Energy Transduction in Escherichia coli

GENETIC ALTERATION OF A MEMBRANE POLYPEPTIDE OF THE (CA\(^{++}\),MG\(^{++}\)) \textsuperscript{ATPase} COMPLEX

(Received for publication, July 2, 1975)

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Recent genetic analyses of the membrane components involved in energy transduction in Escherichia coli have concentrated on the (Ca\(^{++}\),Mg\(^{++}\)) \textsuperscript{ATPase} complex (EC 3.6.1.3). Many mutants have been described with altered biochemical properties and defects in energy-requiring processes such as oxidative phosphorylation, transhydrogenase activity, and active transport of several solutes.

This report describes the isolation of a mutant strain of E. coli that is defective in several energy-requiring processes. The strain BG-31 was obtained by "localized mutagenesis" using phage P1c1. The mutation maps at approximately 73.5 min on the E. coli chromosome. Reversion and suppression analyses indicate that the defect is the result of a single amber mutation. This strain is unable to utilize succinate, d-lactate, or malate for growth. Mutant cells are unable to couple the energy derived from the hydrolysis of ATP to the active transport of proline, although coupling of energy derived from electron transport to solute transport appears normal when examined in both cells and isolated membrane vesicles. Isolated membranes of the mutant are unable to couple the energy derived from the hydrolysis of ATP to transhydrogenase activity while they can utilize the energy generated from electron transport to drive transhydrogenase activity. Extracts of strain BG-31 have normal levels of (Ca\(^{++}\),Mg\(^{++}\)) \textsuperscript{ATPase} activity. The \textsuperscript{ATPase} portion of the complex, bacterial F\(_{1}\) (BF\(_{1}\)), is poorly attached to the membrane portion of the complex. In vitro reconstitution of transhydrogenase activity with stripped membrane fractions and crude preparations of BF\(_{1}\) localize the defect in strain BG-31 to the membrane portion of the complex.

Analysis of membranes of the strain BG-31 by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate demonstrate the absence of a single polypeptide of molecular weight about 54,000 and the appearance of a new polypeptide of lower molecular weight, about 25,000. Analysis of a spontaneous revertant of BG-31 shows complete restoration of the parental phenotype including the gel patterns.

The characterization of this mutant provides the first demonstration of the consequences of a structural gene mutation on a polypeptide in the membrane portion of the complex and represents the initial stages in what we hope will be the biochemical definition and functional characterization of this important energy-transducing system.
The E. coli complex has an $F_1$ component (BF$_5$) that is quite similar to that previously described for mitochondria and chloroplasts. It is easily removed from the membrane by washing with buffer of low ionic strength in the presence of EDTA (stripping procedure). It has five distinct polypeptides as reported initially by Bragg and Hou (5), and the molecular weights of the peptides appear to be approximately $\alpha$-56,000, $\beta$-52,000, $\gamma$-30,000, 5-20,000, and $\epsilon$-10,000. The ATPase activity of the complex is activated by Mg$^{2+}$, which also plays a role in attaching the $F_1$ to the membrane portion of the complex. The ATPase activity of the complex is inhibited by dicyclohexylcarbodiimide and Dio9, but the ATPase activity of the soluble BF$_5$ is not sensitive (6). Therefore a DCCD$^-$ sensitivity-conferring protein has been implicated as a component of the membrane portion of the complex. Removal (stripping) of BF$_5$ from the membrane results in energetic uncoupling that may be due to a proton leak through the membrane portion of the complex, the proton channel (7, 8).

In E. coli this uncoupled state can be recoupled by treatment with DCCD or by reconstitution with BF$_5$ (9-13).

A host of mutants have now been isolated, particularly in E. coli, that have alterations in the ATPase complex. The first of such mutants was reported by Butlin et al. (14); these have been designated unc for uncoupled. This group observed that such mutants were incapable of coupling electron transport to ATP synthesis (oxidative phosphorylation). This original mutant, AN120, was also lacking ATPase activity. This same group later described another class of unc mutants that had normal ATPase activity (15).

Subsequently, a number of mutants both with and without ATPase activity, have been isolated by other workers (12, 16-21). The general phenotype of all unc mutants reported until now can be described as follows: (a) unc mutants are able to utilize fermentable carbon sources such as glucose or glycerol for growth but are unable to utilize carbon sources that yield energy primarily as a result of ATP synthesis via oxidative phosphorylation such as succinate, malate, lactate, etc.; (b) the mutants give low aerobic growth yields when grown on limiting amounts of glucose, or more simply the amount of cell mass produced per amount of glucose utilized is low; (c) unc mutants have normal respiratory activity; (d) the mutants have little or no detectable oxidative phosphorylation; (e) ATPase activity can be present or missing; (f) where determined all unc mutations so far described in E. coli are located at about 73.5 min on the chromosome and are 20% to 50% co-transducible with the $iIu-C$ locus and about 50% co-transducible with the $asn$ locus (14, 15, 18, 20, 21).

This report described the physiological properties of the strain that has a mutation in a structural gene coding for a polypeptide of the membrane portion of the ATPase complex.

**EXPERIMENTAL PROCEDURE**

**Materials**—The following materials were obtained from the indicated sources: Sepharose 4B from Pharmacia; $^{14}$C cholrine from New England Nuclear; ATP, NAD, NADP, and alcohol dehydrogenase (yeast) from Sigma; DNase from Worthington. All other materials were obtained from commercial sources and were the highest purity available.

$^1$ The abbreviation used is: DCCD, dicyclohexylcarbodiimide.
in strain BG-31 was transferred by transduction into a fresh isolate of 1100 ilo− by using P1c1 grown on strain BG-31 as donor and 1100 ilo− as recipient. The selection and screening are the same as in the initial isolation. In this second transduction an ilo− sucinate− transductant was taken as the parental strain and an ilo− sucinate− strain was taken as BG-31. Both were purified by repeated single colony isolation. A revertant of BG-31 was then selected by spreading about 10⁶ cells on a plate containing succinate as carbon source followed by incubation for 4 days at 37°. Several colonies were picked and purified by repeated single colony isolation, and one was retained for further study. Strain BG-31 was tested for the presence of an amber mutation by spreading 10⁶ cells on a plate containing succinate as carbon source, and the plate was incubated at 37° for 3 days. The region of growth was scored as a positive test for an amber mutation. The suppressed strain was purified from the growth region by repeated single colony isolation.

The extensive genetic manipulation described above assure that the strains we are comparing are isogenic except for the region of interest. For the sake of convenience, we will refer to these strains as 1100, the parental strain; BG-31, the mutant strain; BG-31-R, the revertant strain; and BG-31-S, the suppressed strain.

Preparation and Fractionation of Crude Extracts—The methods for isolation of the soluble (Ca²⁺,Mg²⁺)-ATPase (BF₃) and membrane fractions are essentially a composite of a variety of procedures previously reported by others (20-28), but since the subsequent composition and activity of the purified BF₃ appears to be dependent upon the strain and isolation procedure it will be described. Approximately 10⁶ frozen cells of each strain tested were suspended in 50 ml of medium containing Tris-Cl buffer, pH 7.5, containing 0.01 M MgCl₂ and disrupted by sonic oscillation using the large probe of a heat system sonifier at a setting of 40. Sonication was carried out in two 2-min periods with cooling in an ice bath. Unbroken cells were removed by centrifugation at 5,000 × g for 15 min. The supernatant solution was then subjected to centrifugation at 150,000 × g for 90 min, after which the supernatant was discarded, and the membrane pellet was resuspended in 40 ml of the same buffer used for preparing the extract. This fraction is referred to in subsequent procedures as the membrane fraction. The membranes were again isolated by centrifugation at 150,000 × g for 90 min. If a washed membrane fraction was to be tested, the pellet was resuspended again in the lysis buffer, and this fraction is referred to hereafter as washed membranes. Otherwise, the membrane pellet was resuspended in 40 ml of stripping buffer which consists of 1 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM EDTA and 10% glycerol. All steps prior to this were conducted at 0-4°C, and all steps subsequent to this were conducted at room temperature. The membrane suspension was allowed to incubate for 50 min and then subjected to centrifugation at 150,000 × g for 90 min. The supernatant solution usually containing about 80% of the ATPase activity (B₃₇) was saved as the EDTA extract, and the membranes were retained as stripped membranes. All membrane fractions were used the day they were prepared.

ATP was added to the crude strip fluid to a final concentration of 1 mM, and solid ammonium sulfate was added to a final concentration of 0.3 to 0.4 mg of protein was incubated for 15 min at 23°C, and the precipitate was collected by centrifugation at 150,000 × g for 90 min. The precipitate was dissolved in 100 ml of the stripping buffer, and the supernatant solution was subjected to centrifugation at 150,000 × g for 90 min, after which the supernatant was discarded, and the membrane pellet was resuspended in 40 ml of the same buffer used for preparing the extract. This fraction is referred to in subsequent procedures as the membrane fraction. The membranes were again isolated by centrifugation at 150,000 × g for 90 min. If a washed membrane fraction was to be tested, the pellet was resuspended again in the lysis buffer, and this fraction is referred to hereafter as washed membranes. Otherwise, the membrane pellet was resuspended in 40 ml of stripping buffer which consists of 1 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM EDTA and 10% glycerol. All steps prior to this were conducted at 0-4°C, and all steps subsequent to this were conducted at room temperature. The membrane suspension was allowed to incubate for 50 min and then subjected to centrifugation at 150,000 × g for 90 min. The supernatant solution usually containing about 80% of the ATPase activity (B₃₇) was saved as the EDTA extract, and the membranes were retained as stripped membranes. All membrane fractions were used the day they were prepared.

Transport Experiments—Cells were grown, harvested, and washed as described above. Transport systems containing 5 μl of minimal salts solution, 0.2% glucose, 1 mM KCN (when present), and cells (0.3 to 0.4 mg of protein) were incubated for 15 min at 23°C, and the reaction was stopped by the addition of 1 ml of 5% (v/v) trichloroacetic acid (TCA) prepared in isotonic saline. Aliquots of the incubation mixture were removed at the indicated times and filtered under vacuum at 0.45 μm filters (Millipore). Each filter was washed once with 1 ml of minimal salts solution at 23°C. Filtration and washing was completed in 5 to 6 s. The filters were dried under a heat lamp and the radioactivity obtained from zero time controls are subtracted for each cell type tested.

Membrane vesicles for transport studies were prepared, and transport assays were conducted as previously described (29). [¹⁴C]Proline (233 Ci/mol) was used at a final concentration of 5 μM and d-lactate at a final concentration of 10 mM.

Polyacrylamide Gel Electrophoresis—Denatured samples of membranes from each strain were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using the discontinuous buffer system of Laemmli (30), a slab gel apparatus similar to that described by Studier (31) and according to the procedure of Ames (32). Samples were prepared by incubation of the membranes in a solution that contained 1% sodium dodecyl sulfate, 2 mM urea, and 1% mercaptoethanol and a pinch of bromphenol blue. Samples containing 2 mg/ml of protein were incubated at 100°C for 2 min. Twenty microliters were applied to each slot of the gel and run at a current of 20 mA for about 3 hours. Protein was detected by staining with Coomassie blue according to the procedure of Fairbanks et al. (33).

RESULTS AND DISCUSSION

Properties of the Mutant BG-31

Growth Properties—The growth properties of BG-31 are essentially those reported for other unc type mutants (12, 16-21). It fails to grow on carbon sources that require the oxidative synthesis of ATP for energy production such as succinate, d-lactate, and malate. It does grow reasonably well on fermentable carbon sources such as glucose, glycerol, galactose, lactose, maltose, and pyruvate. The mutant retains its sensitivity to the antibiotic Neomycin in contrast to unc mutants that have been selected as Neomycin-resistant. It is interesting that in our experience only those unc mutants selected on Neomycin are resistant to the antibiotic suggesting that this selection procedure may bias the type of mutants obtained.

It was largely for this reason we have employed the phage mutagenesis procedure which avoids any selection pressure. The revertant strain BG-31-R has essentially the same growth properties as the parental strain.

Map Location—The isolation procedure for BG-31 ensures that the defect occurs in the unc region of the chromosome. In separate experiments we have determined that the lesion in this strain is approximately 20% co-transducible with the ilo-C locus. The defect is the result of a single point mutation as indicated by a reversion frequency of about one in 10⁷. Twelve revertants were screened for their ability to grow on various carbon sources, and all appeared indistinguishable from the parental strain.

Since we hoped to be able to demonstrate an altered polypeptide by a technique that discriminates on the basis of size, we screened each mutant isolated for the presence of nonsense or chain-terminating mutations. This was done by treating each strain with phage ϕ80 that carried the amber suppressor su-3. Strain BG-31 was one of several strains tested that gave a positive test when treated with this phage.

(Ca²⁺,Mg²⁺)-ATPase Activity—The ATPase activity of strain BG-31 was examined in extracts at various steps in the fractionation procedure. The results are presented in Table I. Compared to the parental strain and the revertant, BG-31 has essentially normal amounts of ATPase activity if the crude sonic extract is examined. However, as the membranes of BG-31 are isolated and washed, only about 40% of the initial activity is recovered in the washed membrane fraction. This is in marked contrast to a recovery of over 80% of the activity in washed membranes of the parental and revertant strains. The loss of activity from the membrane fraction of strain BG-31 is dependent on the volume of buffer used in the sonication and wash steps and can be varied from 10% with small volumes to as high as 80% with larger volumes. From this initial
observation, it seems clear that the attachment of BF₁ to the membrane is altered in this strain. The revertant strain has recovered the binding capacity and appears similar to the parental strain.

Sensitivity of ATPase Activity to Dicyclohexylcarbodiimide—Dicyclohexylcarbodiimide is a relatively specific inhibitor of the ATPase activity in a variety of systems and is effective only when the F₁ portion is associated with the membrane portion of the complex (6). There is also evidence, particularly in mitochondrial systems, that DCCD reacts specifically with one polypeptide in the membrane portion of the complex (34, 35). The ATPase activity of strain BG-31 is insensitive to inhibition by DCCD over a wide concentration range (see Fig. 1). One possible explanation of this insensitivity is that the strain is defective in the DCCD reactive protein. A more likely possibility would be that the poor attachment of BF₁ to the membrane renders it insensitive to the effects of DCCD. A final resolution of these alternatives must await direct DCCD binding experiments.

Transhydrogenase Activity—The functional state of the isolated membranes can be conveniently tested by measuring energy-dependent transhydrogenase activity. Energy can be provided for this reaction by both electron transport and hydrolysis of ATP by the ATPase. These two types of transhydrogenase activity are measured in BG-31, and the results are presented in Table II. This strain has nearly normal levels of the electron transport-dependent activity but is totally lacking the ATP-driven activity. This result is similar to that reported for a number of similar mutants (13, 20). The revertant strain has complete restoration of the ATP-driven activity. Thus it appears that even though this strain is capable of normal rates of ATP hydrolysis it cannot couple the resultant energy to this energy-dependent process.

It is also interesting to note that these strains show neither a decrease in respiratory-driven transhydrogenase as a consequence of removal of BF₁ nor a stimulation of this activity by treatment with dicyclohexylcarbodiimide. This is in contrast with results obtained with another strain (13). This inconsistency may be due to a strain difference and/or a difference in the procedures used to remove BF₁. This question is of considerable importance since removal or abnormal attachment of BF₁ to the membranes has been shown to increase proton permeability and result in reduced energy coupling (9, 13). We are currently attempting to resolve this difference.

The transhydrogenase system allows us to perform in vitro complementation tests to attempt to localize the defect in this strain to the membrane portion of the BF₁ portion of the complex. This can be done because it is possible to remove the BF₁ portion from the membrane by the stripping procedure.

### Table I

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fraction</th>
<th>ATPase activity (units)</th>
<th>Protein (milligrams)</th>
<th>Specific activities</th>
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<td>536</td>
<td>0.70</td>
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<td>Membranes</td>
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<td>188</td>
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</tr>
<tr>
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<td>Washed membranes</td>
<td>300</td>
<td>1/4</td>
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<td>0.67</td>
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<td>214</td>
<td>1.30</td>
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<tr>
<td>BG-31 (mutant)</td>
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<td>192</td>
<td>0.80</td>
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<tr>
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<td>490</td>
<td>216</td>
<td>2.10</td>
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FIG. 1 (left). The effect of dicyclohexylcarbodiimide on the ATPase activity of membranes from strains 1100, BG-31, and BG-31-R. Approximately 30 μg of the membrane fraction from each strain were incubated in 1.0 ml of 0.05 M Tris-Cl buffer, pH 7.5, 1 mM MgCl₂, and the indicated amounts of DCCD. The DCCD was added from an ethanol stock solution, and the highest concentration of ethanol was 0.12%, which in itself was not inhibitory. After preincubation of this mixture for 10 min at 25°C, the assay was initiated by the addition of ATP to a final concentration of 2.5 mM. 0, strain 1100 (parent); ●, membranes (1100) + BF₁ (BG-31); ○, membranes (1100) + BF₁ (BG-31-R); □, membranes (BG-31-R) + BF₁ (1100); and ▲, membranes (BG-31) and BF₁ (1100).

FIG. 2 (center). Reconstitution of ATP-driven transhydrogenase activity in strains 1100, BG-31, and BG-31-R. The stripped membrane fraction, approximately 1 mg, and EDTA-extract (containing BF₂, amount shown on figure) were added to the transhydrogenase incubation mixture, prior to the addition of NADP, and incubated for 15 min at 25°C. The assays were then conducted as described under “Experimental Procedure.” 0, membranes (1100) + BF₁ (1100); ●, membranes (1100) + BF₁ (BG-31); ○, membranes (1100) + BF₁ (BG-31-R); □, membranes (BG-31-R) + BF₁ (1100); and ▲, membranes (BG-31) and BF₁ (1100).

FIG. 3 (right). Binding of BF₁ (1100) to stripped membranes of strains 1100, BG-31, and BG-31-R. Various amounts of EDTA extract (BF₂) from strain 1100 were incubated for 15 min at 23°C with 1 mg of stripped membranes isolated from each of the three strains tested in 1.0 ml of 0.05 M Tris-Cl buffer, pH 7.5, containing 1 mM MgCl₂. The mixture was subjected to centrifugation at 150,000 g for 60 min, after which the supernatant solution was removed, and the membrane pellet was washed by resuspension in the incubation buffer and resedimented as above. The washed membrane pellet was resuspended, and the amount of ATPase activity was determined, as described under “Experimental Procedure.” In all incubations the total recovery of added membrane protein and added ATPase activity were >90%. Symbols same as in Fig. 1.
with concomitant loss of the ATP-driven transhydrogenase activity. If the EDTA extract containing BF₁ (or pure BF₁) is then added back to the stripped membranes under appropriate conditions, ATP-driven transhydrogenase activity can be restored (26). It should be pointed out that the demonstration of normal ATPase activity does not rule out the possibility of an alteration in BF₁ (27). As shown in Fig. 2, BF₁ isolated from strain BG-31 is as effective as BF₁ isolated from the parental strain in restoring ATP-driven transhydrogenase activity to stripped membranes of the parental strain. In contrast, stripped membranes of BG-31 are inactive for reconstitution regardless of the source of BF₁. It seems clear that the defect in BG-31 resides in the membrane portion of the complex. Membranes isolated from the revertant have fully restored capacity for reconstitution.

**Binding of BF₁ to Stripped Membranes**—The evidence that BF₁ of strain BG-31 is poorly attached and that membranes isolated from BG-31 are inactive in transhydrogenase reconstitutions suggested that rebinding of BF₁ to the membrane may be defective in this strain. This was tested directly in binding experiments, and the results are shown in Fig. 3. Membranes of BG-31 do not bind appreciable amounts of any BF₁ relative to membranes of the parental strain. It therefore appears that the membrane defect in strain BG-31 results in poor attachment of BF₁ as determined both by the ease with which it is lost during fractionation and by direct binding. The revertant strain has recovered the ability to bind BF₁.

**Transport Studies**—The effect of a number of unc mutations on the active transport of a variety of solutes has been examined previously (10, 12, 16, 17, 20, 21, 36). It was largely from such studies that it became clear that energy coupling to active transport in yeast cells is an energy-driven process. In order to assess the effect of the membrane defect in BG-31 on solute transport we have examined the uptake of [14C]proline by cells and isolated membrane vesicles. The data presented in Fig. 4 demonstrate that cells of BG-31 are able to transport proline at rates equivalent to the parent. If, however, the cells are treated with KCN to inhibit electron transport activity, the solute transport capacity is abolished. In contrast, the parental strain is only inhibited about 50% by treatment with KCN. The amount of transport retained by the parental strain in the presence of the electron transport inhibitor is a reflection of the energization of the transport by ATP. It is therefore clear that the mutant cannot utilize ATP for transport.

We have examined the transport capacity of isolated membrane vesicles where the only source of energy is provided by an exogenous substrate of the electron transport system, D-lactate. As shown in Fig. 5 the electron transport-driven uptake of proline appears normal in membrane vesicles.

**Polypeptide Analysis of Membranes of BG-31**—Although many unc mutants have been described and a variety of physiological studies have been conducted there is to our knowledge no direct evidence that any of the mutations occur in the structural genes coding for the various polypeptide components of the complex. We have examined membranes of BG-31 in order to determine if an altered

<table>
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<tr>
<th>Strain</th>
<th>Membrane fraction</th>
<th>Respiratory-driven (nmol NADPH formed/min/mg membrane protein)</th>
<th>ATP-driven (nmol NADPH formed/min/mg membrane protein)</th>
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<tr>
<td>1100</td>
<td>Membranes</td>
<td>20.9</td>
<td>22.9</td>
</tr>
<tr>
<td>1100</td>
<td>Membranes + DCCD</td>
<td>22.1</td>
<td>2.1</td>
</tr>
<tr>
<td>1100</td>
<td>Stripped membranes</td>
<td>16.3</td>
<td>0</td>
</tr>
<tr>
<td>1100</td>
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<td>18.1</td>
<td>0</td>
</tr>
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<td>Membranes</td>
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</table>
gene product could be detected. We have used polyacrylamide slab gels run in the presence of sodium dodecyl sulfate for this analysis since this technique provides good separation of membrane polypeptides and easy comparison of different strains. Since we have concentrated on nonsense mutations we would expect to see an alteration in the size of a specific polypeptide. The gel patterns obtained are presented in Fig. 6. Stripped membranes of BG-31 appear to be missing a polypeptide of molecular weight approximately 54,000. In addition, a new peptide with a molecular weight of approximately 25,000 appears in the pattern and presumably represents that fragment of the polypeptide synthesized prior to the chain-terminating mutation. It is most significant that the gel pattern of the revertant is indistinguishable from that of the parental strain, and it is possible that both the disappearance of the 54,000 molecular weight peptide and the appearance of the 25,000 molecular weight species are the result of physiological regulation and are not a reflection of the mutational event. In order to minimize this possibility we have examined the membranes from one unc- ATPase- strain and three unc ATPase+ strains and have detected no differences in the gel patterns from that obtained with membranes from the parental strain. We feel therefore that the defect observed in BG-31 is not the result of an altered physiological state.

CONCLUSIONS

We have isolated and partially characterized a mutant strain of E. coli that has an altered polypeptide constituent of the membrane portion of ATPase complex. This defect results in poor attachment of BF to the membrane portion of the complex; resistance of the ATPase activity to the inhibitory effects of DCCD, and the inability to couple energy from the hydrolysis of ATP to either transhydrogenase activity or solute accumulation. The interrelationship of these functional parameters, however, makes it impossible to assign a specific function to this membrane component. We are continuing to analyze other mutants in the membrane portion of the complex with the eventual goal of defining the complex and assigning specific functional roles to each polypeptide component.

Acknowledgments—We are very grateful to Dr. E. Murgola for providing the phage mutagenesis procedure prior to publication and to Dr. C. Yanofsky for his helpful discussions and continued interest in this work.

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