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Properties of *Escherichia coli* Mutants with Alterations in Mg^{2+}-Adenosine Triphosphatase

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A mutant *Escherichia coli*, selected for resistance to the antibiotic neomycin, was unable to utilize nonfermentable carbon sources for growth. Two strains were selected from this mutant on the basis of their ability to grow utilizing succinate as a carbon source. All three strains had approximately equal amounts of the Mg^{2+}-adenosine triphosphatase (ATPase) (EC 3.6.1.3) protein, but the activity of the enzyme differed in each strain. The Mg^{2+}-ATPase from each of the three strains lost activity upon solubilization and appeared to undergo rapid dissociation once solubilized. This dissociation is similar to that described for the wild type after cold exposure.

Various laboratories have isolated mutants of *Escherichia coli* that are defective in the Mg^{2+}-adenosine triphosphatase (ATPase) (BF_1) (1, 6, 11-15, 18, 20). Some of these strains were selected for inability to utilize nonfermentable carbon sources for growth (1, 13-15), whereas others were selected for resistance to antibiotics such as neomycin and streptomycin (6, 11, 12, 14, 18, 20).

Many of the strains selected for antibiotic resistance have been found to be defective in their ability to couple oxidative energy to active transport of small molecules (11, 14, 19, 20). The question arises as to whether loss of enzymatic activity of the BF_1 is directly related to the inability to concentrate solutes and whether resistance to antibiotics in BF_1 mutants can be correlated with either BF_1 activity or ability to couple oxidation to transport.

This paper reports some physiological and biochemical properties of a neomycin-resistant mutant that has previously been shown to be defective in the BF_1 and to be unable to couple respiratory energy to active transport (12). The properties of two partial revertants of this mutant isolated by ability to utilize succinate for growth (12) are also explored. The data suggest that a correlation can be made between sensitivity to neomycin and ability to catalyze active transport, but that a similar correlation between Mg^{2+}-ATPase activity and active transport activity may not exist.

MATERIALS AND METHODS

Bacteria and media. Cultures were grown in a basal salts medium (16) supplemented with various carbon sources as noted. Solid media consisted of nutrient agar, neomycin agar (basal salts medium containing 11 mM glucose and neomycin sulfate supplemented with 2% agar), and succinate agar (basal salts medium containing 85 mM sodium succinate supplemented with 2% agar).

The strains used were *E. coli* K-12 strain 7 (5) and the BF_1-deficient derivatives NR70 (11) and NR76 (12). The isolation of the spontaneous partial revertants NR76A and NR76B has been described previously (12).

Preparation of membrane-bound and soluble BF_1. Membrane vesicles with bound BF_1 were prepared by lysis of intact cells with a French pressure cell as previously described (17). Cytoplasmic fractions refer to the supernatant solutions obtained after centrifugation of the lysed cells at 105,000 × *g* for 1 h. Ethylenediaminetetraacetic acid (EDTA)-treated membranes (stripped membranes) and EDTA extract (crude soluble BF_1) were prepared from washed membrane vesicles as described previously (17).

ATPase assays. Three procedures were utilized for the assay of ATPase activity, as described previously (17). The first assay, performed on white-spot plates, gave a qualitative estimate of the ATPase activity of column fractions. The second assay was used for a rough quantitation of ATPase-containing solutions. The third assay was used for the more accurate determination of activities and kinetic constants. It consisted of the same assay mixture as used in the other two assays with the addition of an ATP-regenerating system. The reaction was linear with both time and protein concentration.

Immunological assays. Antiserum was prepared against a purified preparation of BF_1 from strain 7 as described previously (17). Specificity of the antisera toward the BF_1 was verified by the presence of a single precipitin arc on crossed immunoelectrophoresis of crude soluble BF_1 against the antiserum according to the method of Laurell (8). The localization of the precipitin arc corresponded to the locali-

zation of azide-inhibitable ATP-hydrolyzing activity determined by histochemical staining of the slides for inorganic phosphate (7).

Immunodiffusion and immunoelectrophoresis assays were performed using microscope slides covered with 3 ml of 1% agarose in 0.05 M barbital-hydrochloride buffer, pH 8.6. In the immunoelectrophoresis assay, the proteins were allowed to migrate toward the cathode for 4 h at 100 V. In both assays, diffusion was allowed to proceed for 12 to 16 h at 23°C, followed by two washes with 0.9% NaCl. The slides were stained for 15 min at 23°C in 0.1% naphthol blue black in 7.5% acetic acid, followed by destaining in 7.5% acetic acid. For the localization of cross-reacting material in column fractions, the density of the precipitant arcs was estimated by eye without washing or staining.

Ammonium sulfate fractionation and agarose column chromatography of the BF_1. Solid ammonium sulfate (430 g/liter) was added to crude soluble fractions containing the BF_1 with stirring over a period of 15 min. The suspension was stirred for an additional 15 min, followed by centrifugation at 12,000 × *g* for 10 min. The pellet was resuspended in 1 ml of a buffer consisting of 0.05 M tris(hydroxymethyl)aminomethane - hydrochloride, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and 10% glycerol (vol/vol). The solution was clarified by centrifugation and applied to 1.6-cm-diameter column filled to 90 cm with BioGel A-1.5M. The column was eluted at 12 ml/h with the same buffer. Fractions of 2 ml were collected. All steps of the procedure were performed at 23°C.

Transduction. The generalized transducing bacteriophage 363 was used for transduction experiments (4). The bacteriophage was grown on strain MA96 (*ilvA*, obtained from W. Maas) and used to infect strains unable to utilize succinate for growth. Dilutions were spread onto succinate plates containing isoleucine and valine. Colonies were then scored for the ability to grow in the absence of isoleucine and valine.

Protein assays. The protein content of column fractions was estimated by using a nomogram (2) based on the absorbances at 280 and 260 nm. Other protein determinations were performed according to a modification of the method of Lowry et al. (9).

Chemicals. Neomycin sulfate, rabbit skeletal muscle pyruvate kinase (type II), and naphthol blue black were obtained from Sigma Chemical Co. BioGel A-1.5M was purchased from BioRad Laboratories, special enzyme grade ammonium sulfate from Schwarz/Mann, and dicyclohexylcarbodiimide (DCCD) from Eastman Kodak Co. All other chemicals were reagent grade.

RESULTS

Growth properties of the mutant and its revertants. The mutant NR76, selected for spontaneous resistance to 50 μg of neomycin sulfate per ml, is unable to grow in basal salts medium using succinate, D-lactate, acetate, α-ketoglutarate, malate, or pyruvate as carbon source. By selection for spontaneous growth on succinate agar plates, it was possible to isolate strains derived from NR76 that had regained the ability to utilize those carbon sources. Revertant strains with wild-type characteristics, as well as partial revertants, were obtained. Neither of the two strains chosen for further study completely regained the wild-type character. NR76B showed visible colonies on succinate agar plates after 2 days, whereas NR76A required 3 to 4 days. The parent, strain 7, exhibited visible growth overnight on such plates. The ability of these strains to utilize fermentative and aerobic carbon sources in liquid medium (Table 1) was similar to the results found on agar plates. Strain 7 showed the fastest growth rate on each carbon source, followed closely by NR76B. NR76A grew less well, but better than NR76.

Each strain was examined for sensitivity to neomycin (Table 2). The cultures were streaked onto agar plates supplemented with varying concentrations of neomycin. After 40 h, each strain showed varying amounts of growth on plates with no neomycin, and the size of the colonies was roughly proportional to the generation time on basal salts medium supplemented with glucose. Strain NR70 (11) was used as a control-resistant strain. NR76B showed no growth on plates containing as little as 5 μg of neomycin per ml, and the colonies of strain 7 were greatly reduced in size. On the other hand, no reduction in colony size was noted for the other three strains. Increasing concentrations of neomycin caused reductions in colony

TABLE 1. *Growth of strains 7, NR76, NR76A, and NR76B on various carbon sources*[a]

Strain	Doublings/h		
	100 mM succinate[b]	11 mM glucose	55 mM glycerol
7	0.75	1.67	1.25
NR76B	0.40	1.43	0.67
NR76A	0.00[c]	1.11	0.56
NR76	0.00[d]	0.71	0.42

[a] Overnight cultures of each strain were washed under sterile conditions and inoculated into flasks containing basal salts medium supplemented with 100 mM succinate, 11 mM glucose, or 55 mM glycerol. Growth was estimated by turbidity as measured with a Klett-Summerson colorimeter equipped with a no. 66 filter.

[b] Carbon source.

[c] Growth was detectable after 3 days on succinate agar plates.

[d] No growth was detectable after 7 days on succinate agar plates.

TABLE 2. *Sensitivity to neomycin sulfate*

Strain	Relative growth on minimal-glucose agar				
	0[a,b]	5[a,b]	10[a,b]	25[a,c]	50[a,c]
7	++++	+	−	−	−
NR76B	+++	−	−	−	−
NR76A	++	++	+	±	−
NR76	++	++	++	+	−
NR70	+	+	+	++	+

[a] Neomycin sulfate (micrograms per milliliter).
[b] Scored after 40 h at 37°C.
[c] Scored after 60 h at 37°C.

sizes for those strains as well, in the order NR76A, NR76, and NR70.

Similar results were obtained in liquid cultures. Strains NR76 and NR76A were unaffected by 15 μg of neomycin sulfate per ml in basal salts medium supplemented with 0.2% glucose. Strains 7 and NR76B ceased growth within one doubling after the addition of neomycin. Thus, in these strains an inverse relationship exists between the ability to utilize aerobic carbon sources and resistance to neomycin. The strains that were resistant to high concentrations of neomycin grew poorly or not at all using succinate, whereas the strains that grew well on succinate were sensitive to low concentrations of neomycin. Thus selection for succinate-utilizing strains from a neomycin-resistant strain resulted in the selection of neomycin-sensitive strains.

Genetics of the mutants. A number of mutants defective in aerobic metabolism have been reported to be located at 73.5 min on the *E. coli* chromosome (1, 6, 11, 13, 14, 18, 20). The ability to utilize nonfermentable carbon sources was co-transducible with *ilv* with frequencies of 25 to 30% in strains NR70, NR76, and NR76A. Co-transducibility was not measured with strain NR76B because of its ability to utilize succinate. These results together with those showing a defective BF_1 (see below) and a normal membrane site or BF_0 (12) suggest that the lesion in these strains are in the *uncA* region of the chromosome.

Activity of the membrane-bound and soluble BF_1. Each of the strains was examined for ATPase activity in membranes prepared by lysis with a French pressure cell. The partial revertant NR76B was found to have about 8% of the membrane-bound BF_1 activity of the original wild type, strain 7. The slower-growing partial revertant, NR76A, and the mutant NR76 each showed about 1% of the activity of strain 7.

The diminished membrane-bound Mg^{2+}-ATPase activity found in the mutants and revertants could result from one of a number of possible sources: (i) the enzyme might bind poorly to the membrane and exist predominately in the cytoplasm; (ii) the K_m of the enzyme might be increased; (iii) there might be a decrease in the amount of enzyme; or (iv) the V_{max} might be reduced.

It can be readily seen from the data in Table 3 that the activity is not localized in the cytoplasm of the mutant or revertants, since the cytoplasms contained only small amounts of ATPase activity when compared with the total amount of activity found in the wild type.

The BF_1 could be solubilized by treatment of the membrane with a buffer of low ionic strength containing EDTA (12). Approximately 50% of the activity of the wild type was found in the supernatant solution after centrifugation, with about 20% remaining on the stripped membranes (Table 3). In other experiments, up to 70% of the activity was solubilized, with as little as 5% remaining on the stripped membranes. On the other hand, only about 10 to 15% of the activity of the mutant and the two revertants could be found in the supernatant solution (Table 3), with negligible activity remaining on the stripped membranes (data not shown). Thus, BF_1 from each of the three strains lost 80 to 90% of its activity when removed from the membrane.

It was not possible to determine accurately the kinetic parameters of the BF_1 of NR76 and NR76A because both the membrane-bound and soluble fractions contain a large amount of ATP-hydrolyzing activity not due to the BF_1, as demonstrated by the lack of inhibition by the specific antiserum or by azide.

To eliminate the possibility that there were different amounts of BF_1 protein in the mutant or revertants, those amounts were quantitated. Varying amounts of membranes from strains 7, NR76, and NR76A were incubated with a constant amount of an antiserum that had been prepared against the purified BF_1 of strain 7. After centrifugation, the supernatant solutions were assayed for the ability to inhibit Mg^{2+}-ATPase activity in membranes of strain 7 (Fig. 1). The results indicate that there are roughly equal quantities of cross-reacting material on the membrane of the three strains, suggesting that there is an intrinsic difference in the proteins themselves. In support of this possibility, it was found that the cross-reacting material from NR76 and NR76A exhibited a reduced electrophoretic mobility in immunoelectrophoresis assays. This observation will be discussed in greater detail in a later section.

Thus, it appears that the most likely explanation for the reduced Mg^{2+}-ATP activities is

TABLE 3. *Mg^{2+}-ATPase activities of cytoplasmic, membrane, and crude soluble BF_1 fractions*

Strain	Enzyme source	ATP-hydrolyzing activity[a]	Mg^{2+}-ATPase activity[b]	% of wild-type membrane bound[c]	% of membrane bound[d]
7	Cytoplasm	1260	320	21	21
	Membranes	3130	1510	100	100
	Soluble BF_1	970	970	52	52
NR76B	Cytoplasm	960	30	2	24
	Membranes	168	126	8	100
	Soluble BF_1	13	11	1	9
NR76A	Cytoplasm	910	10	0.7	59
	Membranes	60	17	1	100
	Soluble BF_1	6	2.4	0	14
NR76	Cytoplasm	490	0	0	0
	Membranes	8	12	1	100
	Soluble BF_1	4	1.8	0	15

[a] Values are given as nanomoles of inorganic phosphate per minute per milligram of membrane protein. Since the volume of the crude soluble BF_1 and cytoplasmic fractions were the same as the membranes, the values were normalized by calculating specific activities using the protein values of the membranes. Since the protein concentrations of the vesicle preparations of different strains varied, this treatment allows comparisons to be made between the various strains.

[b] Mg^{2+}-ATPase activity was determined by assaying the membranes and cytoplasms in the presence of BF_1 antiserum and the crude soluble BF_1 in the presence of 10 mM NaN_3. The amount of ATP-hydrolyzing activity inhibitable by NaN_3 or antiserum represents Mg^{2+}-ATPase activity. Values are given as nanomoles of inorganic phosphate per minute per milligram of membrane protein. Normalization was as described in *a*.

[c] Calculated as [(activity of fraction)/(activity of strain 7 membranes)] × 100.

[d] Calculated as [(activity of fraction)/(activity of membranes)] × 100.

an alteration in the protein resulting in a decreased V_{max}, although an effect on the K_m cannot be discounted.

Binding of the soluble BF_1 to stripped membranes. As shown above, the BF_1 of the mutant and the two partial revertants lost most of its activity upon solubilization. When the solubilized BF_1 from NR76B was added to stripped membranes from NR76B in the presence of Mg^{2+}, it bound to the membrane and exhibited an activity near that of the original NR76B membrane-bound BF_1 (Table 4). Since it is possible that only a portion of the protein was removed by solubilization, several variations of the reconstitution experiment were performed. It was found that BF_1 from strain 7 bound normally to the membrane of NR76B, and that the BF_1 from NR76B bound to stripped membranes of strain 7 with a reversal of the inactivation. The same experiments were performed using stripped membranes from strain NR70 (Table 4). Membranes from NR70 were used because of the lack of any residual Mg^{2+}-ATPase activity. These experiments suggest that it is unlikely that a subunit of the BF_1 of NR76B remains on the stripped membranes since the soluble fraction from that strain is able to bind to stripped membranes from strains 7 or NR70 with a restoration of activity. Moreover, there is no impediment to the binding of the BF_1 from strain 7 to stripped membranes of strain NR76B. It also appears that the defect in strain NR76B resides in the soluble BF_1 and not in BF_0.

Effect of ethanol and DCCD on the membrane-bound BF_1. Another property of the membrane-bound BF_1 of *E. coli* is its sensitivity to DCCD (10). It was of interest to determine whether the sensitivity to DCCD differed among the various strains. Since DCCD is only sparingly soluble in aqueous solutions, it is usually added in ethanol. Ethanol itself was found to have a stimulatory effect on the BF_1 at concentrations below 0.06 M (0.01 ml of ethanol in an assay mixture of 0.3 ml) and an inhibitory effect at higher concentrations. To prevent any complicating effects of ethanol on interpretation of the results with DCCD, all assay mixtures in which DCCD was used contained a constant amount of ethanol (0.01 ml per assay).

The activity of the membrane-bound BF_1 from strain 7 was found to have a different sensitivity to DCCD than that from strain NR76B. At neutral pH the inhibition caused by DCCD became constant at concentrations of DCCD greater than 50 μM (Fig. 2A and B). However, treatment and assay at basic pH resulted in a reversal of DCCD inhibition at con-

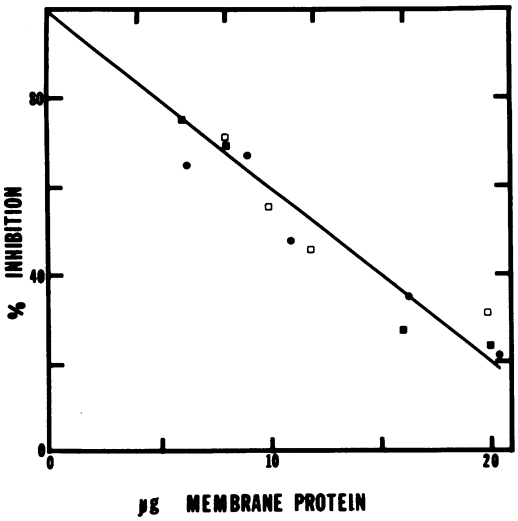

FIG. 1. *Estimation of the relative concentrations of BF_1 on the membranes of strains 7, NR76, and NR76A. Various amounts of control membranes, as indicated, from strains 7 (●), NR76 (■), and NR76A (□) were mixed with 5 μl of anti-BF_1 serum, adjusted to a total volume of 0.1 ml with TKMG buffer, and incubated for 15 min at 23°C. The suspensions were centrifuged at 105,000 × g for 1 h at 4°C. The supernatant solutions were carefully removed, and 10 μl of control membranes from strain 7 (3.5 U/ml) was added to each supernatant solution. After 15 min at 23°C, duplicate 30-μl aliquots were removed and assayed for ATPase activity by assay three, as described in the text.*

centrations greater than 50 μM in the case of strain 7 (Fig. 2A), whereas no such reversal was found for the membrane-bound BF_1 of strain NR76B (Fig. 2B). When the solubilized BF_1 from strains 7 and NR76B, respectively, were bound to the membrane of strain NR70, identical results were obtained, showing that the difference in DCCD sensitivity resides in the BF_1 and not in the BF_0. It is clear, then, that there are significant differences between the BF_1 proteins of these strains. The significance of the effect of DCCD is not clear, especially since the reversal of the inhibition by high concentrations of DCCD has not been observed by other investigators. It does not appear that DCCD at high concentrations solubilizes the protein, since centrifugation of the DCCD-treated membranes removes all ATPase activity from the supernatant. A loosening of the bond between the BF_1 of the wild type and the membrane cannot be ruled out.

Immunological characterization of the soluble BF_1 from strains 7, NR76, NR76A, and NR76B. On immunoelectrophoresis, soluble BF_1 from the mutant NR76 and the revertant NR76A invariably yielded a precipitin arc that corresponded to an antigen with reduced electrophoretic mobility when compared with strain 7 (Fig. 3). On the other hand, the precipitin arc observed for the partial revertant NR76B corresponded to a protein with an electrophoretic mobility equal to that of the wild type. When immunoelectrophoresis slides were stained for ATPase activity, all four strains yielded a band of activity that corresponded to the location of the precipitin arc of the wild type, although the activity bands for the three mutants were much fainter than that of strain 7. Moreover, no activity was observed at a position corresponding to the more slowly migrating antigen. These results suggest that the BF_1 proteins of NR76 and NR76A dissociate rapidly upon removal from the membrane into a form retaining immunological but not catalytic ac-

TABLE 4. *Effect of solubilization and reconstitution[a] on Mg^{2+}-ATPase activity of strains 7 and NR76B*

Membrane	Conditions	Source of added soluble BF_1	ATPase activity[b]
Strain 7	Control	–	3.16
Strain 7	Stripped	–	0.14
–	–	Strain 7	2.32
Strain 7	Stripped	Strain 7	2.04
NR76B	Stripped	Strain 7	2.07
NR76B	Control	–	0.231
NR76B	Stripped	–	0.014
–	–	NR76B	0.059
NR76B	Stripped	NR76B	0.185
Strain 7	Stripped	NR76B	0.295
Strain 7	Control	–	2.93
–	–	Strain 7	2.94
NR70	Stripped	Strain 7	2.26
NR76B	Control	–	0.196
–	–	NR76B	0.018
NR70	Stripped	NR76B	0.175

[a] Stripped membranes and soluble fractions were mixed in a volume ratio of 1:3, and $MgCl_2$ was added to 10 mM. The suspensions were centrifuged for 1 h at 105,000 × *g* at 4°C, washed once with buffer, and resuspended to 3 to 5 mg of protein per ml.

[b] Activity is expressed as nanomoles of inorganic phosphate per minute per milligram of membrane protein, using the protein value of the appropriate control membrane suspension. Since the stripping and reconstitution procedures cause significant alterations in the protein content of the vesicles, this normalization treatment allows for comparison of the various membrane preparations with each other. By normalizing the soluble BF_1 solutions in the same way, it is possible to compare the amount of soluble BF_1 added to the amount bound by stripped membranes.

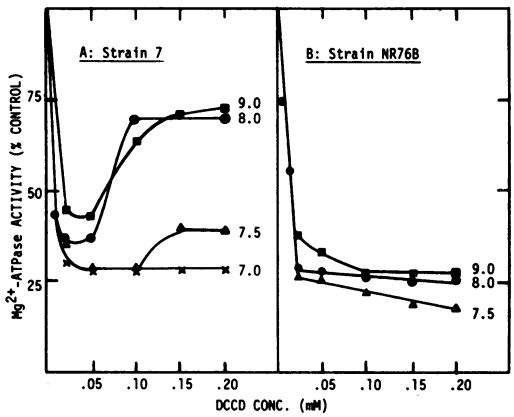

FIG. 2. *Effect of DCCD on Mg^{2+}-ATPase activity in strains 7 and NR76B. Membranes were diluted into assay buffer adjusted to various pH values and containing an ATP-regenerating system. Incubation was carried out for 15 min in the presence of varying concentrations of DCCD, as indicated, before initiation of the reaction by addition of ATP. Assays were performed at pH 7.0 (×), 7.5 (▲), 8.0 (●), and 9.0 (■).*

tivity. However, a small amount of the active protein remained, although there was too little to be seen as a precipitin arc.

Since it was possible that the BF_1 proteins of strains NR76 and NR76A dissociated during the process of preparation, two variations of the immunoelectrophoresis were performed. In the first instance, membranes of the four strains were resuspended in the stripping buffer and immediately were subjected to immunoelectrophoresis without first centrifuging to remove the stripped membranes. In the second instance, membranes of the four strains were solubilized by incubation with 10% Triton X-100 for 15 min at 37°C, and the resulting solution was subjected to immunoelectrophoresis. In both cases, the precipitin arcs were at the same location as found for immunoelectrophoresis of the soluble BF_1 described above.

The results of immunodiffusion of the soluble BF_1 of the four strains against the BF_1 antiserum are shown in Fig. 4. Immunodiffusions of freshly prepared soluble BF_1 from NR76 and NR76A displayed precipitin arcs that showed partial identity with that of the wild-type, whereas NR76B displayed two precipitin arcs, one showed identity with that of the wild type, and the other showed identity with the arcs of the other strains (Fig. 4A). Immunodiffusion of the soluble BF_1 proteins 48 and 96 h after preparation, respectively, showed that the arc of NR76B that showed immunological identity with that of the wild type became fainter and disappeared by 96 h, whereas the arc with immunological identity with the other mutants became correspondingly stronger (Fig. 4B and 4C).

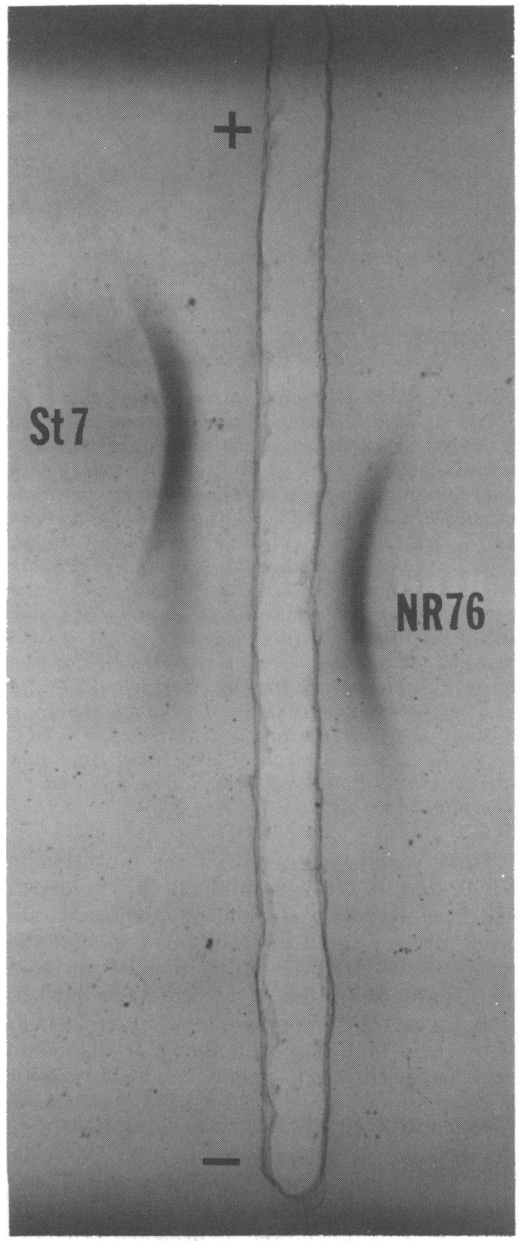

FIG. 3. *Immunoelectrophoresis of crude soluble BF_1 from strains 7 and NR76. Approximately 5 μg of crude BF_1 from the two strains was subjected to electrophoresis as described in the text, after which 50 μl of anti-BF_1 serum was added to the central trough. Diffusion was allowed to proceed overnight. The slide was then incubated with several changes of 0.9% NaCl to remove unreacted protein, dried, and stained with naphthol blue black.*

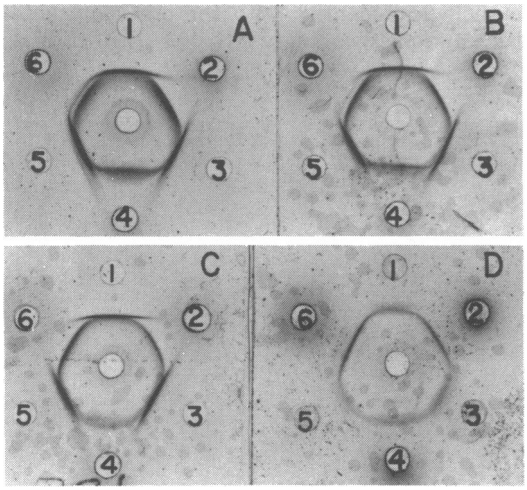

FIG. 4. *Time-dependent dissociation of soluble BF_1. Immunodiffusion was performed as described in the text. After diffusion, the slides were treated as given in the legend to Fig. 3. The center well in each slide contained 5 μl of anti-BF_1 serum. In each slide wells 2, 4, and 6 contained 25 μg of BF_1 from strain NR76, NR76A, and NR76B, respectively, while wells 1, 3, and 5 contained 25 μg of BF_1 from strain 7. (A) BF_1 fractions were used immediately after extraction from the membrane. (B) Immunodiffusion was performed 48 h after isolation of the protein fractions. The proteins were stored at 23°C for that period. (C) The protein fractions were left at 23°C for 96 h before immunodiffusion. (D) Soluble BF_1 from strains NR76, NR76A, and NR76B were left at 23°C for 96 h. BF_1 from strain 7 was frozen in liquid nitrogen immediately after isolation, followed by slow thawing at 23°C.*

These results suggest that the mutation in NR76 and NR76A results in BF_1 proteins which, when removed from the membrane, are prone to very rapid dissociation, and that the reversion in NR76B results in a BF_1 protein more stable than that of the other two but less stable than that of the wild type. Since the BF_1 of the wild type has been shown to dissociate upon freeze-thaw treatment, it was of interest to determine whether the apparent dissociation of NR76, NR76A, and NR76B corresponded to the dissociation of the freeze-thawed wild-type BF_1. When the soluble BF_1 of strain 7 was subjected to freeze-thaw treatment, the resulting precipitin arc showed identity with those of NR76 and NR76A and with the arc of NR76B after 96 h (Fig. 4D). These results are consistent with the hypothesis that the mutation in NR76 and NR76A results in a BF_1 protein that dissociates very readily when removed from the membrane and is indistinguishable from the dissociated BF_1 of the wild type produced by freeze-thaw by the criterion of immunological cross-reactivity. The reversion in the case of NR76B results in a BF_1 that is more stable than that of strains NR76 and NR76A, yet less stable than that of the wild type.

Partial purification of the BF_1 from strains 7, NR76, NR76A, and NR76B. An attempt was made to purify the BF_1 of each of the four strains in order to characterize the differences in these proteins. The crude soluble BF_1 from 8 g of each strain was precipitated by the addition of solid ammonium sulfate to a final 65% of saturation. The pellets were dissolved and applied to BioGel A-1.5M columns, as described under Materials and Methods. The fractions were assayed for protein, ATPase activity, and precipitation by the BF_1 antiserum (Fig. 5). The elution profile for the wild-type enzyme showed a sharp peak of antibody-precipitable material that coincided with the ATPase activity at a position corresponding to a molecular weight of approximately 350,000. No enzymatic activity was found in the NR76 and NR76A column fractions, but a peak of antibody-precipitable material was found at a position corresponding to a lower molecular weight. The eluate from the agarose column containing the NR76B soluble fraction contained two peaks of antibody-precipitable material. The first peak coincided with the ATPase activity and appeared at the same position as that of the wild-type enzyme. The smaller second peak was devoid of enzymatic activity and appeared at the same position as those of NR76 and NR76A.

Immunodiffusion assays of the antibody-precipitable material from each column (Fig. 6) yielded a single arc for the peak fractions of the NR76 and NR76A material. Both peaks from gel filtration of the NR76B material gave lines of identity with each other and with the arcs of the NR76 and NR76A fractions. The peak tube from the separation of the strain 7 BF_1 yielded two arcs, a heavy outside line and a fainter inner arc. The latter showed a line of identity with the arcs from the other three strains. Immunoelectrophoresis of the material from each peak of antibody-precipitable material revealed that the antigens from NR76 and NR76A and both antigens from NR76B migrated at a slower rate than the major antigen from strain 7. The immunoelectrophoretic patterns obtained were identical with those shown in Fig. 3 for the crude soluble BF_1 except that a second fainter arc in the material from the wild type appeared at the same mobility as the antigen from the mutants.

Thus it appears that the BF_1 protein dissociates upon solubilization from the membrane. The dissociation is relatively slow in the case of the wild-type protein, more rapid in the case of

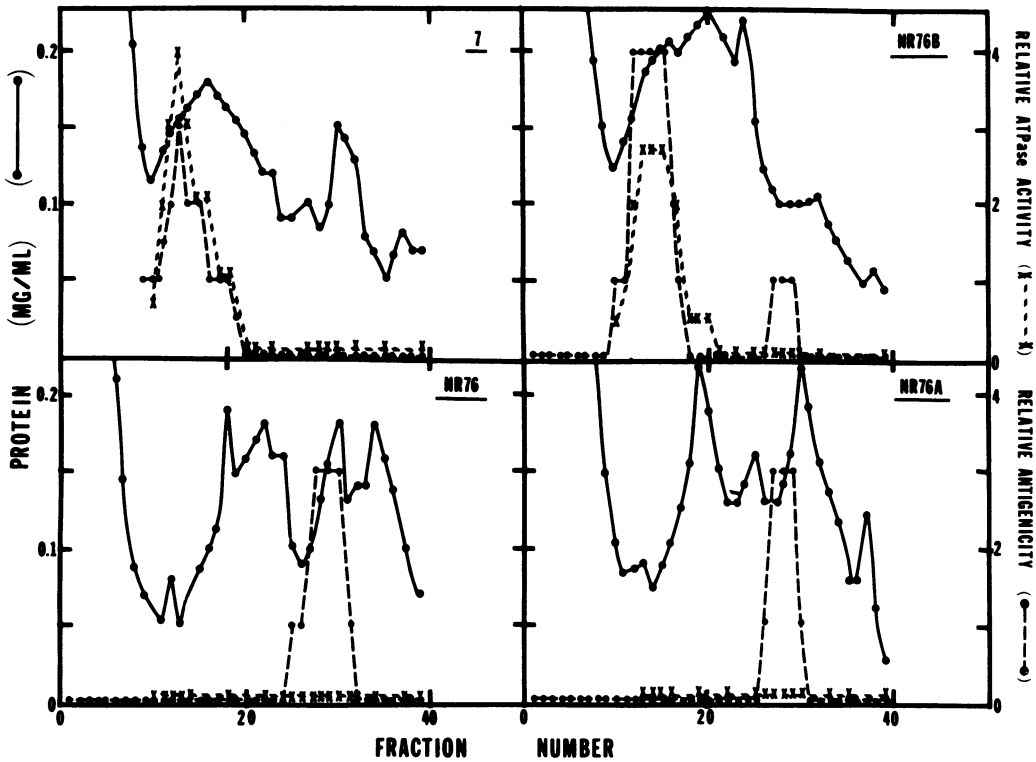

FIG. 5. *Agarose chromatography of the soluble fractions from strains 7, NR76, NR76A, and NR76B. Agarose chromatography was performed on the ammonium sulfate-treated soluble fractions from each strain as described in the text. The fractions were assayed for protein, relative ATPase activity, and relative antigenicity as described in the text.*

the partial revertant NR76B, and even more rapid in the case of the protein from strains NR76 and NR76A. The lack of activity on activity stains of immunoelectrophoresis slides of the mutants and the lack of activity of the smaller-molecular-weight peaks from BioGel chromatography suggest that the dissociation leads to a loss of catalytic activity. Thus the inactivation of the BF_1 proteins of the mutants upon solubilization from the membrane is most likely a result of the dissociation of the proteins. This dissociation may be reversible, as indicated by the reactivation of the BF_1 of strain NR76B upon rebinding to the membrane.

DISCUSSION

Two selection procedures have been reported for the isolation of mutants of *E. coli* with defects in aerobic metabolism. One is a direct selection for strains that cannot utilize for growth substrates of the electron transport chain (1, 13, 15). The second method involves the selection of antibiotic-resistant strains (6, 11, 12, 14, 18). Why the latter method yields mutants with defects in energy metabolism is unclear. Although not all strains have been examined in detail, it appears that strains selected for antibiotic resistance show a decreased ability to accumulate small molecules (11, 14, 19, 20). This suggests that *E. coli* might become resistant to neomycin or streptomycin due to a loss of ability to concentrate these antibiotics.

Since many mutants with defects in aerobic metabolism contain a defective BF_1 (1, 6, 11, 13, 15, 18, 20), it was of interest to examine that protein in several strains with varying degrees of neomycin sensitivity and Mg^{2+}-ATPase activity. Our results show that the two are not directly related. Strain NR76 exhibits about 1% of the wild-type Mg^{2+}-ATPase activity and is resistant to high concentrations of neomycin. Yet strain NR76B, which has only about 8% of the wild-type Mg^{2+}-ATPase activity, is as sensitive to neomycin as is the wild type. Although strain NR76B was selected from strain NR76 by ability to grow on succinate agar, it is still defective compared to strain 7. Thus, a defect in the BF_1 may result in inability to grow on succinate, malate, and acetate but not in neo-

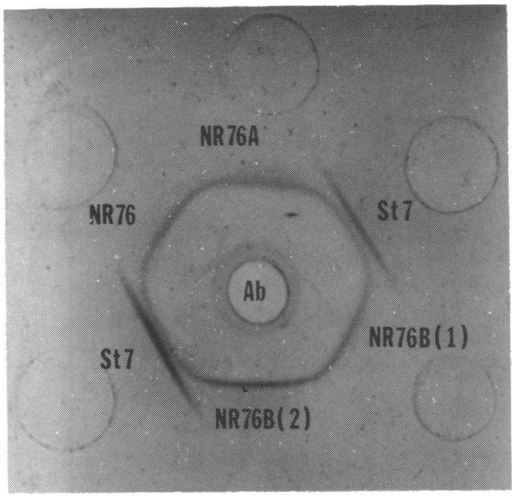

FIG. 6. *Immunodiffusion assays of the antigenic material derived from strains, 7, NR76, NR76A, and NR76B. Immunodiffusion assays were performed as described in the text. The center well contained 5 μl of anti-BF_1 serum. The outer wells contained 5 μl from the tubes of the columns that were identified as containing antigenic material, as described in the legend to Fig. 3. NR76B(1) and NR76B(2) refer to the high- and low-molecular-weight peaks, respecitvely.*

mycin resistance. Of interest is the fact that the ability of these strains to actively transport nutrients occurs in the following order: strain 7 > NR76B > NR76A > NR76 > NR70 (12). The same order is found for the impermeability of the membrane to protons (12), and, as shown above, sensitivity to neomycin. Again, these facts suggest that resistance to neomycin is a result of increased proton permeability, which causes dissipation of the electrochemical gradient of protons, leading to decreased ability to accumulate solutes including neomycin.

The biochemical nature of the mutation leading to the NR76 phenotype and the nature of the subsequent reversions leading to the NR76A and NR76B phenotypes must still be determined. One of the original goals of this investigation was the purification of the BF_1 from the strains described in this report. Of interest is the identity of the altered subunit(s). The apparent dissociation of of the protein from strains NR76, NR76A, and NR76B has rendered this objective more difficult.

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