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CHARACTERIZATION OF A RESPIRATORY MUTANT OF *ESCHERICHIA COLI* WITH REDUCED UPTAKE OF AMINOGLYCOSIDE ANTIBIOTICS

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A strain of *Escherichia coli* (NSW77) which is partially resistant to streptomycin was isolated by selecting for growth on plates supplemented with 12.5 μg/ml streptomycin, a concentration which completely inhibits growth of wild-type strains. The low-level resistance of the mutant appears to result from a reduced ability to accumulate streptomycin intracellularly. In addition, the mutant strain is unable to use succinate for growth because of a defective respiratory chain. Thus, membranes of the mutant strain were found to have approximately half the NADH and D-lactate oxidase activity of the parent strain. Succinate oxidase activity was reduced more drastically, to a level of 7% that of the parent strain. Moreover, membranes of the mutant were found to contain demethylmenaquinone and, in place of ubiquinone, a structural analogue, 2-octaprenyl-3-methyl-6-methoxy-1,4 benzoquinone. The mutation responsible for both the Suc^- phenotype and partial resistance to streptomycin was found to be located near minute 15 on the bacterial chromosome. Both the biochemical and genetic evidence suggests that the mutation in strain NSW77 resides in the *ubi F* gene. Another previously characterized *ubi F* strain was also found to have a reduced capacity to take up an aminoglycoside antibiotic (gentamicin). These results suggest that the respiratory defects in *ubi F* strains are responsible for the reduced capacity of such strains to accumulate aminoglycosides.

Introduction

One approach for investigating the way in which aminoglycoside antibiotics gain entry into bacterial cells is to isolate mutants which are altered in the mechanism of uptake of these antibiotics. Such mutants can be obtained by selecting for strains with either enhanced or reduced sensitivities to aminoglycosides. Characterization of these mutant strains may lead to the recognition of cellular components involved in the transport of aminoglycosides into bacteria.

In a previous publication [1], we showed that mutants of *Escherichia coli* K12 selected as being hypersensitive to the aminoglycoside, streptomycin, take up the antibiotic with increased efficiency. A significant proportion of these hypersensitive strains was found to be defective in the coupling of electron transport to oxidative phosphorylation due to lesions in the Mg^{2+}-ATPAse (*unc* mutants). It is not understood as yet why mutations in *unc* genes result in increased cellular uptake of aminoglycosides. Not all *unc* mutations exhibit this effect; indeed, resistance to low levels of neomycin has been used quite extensively as a method of selecting uncoupled mutants [2]. In these strains it seems that partial resistance to neomycin results from reduced accumulation of the antibiotic.

Strains isolated to date showing resistance to

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Abbreviations: MMQ_8, 2-octaprenyl-3-methyl-6-methoxy-1, 4-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

streptomycin fall into three categories. In most clinical isolates, resistance can be attributed to the expression of extrachromosomal genes residing on R plasmids. Secondly, there are those strains with mutations mapping at the *rps L* (*str A*) gene, of which the protein product is responsible for ribosomal binding of streptomycin [3]. Both of the above classes of strains are generally resistant to high levels of streptomycin (above 100 μg/ml) whereas members of the third class of resistant strains tolerate lower levels of the antibiotic only (5–20 μg/ml). In most cases partial resistance appears to be due to reduced accumulation of aminoglycosides.

Some partially resistant strains have been shown to harbour defects in the cell's energy-transducing machinery [2,4,5], others possess abnormalities in the structure of the cell envelope (the suggested explanation of low-level resistance in *str C* (*str B*) mutants [6]). A further class of strains which are partially resistant to streptomycin are those affected in catabolite repression having either reduced levels of cyclic AMP (because of a mutation at *cya*) or with inactive cyclic AMP-receptor proteins [7–9].

This communication describes the isolation and characterization of a mutant of *E. coli* that is partially resistant to streptomycin because of a change in one of the components of the respiratory chain.

Materials and Methods

Bacterial strains. Strains of *E. coli* K12 used were JP2140 (*ilv-1, his-29(am), trp A9605(am)*, obtained from J. Pittard); NSW77 (derived from JP2140 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment); NSW70 (*nag B, tyr A352, his, arg, thi, lac, lam*^{R}); AN146 (*upp, gal K, ubi F 411, rps L*) and AN147 (*upp, gal K, rps L*). NSW67 (*sdh-O, tyr A352, his, arg, thi, lac, lam*^{R}) and NSW68 (*tyr A352, his, arg, thi, lac, lam*^{R}) were *gal*$^+$ transductants arising from a cross using JRG1002 (*sdh-O, trpA, trp*^{R}, *ic*^{R}, *rpsL*) as donor and AT2273 (*gal, tyr A352, his, arg, thi, lac, lam*^{R}) as recipient. Genetic nomenclature is that used by Bachmann et al. [10].

Media and growth of cells. For all except transduction and proline uptake experiments, cells were grown in nutrient broth (Oxoid) enriched with 0.5% brain/heart infusion (Oxoid). For agar plates, 2% agar (Difco) was added to brain/heart infusion broth. Broth cultures for uptake experiments were grown at 37°C in a reciprocating shaking water bath (Model RW18, Paton Industries, S.A.) at a speed setting of 100 oscillations/min. Cultures for estimation of oxidase rates, quinone concentrations and atebrin fluorescence quenching were grown in 1-l quantities in 3-l baffled flasks with vigorous aeration in a New Brunswick gyrotory shaker at 37°C. Cultures from transduction experiments were grown on plates consisting of medium A [11] with 2% agar and supplemented with glucose or succinate (each 30 mM), or *N*-acetylglucosamine (0.2%), thiamine hydrochloride (0.0005%), L-isoleucine (0.32 mM), L-valine (0.36 mM), L-histidine (0.28 mM), L-tryptophan (0.2 mM). Cultures for proline-uptake experiments were grown in aminoglycoside uptake medium [4] with the addition of glucose, thiamine, casamino acids (0.1%) and the above amino acid supplements as required. Cell growth was monitored as absorbance at 600 nm (A_{600}) using a Spectronic 20 spectrophotometer where 1 ml of culture at an A_{600} value of 0.5 is equivalent to 0.2 mg dry weight bacteria.

Aminoglycoside uptake experiments. Uptake experiments were carried out as previously described [1] except that the final concentration of streptomycin in the culture medium was 12.5 μg/ml and the final specific activity of [H^3]dihydrostreptomycin was 169 dpm/ng streptomycin. Gentamicin was added to a final concentration of 10 μg/ml and the specific activity of [H^3]gentamicin was 0.8 μCi/ml.

Measurement of atebrin fluorescence quenching in membranes. Membranes were prepared as previously described [12] except that cells were washed and smashed in a medium containing 300 mM KCl, 15 mM $MgCl_2$ and 10 mM 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid-KOH, pH 7.5. Quenching of atebrin fluorescence by membranes was estimated by the method of Haddock and Downie [13] using an Aminco SPF 500 Spectrofluorimeter.

Proline uptake experiments. The Method of Pugsley and Schnaitman [14] was used. The final concentration of [H^3]proline was 1 μCi/ml culture and unlabelled proline was added to a final concentration of 0.001%.

Estimation of oxidases in membranes. Membrane preparation and estimation of specific activities of

oxidases in membranes were carried out as described previously [12].

Estimation of protein. Protein concentrations were measured by the method of Lowry et al. [15] using bovine serum albumin as standard.

Estimation of quinone concentrations in whole cells. Lipids were extracted from 10 g (approximate wet weight) of cells using hot acetone in a continuous Soxhlet extractor. Quinones were separated from other lipids by chromatography on silica gel G plates using a solvent of chloroform/light petroleum (7 : 3, v/v) as described previously [12]. Bands with R_f values corresponding to those of quinones were eluted with ethanol and the concentrations of benzoquinones, demethylmenaquinone and menaquinone were determined by ultraviolet spectroscopy using a Cary Model 118 spectrophotometer [12].

Isolation and characterization of MMQ_8. Lipids were extracted as described above from 43 g of cells (wet weight) of strain NSW77. MMQ_8 was separated from other quinones by thin-layer chromatography using four different solvent systems according to the method of Young et al. [16]. After purification, MMQ_8 was identified by ultraviolet spectroscopy and mass spectrometry. Methane chemical ionization mass spectra were recorded with a Finnigan Model 3200 Quadruple instrument interfaced to the same manufacturer's Incos Model 2300 data system. Methane was used as the reactant gas at an ion source pressure of 0.7 Torr. The ion source temperature was 200°C and the sample was volatilised from the solid probe.

Transduction experiments. The generalized transducing bacteriophage Plkc was used for transduction experiments as described by Pittard [17].

Results

Isolation of mutants partially resistant to streptomycin

A culture of the streptomycin-sensitive strain, JP2140, was treated with *N*-methyl-N′-nitro-*N*-nitrosoguanidine as described by Adelberg et al. [18]. After phenotypic expression (overnight incubation at 37°C), cells were spread on brain/heart infusion plates containing 12.5 μg/ml streptomycin. At this concentration of streptomycin, JP2140 showed sensitivity. Partially resistant strains were selected for growth at 12.5 μg/ml streptomycin, yet no growth at 100 μg/ml. In this manner, we hoped to eliminate ribosomally resistant (*rps L*) strains. Three strains possessed the partially resistant phenotype of interest. Here, we describe the characterization of one of these mutants, NWS77. Strain NSW77 was chosen, since in addition to being resistant to lower concentrations of streptomycin, it was unable to grow on medium with succinate as sole source of carbon and energy (Suc^- phenotype), suggesting a defect in respiratory metabolism. In nutrient broth, NSW77 showed biphasic growth with a mean generation time of approx. 42 min up to an A_{600} value of 0.32 then, above this cell density, a longer mean generation time (200 min). The mean generation time of the parent strain was 21 min. It is no known why growth of NSW77 is biphasic; a slower growth rate, however, is consistent with a respiratory defect.

Streptomycin uptake in NSW77

Fig. 1 shows that the partially resistant mutant, NSW77, accumulates negligible quantities of streptomycin over a 1 h period (at a final streptomycin concentration of 12.5 μg/ml) in comparison with the parent strain, JP2140. This result is consistent with the partial resistance observed on plates containing

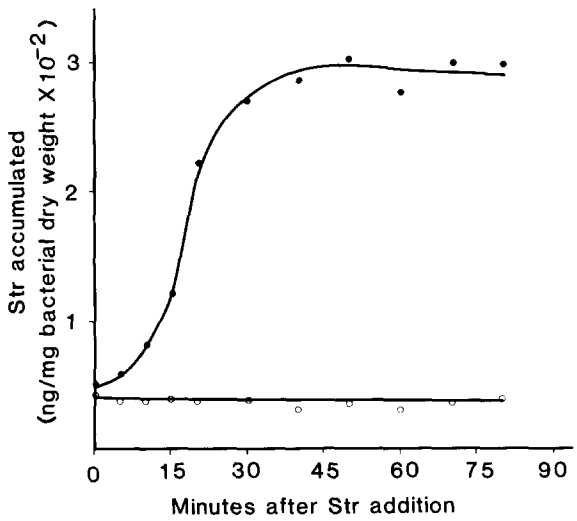

Fig. 1. Kinetics of streptomycin (Str) accumulation for JP2140 and NSW77. Cells were grown in nutrient broth supplemented with 0.5% brain/heart infusion. The final concentration of streptomycin was 12.5 μg/ml. JP2140 (parent), •——•; NSW77 (mutant), ○——○.

12.5 μg/ml streptomycin and suggests that membrane permeability to streptomycin is affected in the mutant.

Quenching of atebrin fluorescence by membranes from NSW77

It is now known that agents such as *o*-dinitrophenol and CCCP, which abolish the proton-motive force across the membrane [19], and mutations which increase the proton permeability of membranes serve to reduce the accumulation of aminoglycosides [1,20,21]. Thus, generation of a membrane potential appears to be essential for aminoglycoside uptake. The ability of membrane particles, in the presence of reduced substrates or ATP, to quench the fluorescence of solutions of atebrin has been used to indicate the formation of a transmembrane proton gradient [13,22]. We have used this technique to estimate membrane energization in membranes of both parent and strain NSW77. Fig. 2 shows that with either NADH or ATP as substrates, membranes from NSW77 quench atebrin fluorescence at least as effectively as membranes from the parent strain. With NADH as substrate, quenching of atebrin fluorescence persists for longer with membranes from NSW77 than with those of the parent (Fig. 2A and B), suggesting that NADH is being utilized more slowly in the mutant than in the parent strain. With ATP, the opposite effect is evident; mutant membranes show a more rapid rate of quenching of atebrin fluorescence than do membranes from JP2140 (Fig. 2C and D). This result suggests that ATP hydrolysis may be the preferred way in which a proton-motive force is established across the membrane in NSW77. We conclude that reduced uptake of streptomycin in NSW77 does not result from an inability to generate a proton-motive force across the membrane.

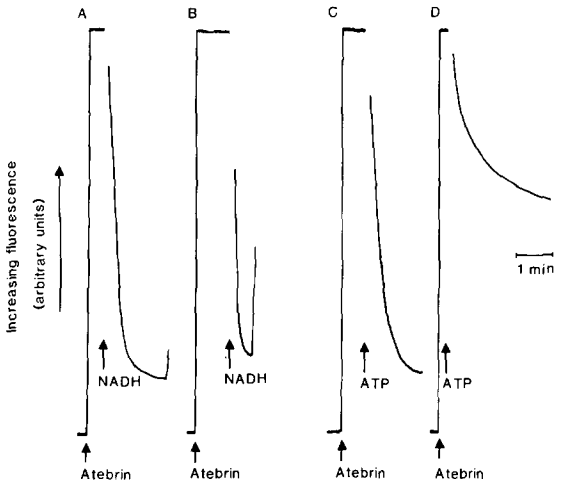

Fig. 2. Atebrin fluorescence quenching with membranes vesicles from strains NSW77 and JP2140. The preparation of membranes was as described in Materials and Methods. Measurements of atebrin fluorescence quenching were carried out as described by Haddock and Downie [13]. Membranes from NSW77 were used for traces A and C at a protein concentration of 1.06 mg/ml and those from JP2140 at 1.1 mg/ml protein (traces B and D). Additions were made as indicated by the arrows: atebrin (4 μM), NADH (0.9 mM) and ATP (0.8 mM).

Proline uptake of NSW77

It seems reasonable to assume that in order to accumulate streptomycin, a bacterial cell must be capable not only of generating membrane potential but also of coupling this energy to the uptake process. Since NSW77 was not defective in generating a proton-motive force across the membrane, we investigated the capacity of the mutant to utilize this energy for the uptake of a normal cellular metabolite, proline. It was found that NSW77 took up proline at a faster rate than the parent strain, JP2140 (Fig. 3), suggesting that the mutant is able to utilize a proton-motive force to drive proline transport. In view of this result, the reduction in streptomycin uptake observed in NSW77 appears to stem not from an inability of the cell to use a

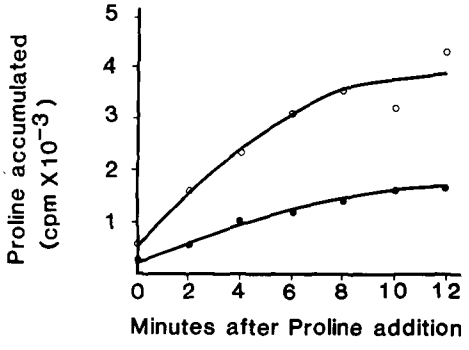

Fig. 3. Kinetics of proline uptake by NSW77 and JP2140. Cells were grown in aminoglycoside uptake medium [4]. JP2140 (parent), •——•; NSW77 (mutant), ○——○.

proton-motive force for uptake processes, but from some other cellular defect.

Estimation of oxidase rates in membranes from NSW77

In *E. coli*, reduction of streptomycin accumulation has been observed in the presence of electron-transport inhibitors, under anaerobic conditions and in strains with mutations affecting activity of the respiratory chain (*hem A* and *ubi D* strains) [4,20, 21]. To check for any alteration of function in the respiratory chain of strain NSW77 we determined oxidase activities in membrane preparations from both the mutant and parent by measuring rates of oxygen uptake with NADH, D-lactate or succinate as added substrates. In membranes from NSW77, the specific activities of both NADH and D-lactate oxidases were reduced to 45% of the activity of parent membranes (Table I). The slow rate of oxidation of NADH explains the persistence of atebrin quenching observed in NSW77 with NADH as substrate (Fig. 2A). The reduction of succinic oxidase to 7% of the parent value is, however, the most significant loss in oxidase activity in mutant membranes. The loss of succinic oxidase activity explains the mutant's inability to use succinate as sole carbon and energy source. We checked to see whether or not the inability to oxidize succinate is directly related to the reduction in uptake of aminoglycosides in *ubi F* strains by examining the accumulation of streptomycin in a strain that lacks succinic dehydrogenase. No difference in streptomycin accumulation between the *sdh* mutant (NSW67), which possesses normal activity in NADH and D-lactate oxidases yet retains only 5% succinic oxidase activity, and an otherwise isogenic *sdh*$^+$ strain (NSW68) was detected.

TABLE I

SPECIFIC ACTIVITIES FOR OXIDASE SYSTEMS

Membranes were prepared from cells grown in nutrient broth supplemented with brain/heart infusion (0.5%). The methods for the preparation of membranes and measurement of oxidase rates as described in Materials and Methods. NADH was used at a final concentration of 0.9 mM; D-lactate (4 mM) and succinate (20 mM were present.

Membranes from	Rate of oxygen uptake (ng atom O/min per mg protein)		
	NADH	D-Lactate	Succinate
JP2140 (parent	467	142	142
NSW77 (mutant)	209	64	10

Strain NSW77 shares phenotypic similarities with a ubiquinone-deficient strain (AN146) that harbours a *ubi F* mutation [23]. *Ubi F* is thought to be the structural gene for the enzyme which converts MMQ_8 to 2-octaprenyl-3-methyl-5-hydroxyl-6-methoxy-1,4 benzoquinone. Thus, AN146 is blocked in the penultimate step of ubiquinone biosynthesis such that MMQ_8 is accumulated in membranes instead of ubiquinone [16,30]. This structurally related precursor differs from ubiquinone in that the benzoquinone ring of MMQ_8 lacks a methoxy group at the C-5 position. MMQ_8 has been shown to function fairly efficiently in NADH and D-lactate oxidation, although activity through succinate oxidase is minimal [23].

Estimation of quinones present in NSW77

Quinones were separated from a lipid extract of cells of strain NSW77 by chromatography in four different solvent systems which have previously been shown to separate MMQ_8 from ubiquinone [16]. The silica gel plates showed a lemon-coloured band with an R_f value characteristic of MMQ_8; the ultraviolet spectrum of material from this band showed an absorption maximum at 270 nm in ethanol, again suggesting the compound to be MMQ_8. The compound was finally identified as MMQ_8 by mass spectrometry, as the spectrum gave the expected molecular ion at *m/e* 697 (the molecular weight of MMQ_8 is 696.54816 [16]). The presence of ubiquinone was not detected in lipid extracts of strain NSW77. It is interesting that the concentration of MMQ_8 in NSW77 (228 nmol/g wet weight of cells) is greater than that of ubiquinone in the parent strain (159 nmol/g wet weight of cells). No MMQ_8 was detected in extracts from the parent strain. It is possible that an increased level of MMQ_8 in the mutant compensates for reduced activity of this analogue as an electron carrier in respiration. No menaquinone was detected in either parent or mutant cells, yet demethylmenaquinone was present in parent and mutant (strain NSW77) at concentrations of 19 and 31 nmol/g wet weight of cells, respectively.

Genetic analysis of NSW77

Analysis of quinones present in NSW77 showed that MMQ_8 replaced ubiquinone in the mutant, suggesting that the strain harbours a mutation at the *ubi F* gene. The *ubi F* gene is located at 15 min on the *E. coli* chromosome and has been shown to be cotransducible with *nag B* at a frequency of 72% [24]. To test for cotransduction between *nag B* and the mutation responsible for the Suc^- phenotype in NSW77, a *nag* B^- strain (NSW70) was used as a recipient for phage Plkc propogated on the donor strain NSW77 (Suc^-). *Nag*$^+$ transductants were selected by growth on media containing *N*-acetylglucosamine as sole carbon source. Of 61 *nag*$^+$ transductants examined, 41 were unable to grow on succinate. This represents a cotransduction frequency of 67% which is consistent with the value reported in the literature. All Suc^- transductants were partially resistant to streptomycin and all transductants capable of growth on succinate showed normal streptomycin sensitivity. There was thus complete correlation of the Suc^- phenotype with partial resistance to streptomycin. These results show firstly that the mutation in NSW77 is at, or near to, the *ubi F* gene and secondly that this mutation is responsible for the phenotype of partial resistance to streptomycin.

Sensitivity of another ubi F strain to aminoglycosides

Both biochemical and genetic evidence (see above) is consistent with NSW77 harbouring a mutation at *ubi F*. In order to confirm that low aminoglycoside uptake is a general characteristic of *ubi F* strains, we examined the uptake of gentamicin in a previously characterized streptomycin-resistant (*rps L*) *ubi F* strain AN146. Fig. 4 indicates that negligible uptake of gentamicin occurs (at 10 μg/ml gentamicin, final concentration) in AN146 compared with the wild-type strain, AN147. This result is consistent with that obtained for streptomycin uptake in NSW77 (see Fig. 1).

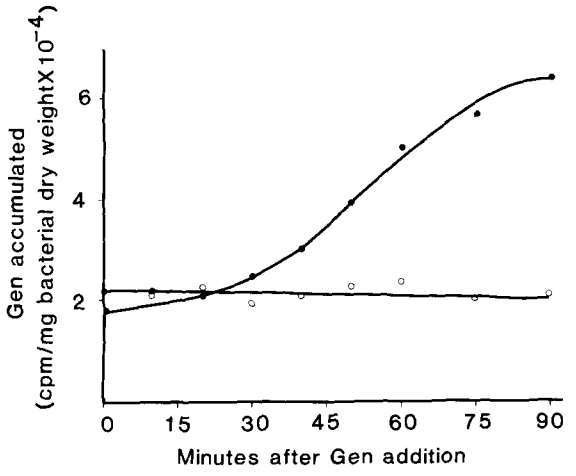

Fig. 4. Kinetics of gentamicin (Gen) accumulation by a *ubi F* strain (AN146). The final concentration of gentamicin was 10 μg/ml. AN147 (*ubi*$^+$), •———•; AN146 (*ubi F*), ○———○.

Using aminoglycoside antibiotic discs it was found that zones of growth inhibition with discs of gentamicin, kanamycin or neomycin were smaller for both strains AN146 (*ubi F*) and the mutant NSW77 than for the corresponding wild-type strains. It appears therefore that partial resistance to aminoglycosides is associated with the *ubi F* genotype and that the partial resistance observed in these mutants is due to reduced antibiotic accumulation.

Discussion

The biochemical and genetic data presented in this paper support the conclusion that the mutation in strain NSW77 is at *ubi F*, a gene thought to encode the enzyme catalysing the penultimate step in the biosynthetic pathway to ubiquinone [30]. Thus, a structural analogue, MMQ_8, was found to replace ubiquinone in the mutant strain, and the mutation responsible for the Suc^- phenotype was found to map in a position on the chromosome that is consistent with it being at the *ubi F* gene. In transduction experiments using NSW77 as donor, all Suc^- colonies tested were found to possess the phenotype of partial resistance to streptomycin, while Suc^+ transductants displayed normal sensitivity. It seems reasonable to conclude, therefore, that in NSW77 the mutation at *ubi F* is responsible for the phenotype of partial resistance to streptomycin.

Evidently, partial resistance to streptomycin in NSW77 is due to reduced accumulation of this antibiotic, since the mutant strain was found to take up streptomycin much less efficiently than the parent strain (Fig. 1). Tests with antibiotic discs suggest that the mutation at *ubi F* affects the accumulation of a number of aminoglycoside antibiotics. Moreover, the effect is not confined to NSW77 alone, since a

previously characterized *ubi F* mutant (AN146) was found to have a reduced uptake of gentamicin (Fig. 4). The suggests that the phenotype of partial resistance to aminoglycosides is characteristic of *ubi F* strains in general.

Why is it that replacement of ubiquinone with a structural analogue (MMQ_8) in cytoplasmic membranes of strains AN146 and NSW77 results in reduction in the rate of uptake of aminoglycosides such as gentamicin and streptomycin?

Previous experimental results suggest that uptake of aminoglycosides depends on an active respiratory chain [4,20,21,28,29]. In membranes from the mutant, replacement of ubiquinone with MMQ_8 reduces the activity of NADH and D-lactate oxidases to half that of the activity present in membranes from the parent while the activity of succinic oxidase is almost completely removed by mutation at *ubi F* (Table 1). Thus, the respiratory capability of *ubi F* strains is generally impaired. The specific loss of succinic oxidase does not affect the accumulation of streptomycin in an *sdh* mutant (NSW67) where the activity of other oxidases is normal (see Results). However, in NSW77 where NADH and D-lactate oxidases are functioning at only 45% efficiency, the loss of succinic oxidase may itself serve to reduce the accumulation of aminoglycosides.

As a result of the general reduction in the rate of electron transport to oxygen in NSW77, a decrease in the rate of establishment of fluorescence quenching of atebrin solutions is observed in membranes from NSW77, even though the final level of quenching with NSW77 membrane is the same as that observed with membranes from the parent (Fig. 2). It is interesting to note, however, that proline uptake, which has been shown to use energy derived from the transmembrane proton-motive force [25–27], is not reduced in the mutant strain relative to the parent (Fig. 3). It is possible that aminoglycoside accumulation is more closely linked to the rate of establishment of a transmembrane proton-motive force than is the uptake of proline. Alternatively, the reduction in aminoglycoside uptake in *ubi F* strains may be attributable to some phenomenon other than the reduced rates of energy production in these strains. One alternative explanation involves the idea that ubiquinone is an essential component of the aminoglycoside uptake system in *E. coli* and that because of the difference in molecular structure between the two quinones, MMQ_8 cannot replace ubiquinone in this role. However, although a role for ubiquinone in aminoglycoside uptake has been suggested previously [4], there is as yet no direct evidence for such a function of ubiquinone.

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