Tobramycin Uptake in *Escherichia coli* Membrane Vesicles

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The uptake of tobramycin was measured in *Escherichia coli* membrane vesicles prepared in KMES [K^+ –2-(*N*-morpholino)ethanesulfonic acid] buffer at pH 6.6. Uptake occurred in vesicles energized with ascorbic acid and phenazine methosulfate, in which the electrical potential ($\Delta\psi$) was -120 mV, but not in vesicles energized with D-lactate ($\Delta\psi = -95$ mV). The addition of nigericin to vesicles energized with D-lactate did not induce tobramycin uptake despite an increase in $\Delta\psi$ to -110 mV. However, when $\Delta\psi$ was increased or decreased by the addition of nigericin or valinomycin, respectively, uptake in vesicles energized with ascorbic acid and phenazine methosulfate was stimulated or inhibited, respectively, confirming studies with whole cells showing that uptake of aminoglycosides is gated by $\Delta\psi$ rather than by proton motive force ($\Delta\mu_{H^+}$) or ΔpH . *N*-ethylmaleimide prevented uptake, suggesting that the aminoglycoside transporter is a cytoplasmic membrane protein with accessible sulfhydryl groups. The observation that uptake is gated in vesicles as well as in whole cells suggested that diffusion occurs through a voltage-gated channel. In vesicles preloaded with tobramycin, no efflux occurred after the addition of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone. In susceptible cells, aminoglycosides themselves decreased the magnitude of $\Delta\psi$. We propose a mechanism of aminoglycoside-induced killing in which aminoglycosides themselves close the voltage-gated channel by decreasing the magnitude of $\Delta\psi$. Channel closure causes aminoglycosides accumulated prior to the fall in $\Delta\psi$ to be trapped, which in turn causes irreversible uptake and subsequent bactericidal effects.

We have previously shown that the early phase of uptake of aminoglycoside antibiotics by bacteria is regulated by electrical potential ($\Delta\psi$), the electrical component of the protonmotive force ($\Delta\mu_{H^+}$) (19, 24, 39, 40). A critical threshold of $\Delta\psi$ must be reached for the initiation of aminoglycoside transport. Once this threshold is exceeded, the initial rates of both uptake and killing are proportional to the magnitude of $\Delta\psi$ (7, 9, 11, 17, 20, 24, 27, 32, 39, 40, 53). These observations lent themselves to a chemiosmotic interpretation that active transport of cationic aminoglycosides is driven by $\Delta\psi$ by a uniport mechanism via a cytoplasmic membrane carrier protein (9, 39). However, uptake which is gated by $\Delta\psi$ demonstrates properties of a voltage-regulated channel.

The observations that the early transport of aminoglycosides in whole cells is both energy dependent and gated are difficult to reconcile with most current models of aminoglycoside mechanism of action. One widely accepted unifying hypothesis to explain how the pleiotropic effects of aminoglycosides cause cell death has recently been proposed (18). This hypothesis suggests that aminoglycoside-initiated misreading of mRNA results in altered proteins that fuse to the cytoplasmic membrane, forming many nonspecific channels. These channels are thought to facilitate the uptake or efflux of a variety of substrates, including aminoglycosides themselves. Four key factors are identified in this model: (i) misreading, (ii) secondary membrane damage, (iii) energy-dependent and irreversible uptake, and (iv) initiation blockade. Uptake prior to misreading

is thought to occur via a leak pathway (18). However, the specific and gated bioenergetic requirements of early aminoglycoside uptake (uptake occurring prior to aminoglycosideribosome interactions [energy-dependent phase I {EDPI}]) are not consistent with a nonspecific leak pathway.

Since there are limitations to the study of transport processes in whole-cell systems, we wished to better characterize the bioenergetics of aminoglycoside uptake in cell-free systems and to determine if voltage regulates the opening of membrane channels or drives active transport via carriers. For these studies, we examined tobramycin uptake in membrane vesicles prepared from *Escherichia coli* ML 308-225 (30, 31). Studies of antibiotic transport in vesicles have several advantages over those of transport in cells. For example, in susceptible bacteria, transport of antibiotics takes place simultaneously with cell death and/or alterations in physiology, including inhibition of protein synthesis (18), alteration of growth rate (46), increased membrane permeability (18, 19), and decreased membrane potential (8, 35); each perturbation alters the aminoglycoside uptake process. Moreover, in cells, the presence of an intracellular sink makes it difficult to distinguish between active transport and facilitated diffusion. Although there are data describing aminoglycoside transport in membrane vesicles prepared from *Pseudomonas putida* (54), no previous data have been obtained for the more extensively characterized *E. coli* membrane vesicle system.

Although the membrane vesicle system is a powerful tool for studying membrane-associated events involved in early aminoglycoside uptake, any comprehensive model of aminoglycoside mechanism of action must also consider membrane processes in cells which occur following the interaction of aminoglycosides with the ribosomal target (EDP II). EDP II represents a subsequent, rapid-accumulation phase of uptake. To extend our previous observations on the bioenergetics of

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aminoglycoside uptake and to investigate the mechanism of irreversible uptake in whole cells, we were also interested in analyzing events occurring late in the course of aminoglycoside uptake and in determining the effects of aminoglycosides on membrane potential. Using both *Staphylococcus aureus* and *E. coli* mutants in which membrane potential could be manipulated and easily quantified, we determined whether late uptake (EDP II) was still dependent on $\Delta\psi$ and gated, features that would be consistent with a specific channel-mediated transport pathway rather than a nonspecific leakage through the membrane.

MATERIALS AND METHODS

Bacterial strains. *E. coli* ML 308-225 and AS-1 were generous gifts from H. R. Kaback and N. Hirota, respectively. An *rpsL* mutant of AS-1 was selected by isolating a single colony that grew on nutrient broth agar that contained 1,000 μ g of streptomycin per ml. Bacterial strains with known transposon inserts were obtained from C. Gross (52). The P1_{vir} transductional mapping was carried out essentially as described by Miller (42). Initial transductant selection was on Luria-Bertani plates with 50 mM sodium citrate and 15 μ g of tetracycline per ml. The tetracycline-resistant colonies were subsequently transferred via sterile toothpicks to Luria-Bertani plates with 15 μ g of tetracycline per ml and 250 μ g of streptomycin per ml. The number of streptomycin-sensitive, tetracycline-resistant colonies versus the total number of tetracycline-resistant colonies tested (200) was scored.

Reagents. Tris hydrochloride, 2-(*N*-morpholino)ethanesulfonic acid (MES), potassium hydroxide (KOH), glucose, chicken egg white lysozyme, EDTA, sucrose, magnesium sulfate, bovine pancreatic type I DNase, RNase A, ascorbic acid (ASC), phenazine methosulfate (PMS), D-lactate, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), nigericin, valinomycin, *N*-ethylmaleimide (NEM), toluene, sodium chloride (NaCl), and lithium chloride (LiCl) were purchased from Sigma Chemical Co., St. Louis, Mo. [³H]tetraphenylphosphonium bromide ([³H]TPP⁺) (26 Ci/mol), 7-[¹⁴C]salicylic acid (57.4 mCi/mmol), and L-[2,3-³H]proline (31 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. [³H]tobramycin (4.9 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. Glutathione maleimide was a generous gift from D. Schachter. Nitrocellulose filters (25 mm; 0.45- μ m pore size) were obtained from Millipore Corp., Bedford, Mass.

Preparation of membrane vesicles. *E. coli* ML 308-225 membrane vesicles were prepared according to the lysis method of Kaback (30, 31, 51), using MES buffered to pH 6.6 with KOH (KMES) in place of pH 6.6 potassium phosphate. Briefly, bacteria were grown to late log phase in 10 liters of 5% glucose–M9 salts, harvested at 16,000 $\times$ g for 30 min, and resuspended in 80 ml of 30 mM Tris–20% sucrose (pH 8.0) per g of wet pellet. Lysozyme and EDTA were added at concentrations of 0.5 mg/ml and 10 mM, respectively, and the suspension was incubated at 25°C for 30 min. Formation of spheroplasts was checked by examination under a phase-contrast microscope. The spheroplasts were harvested at 16,000 $\times$ g for 30 min and resuspended in 20 ml of 100 mM KMES–20% sucrose–20 mM MgSO₄ (pH 6.6) by using a glass syringe with a large-bore blunt needle. DNase and RNase A were both added at a final concentration of 2 μ g/ml to 4 liters of 50 mM KMES (pH 6.6) lysis buffer prewarmed to 37°C, to which the spheroplasts were added. After vigorous stirring for 15 min, 15 mM MgSO₄ and 10 mM EDTA were sequentially added, with continuous stirring for 15 min after both additions. The crude vesicle preparation was harvested at 10,800 $\times$ g for 60 min, resuspended in 200 ml of 100 mM KMES–10 mM EDTA (pH 6.6) at 37°C, and centrifuged twice at 800 $\times$ g for 30 min to remove cells and debris, with the pellet discarded both times. The vesicles were harvested at 45,000 $\times$ g for 30 min, and the pellet was resuspended in 100 mM KMES (pH 6.6). The protein content of the vesicle suspension was determined by a modification of the Lowry method (36). The vesicles were diluted to a final protein concentration of approximately 4 mg/ml in 100 mM KMES (pH 6.6) and stored in liquid nitrogen prior to their use.

Characterization of membrane vesicles and measurement of substrate uptake. Vesicles were characterized by measuring their ability to transport proline (30). To measure uptake, 50- μ l aliquots of vesicles were placed in a series of borosilicate tubes, and under continuous oxygenation, 1 μ l of a final concentration of either 20 mM D-lactate or the combination of 20 mM ASC and 0.1 mM PMS, followed by either 8 μ M proline or 200- μ g/ml tobramycin, was added. Uptake was measured at 25°C for proline and 37°C for tobramycin. After various intervals of time, the reaction mixtures were diluted with 2 ml of either 0.1 M LiCl (for proline) or 3% NaCl (for tobramycin) at room temperature to stop uptake and were quickly filtered through 0.45- μ m-pore-size nitrocellulose filters with one wash of the borosilicate tubes. The filter papers were placed into scintillation fluid, and the radioactivity remaining on the filter papers was measured.

The concentration of proline or tobramycin inside the vesicles was calculated by subtracting the background count obtained at time zero from the counts measured at specified time intervals and dividing this number by the specific activity of the proline, the counting efficiency, the volume of vesicles used, the

protein concentration of the vesicles, and a mean vesicular volume of 2.2 μ l/mg of vesicle protein (30).

Determination of the magnitudes of $\Delta\psi$ and Δ pH. $\Delta\psi$ and Δ pH were calculated by measuring uptake of TPP⁺ and salicylic acid (30), respectively, into oxygenated vesicles, using flow dialysis. Flow dialysis has been shown to correlate well with direct measurement of $\Delta\psi$ and Δ pH obtained via vesicle micropuncture (22). After 100 mM KMES (pH 6.6) was run through the flow dialysis apparatus, 400 μ l of vesicles was placed in the top portion of the flow dialysis chamber. Either 20 μ M [³H]TPP⁺ or 20 μ M [¹⁴C]salicylic acid was added to the upper chamber, and after 20 fractions were collected, either 20 mM D-lactate or the combination of 20 mM ASC and 0.1 mM PMS was added. The effects of nigericin, valinomycin, and CCCP on the uptake of TPP⁺ and salicylic acid were also determined. After calculation of the concentrations of TPP⁺ and salicylic acid inside vesicles (*C_i*) and those remaining in buffer (*C_o*), $\Delta\psi$ and Δ pH were determined by using *C_i/C_o* (39).

Effects of toluene and aminoglycosides in whole-cell systems. To study the effects of toluene on aminoglycoside uptake during EDP II, [³H]tobramycin uptake in an *E. coli* AS1 *rpsL* *acrA* mutant was determined by filtration through glass microfiber filters (Whatman GF/C) during logarithmic-phase growth as described previously (24). In these experiments, 2% toluene was added to tobramycin-preexposed cells at 30 min, and the amount of cell-associated tobramycin per unit of cell mass was measured. Simultaneously, the effect of toluene on cell integrity was measured turbidimetrically (24). The effects of streptomycin and tobramycin on the magnitude of $\Delta\psi$ in *E. coli* AS1 *rpsL* *acrA* cells were determined by measuring the distribution of the lipophilic cation TPP⁺ as described previously (24).

RESULTS

Characterization of membrane vesicles energized with ASC and PMS. Studies with whole-cell preparations of *E. coli* and *S. aureus* have shown that aminoglycoside uptake in medium buffered with potassium phosphate is less than that in unbuffered medium at the same pH (unpublished data). This observation, in conjunction with the difficulty encountered by others in preparing *E. coli* membrane vesicles capable of transporting aminoglycosides (9, 16, 26, 54), prompted us to prepare vesicles by using KMES rather than potassium phosphate. Vesicles were energized with ASC and PMS to ensure a maximal electrochemical potential (41). At pH 6.6, $\Delta\psi$ was –120 mV in vesicles energized with ASC and PMS, calculated by measuring TPP⁺ uptake. The inset of Fig. 1 shows the distribution ratios of TPP⁺ following the addition of ASC and PMS, as determined by flow dialysis. Approximately 5 min after the addition of ASC and PMS, $\Delta\psi$ began to fall in the absence of protonophores, reaching baseline values at approximately 20 min. The addition of ASC and PMS at 20 min restored $\Delta\psi$ to –120 mV (fraction 40). The addition of the protonophore CCCP, which dissipates the electrochemical proton gradient $\Delta\mu_{H^+}$ (both $\Delta\psi$ and Δ pH), at 10 min caused immediate TPP⁺ efflux, whereas addition of the ionophore nigericin, which increases $\Delta\psi$, further stimulated uptake (not shown).

Tobramycin uptake in membrane vesicles energized with ASC and PMS. Aminoglycoside uptake was measured at 37°C by using [³H]tobramycin at a concentration of 200 μ g/ml. The use of this concentration was based upon earlier studies by Bryan and Kwan characterizing streptomycin uptake in *E. coli* membrane vesicles which contained the components of protein synthesis (9). We also observed tobramycin uptake at concentrations of 2 and 20 μ g/ml and at temperatures of 25 and 30°C. Although tobramycin accumulation was measured over 10 to 12 min, uptake reached a peak at 3 to 5 min (Fig. 1). The readdition of ASC and PMS at 20 min, which restored $\Delta\psi$ to –120 mV, had no further stimulatory effect on tobramycin uptake. To measure vesicle-associated aminoglycoside in the absence of $\Delta\psi$, energized vesicles were exposed to the protonophore CCCP or were treated with the K⁺ ionophore valinomycin, which decreases $\Delta\psi$ and increases Δ pH (also see Fig. 3). Energy-independent binding of tobramycin was also shown by measuring vesicle-associated tobramycin in the absence of electron donors (unenergized vesicles). As with whole-cell sys-

FIG. 1. Tobramycin uptake in *E. coli* membrane vesicles energized with ASC and PMS. Uptake in *E. coli* membrane vesicles energized with 20 mM ASC and 0.1 mM PMS (solid circles) and in vesicles energized with ASC and PMS and then treated with 20 μM CCCP (closed triangles) or 0.05 mM NEM (open triangles) prior to the addition of 200 μg of tobramycin per ml is shown. Uptake in unenergized vesicles (open circles) and mean data (squares) for vesicles treated with CCCP, vesicles treated with NEM, and unenergized vesicles are also shown. The inset shows steady-state distribution ratios of $[^3\text{H}]\text{TPP}^+$ determined by flow dialysis following the addition of ASC and PMS at fractions 10 and 40 (approximately 5 and 20 min, respectively). TPP^+ was added to the upper chamber of the flow dialysis cell at the beginning of the experiments.

FIG. 2. Tobramycin uptake in *E. coli* membrane vesicles energized with D-lactate. $[^3\text{H}]\text{tobramycin}$ (200 μg/ml) uptake in vesicles energized with 20 mM ASC and 0.1 mM PMS (inverted triangles) and in those energized with 20 mM D-lactate with (squares) and without (diamonds) 0.4 μM nigericin is shown. Uptake in unenergized vesicles (circles) and in those treated with 20 μM CCCP (triangles) is also shown. The inset shows steady-state distribution ratios of $[^3\text{H}]\text{TPP}^+$ following the addition of D-lactate as determined by flow dialysis. TPP^+ was added to the upper chamber of the flow dialysis apparatus at fraction 15 (about 7 min), 0.4 μM nigericin was added at fraction 40 (about 20 min), and CCCP was added at fraction 55 (about 23 min).

tems, energy-independent binding of aminoglycosides to vesicles was virtually instantaneous (10, 43).

Studies with the maleimide NEM were also performed to characterize the effect of sulfhydryl reagents on aminoglycoside uptake; these chemicals are known to inactivate carrier proteins in *E. coli* (14). NEM at 0.05 mM, a concentration previously shown to have no effects on vesicle membrane potential (14), had no effect on initial aminoglycoside binding but blocked subsequent uptake in vesicles energized with ASC and PMS (Fig. 1). Since NEM would not be expected to interfere with nonspecific membrane binding of aminoglycosides, this confirms the studies with unenergized and deenergized vesicles characterizing nonspecific surface binding of aminoglycosides.

Tobramycin uptake in vesicles energized with D-lactate. To determine whether aminoglycoside uptake in vesicles, like that in cells (9, 39), is gated, tobramycin uptake in vesicles energized with D-lactate was compared with that in vesicles energized with ASC and PMS. As shown in Fig. 2, there was no uptake in vesicles energized with D-lactate ($\Delta\psi = -95$ mV), and the addition of nigericin did not stimulate uptake despite an increase in $\Delta\psi$ to -110 mV. These studies show that ami-

noglycoside uptake in membrane vesicles is gated at $\Delta\psi$ between -110 and -120 mV. The inset of Fig. 2 shows the distribution ratio of TPP^+ following the addition of D-lactate with or without nigericin as determined by flow dialysis. In vesicles energized with D-lactate, unlike those energized with ASC and PMS, $\Delta\psi$ was maintained for >20 min until the addition of CCCP (or valinomycin [not shown]).

Tobramycin uptake in vesicles energized with ASC and PMS and then exposed to ionophores. Previous studies with *E. coli* cells and ML 308-225 vesicles have shown that at pH 6.6 and an external K^+ concentration of 100 mM, $\Delta\psi$ can be increased or decreased with the ionophores nigericin and valinomycin, respectively (51). In whole-cell preparations, the opposite effects of these ionophores on $\Delta\psi$ have provided evidence that aminoglycoside uptake is regulated by $\Delta\psi$ and not by ΔpH or $\Delta\mu_{\text{H}^+}$ (39). As in whole-cell systems (37, 39), when 0.1 to 0.4 μM nigericin was added to vesicles energized with ASC and PMS, tobramycin uptake increased, whereas 20 μM valinomycin blocked uptake (Fig. 3). Maximal stimulation occurred at 0.4 μM nigericin. The maximal value of $\Delta\psi$ obtained after addition of 0.4 μM nigericin was -136 mV. These studies are consistent with data obtained with intact cells (8, 20, 39).

FIG. 3. Effects of nigericin and valinomycin on tobramycin uptake in *E. coli* membrane vesicles. [^3H]tobramycin (200 $\mu\text{g}/\text{ml}$) uptake in vesicles energized with 20 mM ASC and 0.1 mM PMS with 0.1 μM (diamonds), 0.2 μM (closed circles), and 0.4 μM (inverted triangles) nigericin is shown. Also shown are uptake in ASC- and PMS-energized vesicles without nigericin (squares) and uptake in vesicles treated with 20 μM CCCP (open circles) and 20 μM valinomycin (triangles).

Proline uptake in vesicles energized with ASC and PMS or with D-lactate. To compare the voltage-gated uptake of tobramycin with active transport through a known membrane carrier, we compared tobramycin uptake with that of proline in vesicles energized with either D-lactate or ASC and PMS (Fig. 4). At pH 6.6, proline uptake was observed in vesicles energized either with ASC and PMS or with D-lactate. In vesicles energized with either electron donor, uptake was proportional to the magnitude of $\Delta\mu_{\text{H}^+}$ and was not gated. Addition of CCCP at 5 min caused proline to efflux from energized vesicles (not shown).

Effects of CCCP and toluene on vesicles and cells preloaded with tobramycin and on unenergized vesicles. Previous studies with cells have shown that aminoglycoside uptake is irreversible; aminoglycoside efflux does not occur following the addition of the protonophore CCCP or 2,4-dinitrophenol (4, 18, 47). We examined the effects of 20 μM CCCP in vesicles that either were energized with ASC and PMS and then preloaded with tobramycin for 10 min or were exposed to tobramycin in the absence of electron donors. CCCP dissipates both $\Delta\psi$ and ΔpH and causes efflux of substrates that have been actively transported via membrane carriers in response to $\Delta\psi$, ΔpH , or both ($\Delta\mu_{\text{H}^+}$). The addition of CCCP was not associated with efflux of preaccumulated tobramycin (Fig. 5) but caused complete efflux of preaccumulated proline (not shown). CCCP did not cause tobramycin to efflux even from energized vesicles exposed to 0.4 μM nigericin.

Compared with the effects of treatment with protonophores such as CCCP, the effects of toluene on aminoglycoside uptake

FIG. 4. Effect of ASC and PMS and of D-lactate on proline uptake in *E. coli* membrane vesicles. Proline uptake in vesicles energized with either ASC and PMS (circles) or D-lactate (squares), as well as uptake in unenergized vesicles (triangles) and those treated with 20 μM CCCP (diamonds), is shown.

in whole cells are more complex. Previous studies with *E. coli* whole-cell systems have shown (2, 3) that the addition of toluene prior to EDP II stimulates aminoglycoside uptake. However, when *E. coli* cells were treated with 2% toluene following the onset of the aminoglycoside-induced rapid accumulation phase (EDP II), efflux did occur (Fig. 6), as others have also observed (4, 48). Following EDP II, the addition of toluene to *E. coli* was associated with a rapid efflux of tobramycin that had a half-life of less than 1 min and reached a nadir at 2 min (Fig. 6A). Toluene also caused cellular lysis, with a half-life for lysis of approximately 30 min (Fig. 6B). Thus, the effects of toluene appear to be due to membrane damage and loss of intracellular components which may bind aminoglycosides, thus explaining efflux in whole cells.

To confirm early studies by Anand et al. (3) suggesting that toluene stimulated aminoglycoside uptake when added prior to the initiation of the rapid phase of streptomycin uptake (EDP II), we examined tobramycin uptake in de-energized vesicles (Fig. 5). The addition of toluene to unenergized vesicles 10 min after the addition of tobramycin caused rapid uptake (influx) of tobramycin. However, toluene addition was not associated with aminoglycoside efflux in vesicles energized with ASC and PMS and then preloaded with tobramycin. In cells or vesicles that are treated with toluene, aminoglycoside translocation bypasses the putative membrane carrier (3, 18), whereas for actively transported substrates, efflux due to CCCP occurs via specific membrane carriers (51).

Effects of aminoglycosides on membrane potential in susceptible *acrA* mutants of *E. coli*. While it has long been known that aminoglycoside uptake is irreversible (4, 19), the mechanism of aminoglycoside trapping is unknown (19, 26). We proposed a mechanism of aminoglycoside trapping in which an

FIG. 5. Effect of toluene on tobramycin uptake in *E. coli* membrane vesicles. The effect of 1% toluene (squares) added at 10 min to unenergized vesicles is shown. Also shown is the effect of 20 μ M CCCP (added at 10 min) in vesicles preloaded with tobramycin and energized either with 20 mM ASC and 0.1 mM PMS (triangles) or with ASC, PMS, and 0.4 μ M nigericin (circles), all added at time zero.

aminoglycoside-associated decrease in the magnitude of $\Delta\psi$ prevents efflux via voltage-gated channels (35). The observations that neither CCCP nor toluene caused tobramycin efflux in vesicles preloaded with tobramycin suggested that tobramycin transport occurred via diffusion through voltage-gated channels rather than by active transport driven by $\Delta\psi$ and mediated by membrane carriers. Previous studies using a variety of methods to measure the effects of aminoglycosides on $\Delta\psi$ in aminoglycoside-susceptible bacteria are contradictory (9, 12, 47), and it has been suggested that the calculation of $\Delta\psi$ by measuring TPP^+ uptake may be inaccurate for *E. coli* treated with aminoglycosides (21). Therefore, we first examined the effects of 0.2 to 2.0 mM streptomycin on TPP^+ uptake in an *rpsL* (high-level streptomycin-resistant) mutant of the *acrA* strain *E. coli* AS-1. AS-1 was used because we have shown that TPP^+ uptake in this strain is equivalent to that in *E. coli* treated with EDTA (15, 34).

In order to confirm that the high-level streptomycin resistance mutation was in the *rpsL* gene, contrasductional studies with P1 phage were performed. The *rpsL* locus is at 72.8 min on the standard *E. coli* genetic map (5). A set of *E. coli* strains with transposon inserts at known locations has been generated by Singer et al. for rapid mapping purposes (52). In this case, P1 phage was propagated on strain CAG18456 (*cysG zhe-3084::Tn10*), whose insert maps at 74.00 min near *rpsL*, and CAG12153 (*zhaT6::Tn10*), whose insert maps at 70.00 min, a distance to *rpsL* greater than the distance that P1 should span (42, 52). Both of these strains are streptomycin sensitive. The P1 lysate from each of these strains was subsequently used to transduce *E. coli* AS-1 (streptomycin resistant) as described previously (42), with initial selection for tetracycline resistance. The resulting colonies were transferred with sterile toothpicks to Luria-Bertani agar with 250 μ g of streptomycin per ml. The number of tetracycline-resistant, streptomycin-sensitive colonies for each set of transductants was scored. In the case of the

FIG. 6. Effect of toluene on tobramycin uptake and lysis in *E. coli* cells. (A) Tobramycin (200 μ g/ml) uptake in *E. coli* AS1 with (squares) and without (diamonds) 1% toluene (added at 30 min). (B) Turbidimetric readings (A_{600}) in the presence (squares) and absence (circles) of toluene.

FIG. 7. Effect of streptomycin on TPP⁺ uptake and $\Delta\psi$ in an *rpsL* mutant of *E. coli*. (A) Uptake of TPP⁺ in *E. coli* AS1 *rpsL* *acrA* with (circles) and without (squares) the addition of streptomycin (100 μ g/ml). (B) Dose-response effects of tobramycin on membrane potential in *E. coli* AS1 *rpsL*.

CAG18456 transducing lysate, cotransduction of tetracycline resistance and streptomycin sensitivity was 16.5% (200 colonies scored). This indicates that the Tn10 insert at 74.00 min is approximately 1 min from the streptomycin resistance marker, which is consistent with the map location of *rpsL*. In contrast, as expected there were no contrasductants observed with the 70.00-min marker, since the distance between this Tn10 marker and *rpsL* is greater than the distance that can be spanned by P1 phage transduction.

The steady-state concentrations of TPP⁺ were identical in cells exposed to 0 to 100 μ g of streptomycin per ml (Fig. 7A). TPP⁺ concentrations were identical at other streptomycin concentrations (data not shown). In cells treated with 0 to 25 μ g of tobramycin per ml, $\Delta\psi$ fell from -168 to -135 mV (Fig. 7B). Higher concentrations of tobramycin were not associated with a further decrease in potential.

Bioenergetics of transport in whole cells during EDP II. To better define the energy requirements of late EDP II, an *acrA* mutant of *E. coli* and a wild-type isolate of *S. aureus* were exposed to conditions previously shown to block early aminoglycoside uptake (EDP I) by decreasing $\Delta\psi$, including treatment with CCCP, which dissipates $\Delta\mu_{H^+}$, and medium acidification, which decreases $\Delta\psi$, increases Δ pH, and maintains $\Delta\mu_{H^+}$ (39, 40). In *E. coli*, both CCCP and medium acidification (changing pH from 7.5 to 5.0) at 45 min blocked late tobramycin uptake during late EDP II (Fig. 8A). To further establish that uptake in late EDP II is gated by $\Delta\psi$, the studies were repeated with the strain *S. aureus* 86. Experiments were done with *S. aureus* to examine the effects of increasing $\Delta\psi$ at pH 5.0 with nigericin (40). Studies with the ionophores nigericin and valinomycin are not possible with *E. coli* *acrA* mutants. While acidification of the medium to pH 5.0 at 15 min (Fig. 8B) was associated with the complete cessation of tobramycin uptake, the subsequent addition of nigericin to these cells at 45 min stimulated uptake to levels seen in cells maintained at pH 7.5. These studies with both *E. coli* and *S. aureus* show that voltage-gated uptake, analogous to that seen in the early phase of

aminoglycoside uptake, occurs in whole cells during late EDP II.

DISCUSSION

Studies of aminoglycoside uptake in *E. coli* membrane vesicles confirm our previous data for cells, showing that uptake is regulated by $\Delta\psi$. As with cells, the initial surface binding of aminoglycosides to unenergized vesicles or to vesicles that are initially energized with ASC and PMS and then de-energized with either CCCP or valinomycin is virtually instantaneous. In vesicles, unlike in cells (24, 25), binding represents a significant fraction (up to 25%) of vesicle-associated tobramycin. Uptake occurs when $\Delta\psi$ reaches a critical threshold of between -110 and -120 mV. This threshold is remarkably similar to that in *E. coli* whole cells, for which the gate has been estimated at between -107 and -125 mV (10). As with cells (37, 39), nigericin increases and valinomycin decreases aminoglycoside uptake by vesicles energized with ASC and PMS, demonstrating that $\Delta\psi$, and not Δ pH or $\Delta\mu_{H^+}$, regulates aminoglycoside uptake.

In vesicles, low concentrations of the relatively lipophilic maleimide NEM (0.05 mM) block the uptake of tobramycin. In ML 308-225 vesicles, 0.05 mM NEM also blocks the uptake of lactose, melibiose, and proline by inactivating carrier proteins but has no effect on the ability of vesicles to generate $\Delta\mu_{H^+}$ (14). We have also performed similar studies with whole-cell preparations of *E. coli* and *S. aureus*. In whole cells, both NEM and the more hydrophilic glutathione maleimide blocked tobramycin uptake at concentrations that do not alter cell growth rate or membrane potential (unpublished data). These studies suggest that the aminoglycoside transporter is a protein with accessible sulfhydryl groups on the external face of the cytoplasmic membrane.

While previous studies examining aminoglycoside uptake in *E. coli* membrane vesicles have had limited success (9, 16, 48, 54), studies using membrane vesicles of *P. putida* (54) have

FIG. 8. Tobramycin uptake during late EDP II in an *acrA* mutant of *E. coli*. (A) Effects of a pH change from 7.5 (triangles) to 5.0 (diamonds) with 1 N HCl and of 30 μ g of chloramphenicol per ml (inverted triangles) on [3 H]tobramycin uptake (10 times the MIC) in an *acrA* mutant of *E. coli* (C12) grown in nutrient broth supplemented with 0.5% glucose and 0.1 M Tris. All manipulations were made at 45 min (i.e., during late EDP II). (B) Effects of 0.2 μ M nigericin on the uptake of tobramycin (10 times the MIC) in *S. aureus* 86. Tobramycin was added at the beginning of the experiment to log-phase cells grown in nutrient broth-yeast extract at pH 6.7. The pH was adjusted to 5.0 by the addition of 1 N HCl at 15 min (circles), and nigericin was added at 45 min (inverted triangles). Tobramycin uptake in control cells (no pH adjustment or nigericin) is also shown (triangles).

shown aminoglycoside uptake kinetics similar to that in our *E. coli* vesicle preparations. When *P. putida* vesicles were energized with ASC and PMS, streptomycin uptake inhibitable by CCCP was observed. As with tobramycin uptake in *E. coli* membrane vesicles, the concentration of streptomycin inside *P. putida* vesicles was less than threefold that in the medium inside cells and was approximately double that in the medium outside cells.

Studies with *E. coli* whole-cell systems have shown that treatment with toluene alters the cytoplasmic membrane, facilitating aminoglycoside uptake or efflux in cells which have been briefly exposed to or preloaded with aminoglycosides, respectively. Anand et al. (3) showed that the addition of 2% toluene prior to the initiation of the rapid phase of streptomycin uptake (EDP II) was associated with virtually instantaneous streptomycin influx, suggesting that toluene-induced membrane damage bypassed the aminoglycoside membrane transport system (3, 19). We have confirmed toluene-associated stimulation of uptake by energized and CCCP-treated cells and also showed that toluene-induced uptake occurred in membrane vesicles as well. In contrast, Andry and Bockrath (4) showed that the addition of 2% toluene to streptomycin-preloaded cells was associated with rapid streptomycin efflux. We confirmed the studies with tobramycin-preloaded cells treated with 1% toluene, but we also demonstrated that toluene exposure was associated with membrane damage which caused rapid lysis. Notably, we were unable to demonstrate tobramycin efflux from toluene-treated preloaded membrane vesicles.

Uptake that is dependent on $\Delta\psi$ has generally been interpreted according to chemiosmotic theory (9, 39, 53). According to this model, aminoglycosides are actively transported through a membrane carrier via a uniport mechanism. Both

voltage-dependent carriers and channels are proteins that traverse the cytoplasmic membrane, and the observation that aminoglycoside uptake by vesicles is voltage gated suggests that the transporter has properties of a channel. While under different conditions, membrane proteins may show properties of carriers or channels, for channels, translocation of substrates occurs at equivalent rates in both directions, is orders of magnitude more rapid, generally has lower K_m values, and is nonconcentrative (55).

In addition to gating, other characteristics of aminoglycoside uptake that are difficult to reconcile with the chemiosmotic model and are consistent with transport through channels (1) include the inability to saturate the carrier at low substrate concentrations, uptake and killing kinetics that are proportional to $\Delta\psi$ for 2 to 4 min but independent of $\Delta\psi$ thereafter, the dependence of the apparent magnitude of the critical threshold for uptake on aminoglycoside concentration, and the irreversibility of uptake.

In general, carriers are saturated at lower substrate concentrations than are channels. While the determination of K_m values for antibiotics in susceptible bacteria is fraught with technical problems (i.e., cells are being inhibited or killed during the antibiotic uptake process), studies by Bryan and Van Den Elzen using *E. coli rpsL* mutants showed that uptake was saturable at high streptomycin concentrations (10). Moreover, the K_m for streptomycin in membrane vesicles of *P. putida* was 15 mM (54), and no known substrates showed competitive inhibition of aminoglycoside uptake. Thus, while streptomycin uptake is saturable in vesicles (and cells), it occurs at high K_m values, consistent with transport via channels rather than via carriers.

Uptake through channels also suggests a mechanism in

which the apparent magnitude of the gate is dependent upon the aminoglycoside concentration tested, as does the observation that the rate of aminoglycoside uptake is proportional to $\Delta\psi$ only for the initial 2 to 4 min (20, 39). Studies by Bryan and Kwan with *Bacillus subtilis*, in which $\Delta\psi$ was regulated by varying the external K^+ concentration, showed that the critical threshold necessary for the initiation of streptomycin uptake was dependent upon the concentration of streptomycin used (9). Studies by Mandel et al. (37) and Eisenberg et al. (20) with *S. aureus* also showed that increasing the magnitude of $\Delta\psi$ with either *N,N'*-dicyclohexylcarbodiimide or medium alkalization lowered the concentration of gentamicin at which uptake occurred. Both groups showed that after several minutes of incubation, increasing the electrical potential (9, 44) did not further enhance aminoglycoside uptake.

On the other hand, when uptake and killing were examined at 2 to 4 min, we observed that both were proportional to $\Delta\psi$ (20, 39). We previously interpreted the early proportionality between membrane potential and both killing and uptake according to the chemiosmotic model, thinking that the absence of this relationship after 4 min was an artifact due to aminoglycoside-induced perturbation of transport. However, uptake via voltage-gated channels offers an alternative explanation for uptake that is only briefly proportional to $\Delta\psi$ and for the apparent concentration dependence of the magnitude of the voltage-dependent gate. Voltage-gated channels do not "open" or "close" at a given potential. Rather, at the single-channel level, gating transitions are stochastic. While the number of open channels per unit membrane fluctuates spontaneously, the likelihood of being in an open configuration varies over a narrow range of membrane potential (13, 28). To understand the apparent alteration of the apparent magnitude of the gate by external aminoglycoside concentration, it is also important to note that following the initiation of uptake, aminoglycosides themselves stimulate a series of events (EDP II) that greatly increase aminoglycoside uptake and are necessary for lethal activity (9, 45). We propose that at a lower magnitude of $\Delta\psi$, sufficient uptake occurs at high aminoglycoside concentrations to initiate EDP II despite fewer channels being in the open configuration. An increase in the magnitude of $\Delta\psi$ would open more channels so that otherwise subinhibitory concentrations of aminoglycosides would initiate EDP II and killing.

This model may also explain why uptake and killing are proportional to membrane potential only for a brief period. Since the percentage of channels in the open configuration is a function of the magnitude of $\Delta\psi$, it follows that at a fixed external aminoglycoside concentration, the initial rate of aminoglycoside uptake is also proportional to $\Delta\psi$. However, since the internal and external aminoglycoside concentrations rapidly reach equilibrium, the apparent relationship between the magnitude of $\Delta\psi$ and the rate of uptake ceases.

Unidirectional, gated transport suggests a mechanism for irreversible aminoglycoside uptake. While studies examining the effect of aminoglycosides on $\Delta\psi$ have been contradictory (6, 9, 12, 26), our data suggest that aminoglycosides decrease $\Delta\psi$. Previous studies have also shown that after several minutes, the addition of either gentamicin (9) or streptomycin (26) to *E. coli* is associated with a fall in the magnitude of $\Delta\psi$ by 20 to 30 mV, which is comparable to that seen when *E. coli* is exposed to tobramycin. Studies comparing the magnitude of $\Delta\psi$ in cells incubated for longer periods either with or without aminoglycosides (26) are difficult to interpret since the magnitude of $\Delta\psi$ falls by more than 20 mV when members of the family *Enterobacteriaceae* reach late-log-phase growth (33).

Davis et al. (18, 19) and Bakker and Busse et al. (6, 12)

recently suggested that aminoglycoside-induced misreading of base pairs during mRNA translation is associated with the formation of nonspecific channels capable of transporting aminoglycosides into cells, and Busse et al. hypothesized that these transient channels cause intracellular trapping of aminoglycosides. However, the observation that aminoglycoside uptake is gated in vesicles prepared from cells that have not been exposed to aminoglycosides, in conjunction with voltage-gated uptake during late EDP II, is difficult to reconcile with uptake via many nonspecific channels. On the other hand, the well-documented loss of cytoplasmic membrane integrity (18) and the fall in membrane potential may be due to the insertion of misread proteins into the cytoplasmic membrane (6, 12, 18, 19).

We propose an alternative model to explain aminoglycoside-induced killing. Aminoglycosides themselves decrease the magnitude of $\Delta\psi$. The closure of the voltage-gated channels would cause irreversible trapping of intracellular aminoglycosides. This hypothesis also might explain why the aminoglycosides are bactericidal whereas other inhibitors of protein synthesis that do not alter $\Delta\psi$ or enter via voltage-gated channels are bacteriostatic. The absence of tobramycin efflux upon the addition of CCCP is also consistent with this model.

Although there are important functional and kinetic differences between channels and carriers, there are common structural themes and evolutionary origins, and it has been recognized for some time that all translocator proteins likely form channels (1, 13, 23, 28, 29, 38, 49, 50, 55). We believe that the aminoglycoside channel may represent a primordial wide channel that is capable of transporting small ions as well as large substrates (29). It is important to note that there is little correlation between channel conductance and the width of a channel. In conjunction with voltage-regulated gating, this may explain why these putative channels are not lethal to bacteria.

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