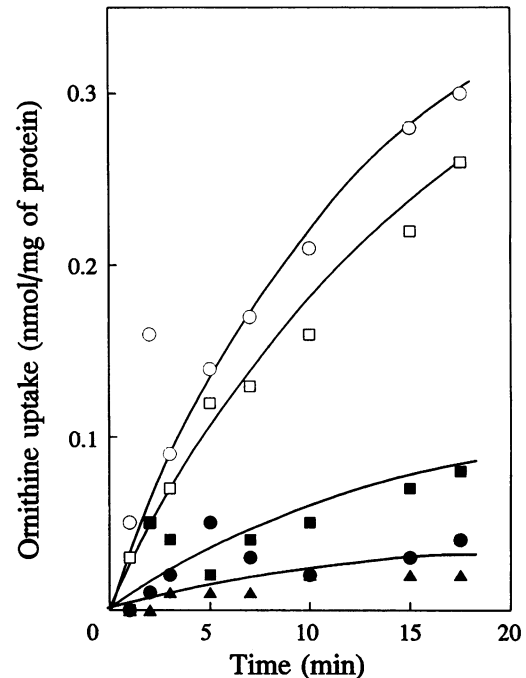


FIG. 3. Effect of nigericin and valinomycin on ornithine transport. Uptake of ornithine by ArcD membrane vesicles was assayed aerobically in the absence (■) or presence (□) of glucose-PQQ with 10 nM nigericin (○), 100 nM valinomycin (●), and both 10 nM nigericin and 100 nM valinomycin (▲). Ionophores and glucose/PQQ were added 1 min prior to the addition of [^{14}C]ornithine. Uptake experiments were performed at pH 7.0 and 25°C.

tential ($\Delta\psi$) increases (8–10). Valinomycin catalyzes electrogenic K^+ transport and collapses $\Delta\psi$ in the presence of a high concentration of K^+ . The reduction of the $\Delta\psi$ is partially compensated by an increase in the magnitude of the ΔpH . Ornithine uptake was almost completely blocked by valinomycin at pH 7.0 (Fig. 3). The addition of nigericin resulted in a small increase of the ornithine uptake, whereas uptake was abolished when both ionophores were present. Similar results were obtained at pH 6.0 and 7.5 (data not shown). These results support the conclusion that the $\Delta\psi$ acts as the driving force for ornithine transport.

To determine the substrate specificity of the ArcD protein, the effect of various L-amino acids on the uptake of [^{14}C]ornithine and lysine was studied. Uptake of [^{14}C]lysine does not depend on the ArcD protein; both ArcD and control membrane vesicles displayed a high level of lysine uptake (Fig. 2). Accumulated [^{14}C]lysine was rapidly chased by an excess of unlabeled lysine. Release of [^{14}C]lysine effected by an excess of ornithine or of arginine was observed only when ArcD was present (Fig. 4). Citrulline, a metabolic intermediate of the arginine deiminase pathway, was ineffective with both membrane vesicle preparations. ArcD membrane vesicles accumulate ornithine in response to the Δp (Fig. 2B). [^{14}C]ornithine was released by an excess of unlabeled lysine, arginine, or ornithine but not citrulline (data not shown). These results demonstrate that the ArcD protein transports arginine, ornithine, and lysine and catalyzes their rapid exchange.

Arginine-ornithine exchange in reconstituted proteoliposomes. Exchange between arginine and ornithine was studied in more detail by using proteoliposomes bearing the recon-

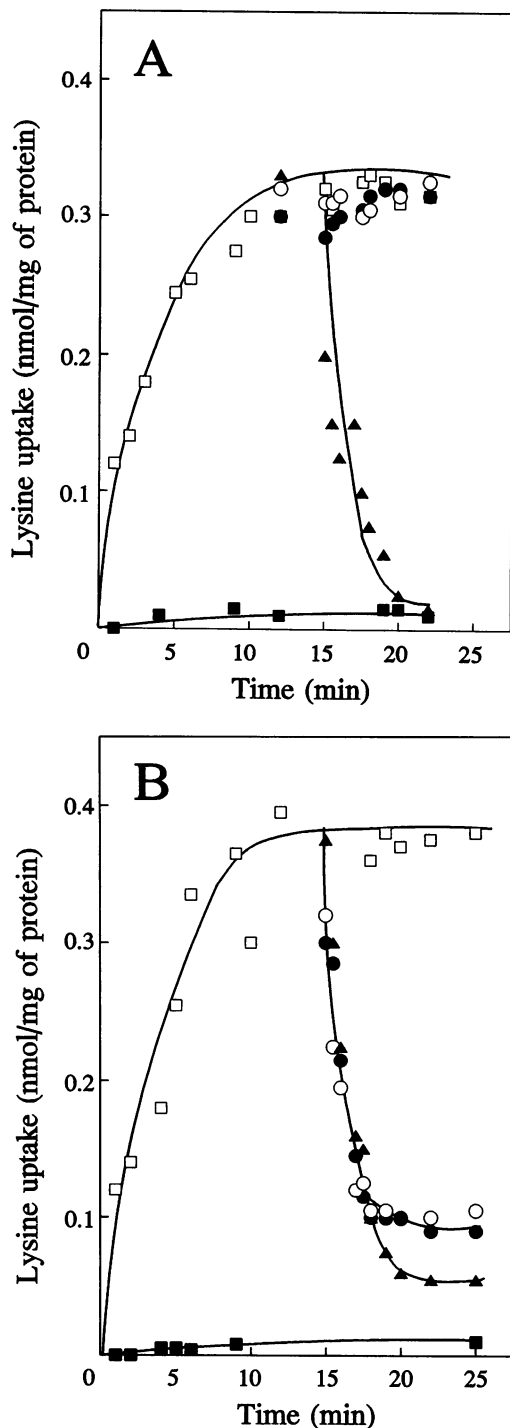


FIG. 4. Release of L-[¹⁴C]lysine by the addition of a 100-fold excess of L-lysine (▲), L-arginine (●), and L-ornithine (○) by control (A) and ArcD (B) membrane vesicles. Membrane vesicles were incubated in the presence (□) and absence (■) of glucose-PQQ and allowed to accumulate L-[¹⁴C]lysine for 15 min, and then the unlabeled amino acids were added. Further experimental details are given in the legend to Fig. 3.

stituted ArcD protein. With this procedure, arginine deiminase is removed, yielding proteoliposomes that are devoid of arginine metabolism (data not shown). ArcD membrane vesicles were solubilized with *n*-octyl- β -glucopyranoside in

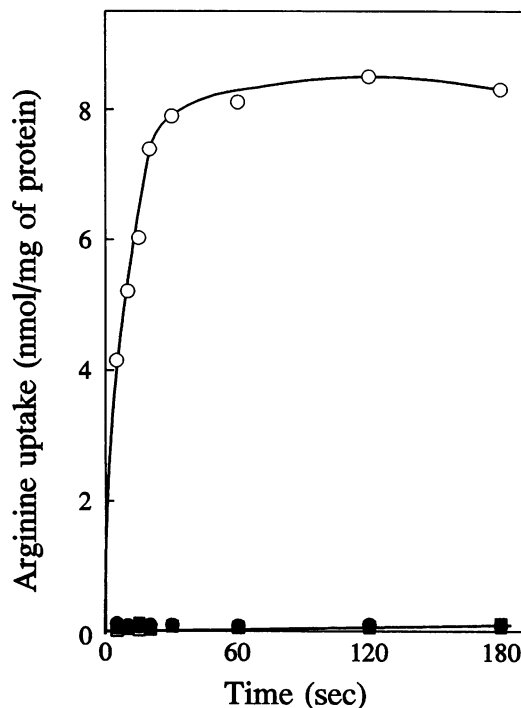


FIG. 5. Reconstitution of arginine-ornithine exchange activity in proteoliposomes obtained from control (■, □) and ArcD (●, ○) membrane vesicles. Proteoliposomes were loaded with 500 μ M ornithine (□, ○) or not loaded (■, ●) and diluted 50-fold in a buffer containing 1.5 μ M L-[¹⁴C]arginine. Uptake was assayed at 25°C.

the presence of *E. coli* phospholipids and the osmolyte glycerol. The extracted proteins were reconstituted into proteoliposomes by detergent dilution. Proteoliposomes were loaded with ornithine and rapidly diluted into a buffer containing [¹⁴C]arginine. Arginine was rapidly accumulated by the proteoliposomes (Fig. 5). There was no detectable uptake when proteoliposomes that were not loaded with ornithine were used. Rapid uptake of [¹⁴C]arginine was also observed when proteoliposomes were loaded with lysine instead of ornithine, whereas citrulline-loaded proteoliposomes were inactive (data not shown). Proteoliposomes prepared from control membrane vesicles were inactive for arginine uptake (Fig. 5). These results further demonstrate that the ArcD protein mediates exchange between arginine and ornithine.

To determine the stoichiometry of arginine-ornithine exchange, proteoliposomes were loaded with 500 μ M [³H]ornithine and diluted into a medium containing 20 μ M [¹⁴C]arginine. Rapid uptake of arginine was accompanied by efflux of ornithine (Fig. 6). In a parallel experiment, [³H]ornithine-loaded proteoliposomes were diluted into arginine-free buffer to assess the slow arginine-independent rate of ornithine efflux. Ornithine-independent uptake of arginine was measured by diluting unloaded proteoliposomes into a buffer containing [¹⁴C]arginine. At each time point, the exchange stoichiometry was calculated from the arginine-stimulated ornithine release and ornithine-dependent arginine uptake (Fig. 6). This value was found to be close to 1 (Fig. 6, inset).

The kinetic constants of arginine-ornithine exchange were estimated from the initial rates of arginine uptake measured for 5 s at different arginine concentrations. Arginine-orni-

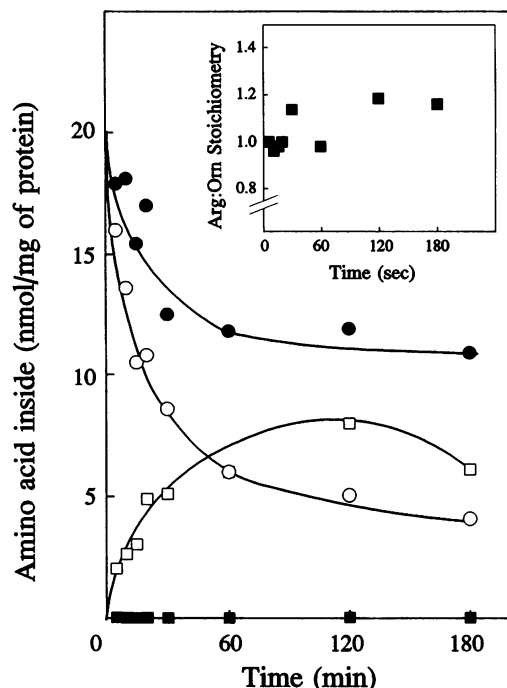


FIG. 6. Stoichiometry of arginine-ornithine exchange in proteoliposomes. Proteoliposomes were loaded with 500 μM [2,3- ^3H]ornithine and diluted 50-fold in 50 mM potassium phosphate (pH 7.0). [^3H]ornithine efflux was assayed in the presence (\circ) or absence (\bullet) of 20 μM [^{14}C]arginine. [^{14}C]arginine uptake by nonloaded proteoliposomes (\blacksquare) and by proteoliposomes loaded with 500 μM [^3H]ornithine (\square) was measured at 20°C. (Inset) Arginine-ornithine stoichiometry as a function of the assay time calculated from arginine-stimulated ornithine efflux and ornithine-stimulated arginine uptake.

thine exchange showed monophasic saturation kinetics. The apparent K_t for arginine uptake was 11 μM with a V_{max} of 121 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ ($n = 3$). Under conditions of homologous ornithine-ornithine exchange, the K_t and V_{max} for ornithine uptake were 60 μM and 132 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$, respectively.

DISCUSSION

In this paper we demonstrated that the ArcD protein of *P. aeruginosa* catalyzes Δp -driven uptake of arginine and ornithine and exchange between arginine and ornithine. This system differs from the previously characterized binding protein-dependent transport systems, which allow the uptake of lysine, arginine, and ornithine under aerobic growth conditions (15). ArcD is an essential protein for anaerobic growth of *P. aeruginosa* on arginine (23, 34). In the arginine deiminase pathway, the system functions as an antiporter that catalyzes a one-to-one exchange between arginine and ornithine.

Arginine and ornithine are positively charged in the neutral pH range. The stoichiometric exchange between arginine and ornithine is therefore electroneutral. Electroneutral exchange has also been demonstrated for the arginine-ornithine antiporter of *L. lactis* and the agmatine-putrescine antiporter of *Enterococcus faecalis* (12, 13). The ArcD protein resembles the arginine-ornithine antiporter of *L. lactis* (1, 12) in many respects but differs in its ability to mediate a slow but significant rate of Δp -driven uptake of

arginine and ornithine. In growing cells, the exchange between arginine and ornithine is driven by the concentration gradients of both amino acids, which are maintained by the metabolism. Compared with a Δp -dependent reaction, exchange is energetically more favorable to the cell, because it does not require the input of additional metabolic energy. By this efficient design, the transport system makes a significant contribution to the overall energy production during anaerobic arginine metabolism.

In recent years, a number of bacterial exchange systems that mediate the inward movement of substrate directly coupled to the outward movement of a product have been described (28). These systems either operate by a strictly coupled antiport reaction or mediate an exchange reaction that also permits net uptake of substrates. Studies with membrane vesicles show that the exchange mediated by the ArcD protein is at least 1,000-fold faster than the rate of Δp -driven uptake (130 and 0.08 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg of protein}$, respectively [36]). For accurate comparison, it will be necessary to perform kinetic experiments on Δp -driven translocation as a function of the Δp . Exchange reactions are favored when both the intra- and extracellular substrate levels are saturating (11). A high intracellular ornithine concentration will force a rapid reoccupation of the binding site when arginine is released on the inner surface of the membrane. Since strictly coupled antiport will not lead to ornithine accumulation, another mechanism is required to maintain a high intracellular concentration of ornithine. In *P. aeruginosa*, Δp -driven uptake may serve as a route to acquire arginine for biosynthetic purposes. The Δp -driven reaction may be necessary to accumulate intracellular ornithine to maximize the rate of exchange during arginine catabolism. In *L. lactis*, ornithine can be replaced by lysine, which is accumulated via a separate Δp -dependent transport system (13).

Our results suggest that $\Delta\psi$ functions as a main driving force for the net ArcD-mediated uptake of ornithine. If $\Delta\psi$ functioned as the sole driving force, a more pronounced effect of nigericin on the rate of ornithine uptake would be expected, because this ionophore causes an increase in $\Delta\psi$ (36). Transport is biased toward the $\Delta\psi$, although the possibility of a role for protons cannot definitely be excluded.

We observed that extensive overexpression of the *arcD* gene product was lethal to *E. coli* cells. Stable expression of *arcD* was only possible when the gene was transcribed in tandem with the *arcA* gene, which codes for arginine deiminase. The reason for this observation is unclear. The domain coding for ArcD is the most unstable part of the mRNA and is rapidly processed (17). Although overexpressed arginine deiminase interfered with arginine uptake experiments in membrane vesicles, problems could be circumvented by the use of proteoliposomes or by the study of ornithine uptake.

The results suggest that, during anaerobic arginine catabolism by *P. aeruginosa*, ArcD serves as an arginine-ornithine exchanger. Uptake of arginine results from the energy-independent electroneutral exchange with intracellular ornithine. This process is driven by arginine metabolism and should allow optimal energy conversion when *P. aeruginosa* cells are exposed to conditions of limited energy supply.

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