Physiological Suppression of a Transport Defect in *Escherichia coli* Mutants Deficient in Ca\(^{2+}\),Mg\(^{2+}\)-Stimulated Adenosine Triphosphatase

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Transport properties of membrane vesicles isolated from two adenosine triphosphatase-deficient mutants of *Escherichia coli*, NR70 and DL54, were compared with those of vesicles prepared from the corresponding parental strains. As reported previously (Rosen, 1973; Altdorf et al., 1974), vesicles prepared from these mutants grown under aerobic conditions exhibited defective amino acid transport, and activity was restored after treatment with dicyclohexylcarbodiimide. In sharp contrast, however, vesicles isolated from the same mutants grown anaerobically in the presence of nitrate exhibited completely normal transport activity when assayed under either anaerobic or aerobic conditions. Suppression of the transport defect was not due to the manner by which the vesicles were prepared, and the adenosine triphosphatase deficiency was not ameliorated by anaerobic growth in the presence of nitrate. Finally, the transport activity of vesicles prepared from the mutants grown under aerobic conditions was relatively resistant to the effect of 1.0 M guanidine hydrochloride extraction, whereas the activity of vesicles prepared from mutants grown anaerobically was totally refractory to the effect of the chaotrope.

Mutants of *Escherichia coli* defective in the membrane-bound Ca\(^{2+}\),Mg\(^{2+}\)-stimulated adenosine triphosphatase (ATPase) complex have been used to study oxidative phosphorylation (5–8), respiration- and adenosine triphosphate (ATP)-dependent transhydrogenase (6, 7, 9, 13, 18, 29), active transport (1–3, 30, 31, 33, 35, 40, 42, 44–46; B. I. Kanner, N. Nelson, and D. L. Gutnick, Biochim. Biophys. Acta, in press), and other energy-dependent phenomena (21, 26, 32, 43, 44). These mutants are defective in both oxidative phosphorylation and ATP-driven transhydrogenase activity, but the precise involvement of the ATPase complex in active transport is not completely clear since hydrolysis of ATP per se is not involved in respiration-linked active transport (17). Moreover, mutants in the Ca\(^{2+}\),Mg\(^{2+}\)-stimulated ATPase complex exhibit a variety of phenotypes with regard to active transport. Thus, one class of ATPase mutants exhibits normal transport activity under aerobic conditions but diminished activity under anaerobic conditions (30, 31, 38–40), and membrane vesicles isolated from these mutants exhibit normal respiration-linked transport activity (33). A second class of mutants exhibits defective active transport under aerobic conditions in both whole cells and isolated membrane vesicles (36; Kanner et al., in press), whereas a third type of mutant (42) exhibits normal aerobic transport activity (3) although this activity is defective in membrane vesicles isolated from the intact cell (1).

According to the chemiosmotic hypothesis of Mitchell (14, 24, 25), the driving force for active transport is an electrochemical gradient of protons (i.e., the "proton motive force") generated as a result of respiration or ATP hydrolysis. By means of this concept, the first class of mutants is easily explained, the defect in active transport becoming manifest only under anaerobic conditions when respiration is inhibited. This explanation, however, is not sufficient to explain the other two types of mutants that exhibit normal respiratory activity. Significantly, it has been demonstrated that the lesion in the ATPase complex in these mutants is associated with a marked increase in the proton permeability of the membrane, and that this defect, as well as the defect in active transport, can be cured by treatment with dicyclohexylcarbodiimide (DCC) (35–37, 45). In keeping with previous work on mitochondria and chloroplasts (11, 34), these observations have led to

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the suggestion that in addition to its catalytic activity, ATPase plays a structural role in the membrane in which the complex masks a proton channel (or carrier) in wild-type membranes, and that the complex is either missing or readily solubilized in these mutants, leading to enhanced proton permeability (1, 37).

The critical importance of membrane permeability to protons has also been demonstrated directly by recent experiments from this laboratory (L. Patel, S. Schuldiner, and H. R. Kaback, Proc. Natl. Acad. Sci. U.S.A., in press). Extraction of isolated membrane vesicles with strong chaotropic agents causes the vesicles to become specifically permeable to protons in a manner that is completely reversed by treatment with a variety of carbodiimides. The extracted vesicles are unable to generate a membrane potential (interior negative) in the presence of ascorbate-phenazine methosulfate, nor are they able to catalyze active transport. Both of these properties are completely restored to the system when the vesicle membrane is made impermeable to protons by treatment with carbodiimides. Moreover, the transport activity of guanidine hydrochloride-extracted membrane vesicles isolated from a DCC-resistant mutant of E. coli that exhibits DCC-resistant ATPase activity (12) is not reactivated by DCC (L. Patel and H. R. Kaback, unpublished observations). These experiments support the hypothesis that the DCC-sensitive component of the ATPase complex is involved in proton permeability.

This report extends the studies of Rosen (36) and Altendorf et al. (1) to include a characterization of active transport in vesicles prepared from Ca\(^{2+}\),Mg\(^{2+}\)-stimulated ATPase mutants NR70 and DL54 grown anaerobically in the presence of nitrate. Surprisingly, the transport activity of these vesicles is completely normal, although the defect in ATPase is still present. In addition, the transport activity of these vesicles is shown to be resistant to inactivation by guanidine hydrochloride.

**MATERIALS AND METHODS**

**Growth of cells and preparation of membrane vesicles.** Parental strains ML 308-225 and 7, derivatives of E. coli ML and K-12, respectively, were used together with the corresponding ATPase-deficient mutants, DL54 (42) and NR70 (35). Strain DL54 was the generous gift of J. Smith of Cornell University, and strains 7 and NR70 were generously contributed by B. Rosen of the University of Maryland School of Medicine. Cells were grown aerobically to late log phase in minimal medium (10) supplemented with 0.1% yeast extract (Difco) and 0.5% glucose under forced aeration. Anaerobically, cells were grown in the same medium supplemented with 50 mM potassium nitrate, 1 µM selenic acid, and 1 µM sodium molybdate as described previously (20; J. Boonstra, M. T. Huttenun, W. N. Konings, and H. R. Kaback, J. Biol. Chem., in press). Samples of each culture were routinely streaked onto agar plates containing minimal medium and either 1% sodium succinate (hexahydrate) or 0.5% glucose before harvesting as a screen against contamination. The mutants were unable to grow on succinate regardless of growth conditions. Membrane vesicles from either aerobically or anaerobically grown cells were prepared according to the procedure of Konings and Kaback (20). Throughout the paper, vesicles prepared from aerobically grown cells are referred to as “aerobic vesicles,” and the preparations from anaerobically grown cells are referred to as “anaerobic vesicles.”

**Transport assays.** Transport was assayed under aerobic conditions (16) or under oxygen-free argon (less than 1 µg of oxygen per ml) (20). For the anaerobic assays, the reaction mixtures were gassed with argon for 5 min before addition of 10 mM sodium formate, 10 mM potassium nitrate, and [\(^{14}\)C]proline. In all cases, [\(^{14}\)C]proline (232 mCi/mmol) was used at a final concentration of 16.6 µM.

Ca\(^{2+}\),Mg\(^{2+}\)-stimulated ATPase activity. Membrane vesicles prepared as described above were centrifuged for 20 min at 48,000 × g, resuspended in 30 mM tri(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) to a final protein concentration of 5.0 mg/ml, and subjected to sonic oscillation at 0 C in a Branson Sonifier (model W140) at maximum output for four periods of 15 s each. Approximately 0.05 mg of membrane protein was incubated with 30 mM tri(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 2 mM magnesium sulfate in a total volume of 0.1 ml for 5 min at 37 C. The reaction was initiated by addition of 10 µl of [\(^{32}\)P]ATP (final concentration 4 mM), containing about 60,000 counts/min and terminated at an appropriate time by addition of 0.4 ml of 1.25% ammonium molybdate in 1.0 N HCl. Inorganic phosphate was extracted into 0.85 ml of isobutanol-benzene-acetone (30), and aliquots of the upper phase were dried on planchets and counted in a Nuclear-Chicago gas flow counter. Results were corrected for the amount of inorganic phosphate present at zero time and for nonenzymatic hydrolysis of ATP.

**Immunodiffusion.** Immunodiffusion was carried out in plastic petri dishes (60 by 15 mm; Falcon Plastics) containing 5.0 ml of 1.0% Noble agar. Aliquots of the vesicle preparations containing 15 mg of membrane protein per ml were dissolved in sodium dodecyl sulfate (0.5% final concentration), and 10 µl was added to the wells as indicated in Fig. 4. Antiserum and preimmune sera were added to separate wells in 10-µl aliquots. Preparations of anti-(α+β), anti-α, anti-β, and anti-γ (29; W. N. Konings and J. Boonstra, J. Curr. Top. Mem. Trans., in press) were the generous gift of N. Nelson of the Technion.

**Guanidine hydrochloride extraction and reactivation with DCC.** Extraction of membrane vesicles with 1.0 M guanidine hydrochloride and subsequent treatment with 70 µM DCC were carried out as described by Patel et al. (Proc. Natl. Acad. Sci. U.S.A., in press).
Protein determinations. Protein determinations were performed according to Lowry et al. (23).

Materials. [U-14C]proline and [γ-3H]ATP were obtained from New England Nuclear Corp. DCC was obtained from Calbiochem. Enzyme-grade guanidine hydrochloride was obtained from Schwartz/Mann Biochemicals. All other chemicals were reagent grade obtained from commercial sources.

RESULTS

Active transport by anaerobic membrane vesicles from E. coli ML 308-225 and DL54. Isolated membrane vesicles from E. coli ML 308-225 catalyze active transport under anaerobic conditions in the presence of formate and nitrate (20; Boonstra et al., in press; Konings and Boonstra, in press). To elicit this phenomenon, the parent cells are grown under conditions that induce the anaerobic electron transfer system, and vesicles are prepared by using a modified procedure designed to prevent the loss of loosely bound components from the vesicles. Measurements of proline uptake by membrane vesicles prepared in this manner from appropriately grown E. coli ML 308-225 and the corresponding ATPase mutant DL54 are shown in Fig. 1. Surprisingly, there was no significant difference in the transport activities exhibited by these vesicles under a variety of conditions. Thus, anaerobic DL54 vesicles catalyzed active proline transport as effectively as anaerobic ML 308-225 vesicles under argon with formate as electron donor and nitrate as acceptor, and with formate or reduced phenazine methosulfate as electron donors and oxygen as the electron acceptor. These results are in contrast with previously reported observations (1, 42) demonstrating that DL54 vesicles isolated from aerobically grown cells are defective in active transport.

Since the methods used to prepare vesicles that catalyze anaerobic transport were significantly different from the standard preparation, it is possible that the results shown above were due to the use of this method. This was not the case, however, as shown by the experiments presented in Fig. 2. Using the modified procedure (20) to prepare vesicles from aerobically grown cells, DL54 vesicles were only 50% as active as ML 308-225 vesicles in the presence of ascorbate-phenazine methosulfate (compare Fig. 2A and B). Although not shown, with d-lactate as electron donor, DL54 vesicles exhibited about 20% of the activity of ML 308-225 vesicles, as reported previously (42). Similar results were also obtained when vesicles were isolated from aerobically grown ML 308-225 and DL54, using the standard procedure for vesicle preparation (data not shown).

Effect of DCC on aerobic and anaerobic proline transport. As shown in Fig. 2B, exposure of aerobic DL54 vesicles to DCC resulted in marked stimulation of proline transport in the presence of ascorbate-phenazine methosulfate, and after treatment with this carbodiimide, the activity of the mutant vesicles was comparable with that of aerobic ML 308-225 vesicles (compare Fig. 2A and B). Although the degree of stimulation by DCC was quantitatively less striking, these results are similar to those reported by Altendorf et al. (1). On the other hand, anaerobic ML 308-225 and DL54 vesicles exhibited similar transport activities in the presence of formate and nitrate, and exposure to DCC elicited little or no effect on the transport activity of either vesicle preparation (Fig. 2C, D). Similar results were obtained when reduced phenazine methosulfate or formate was used as electron donor in the presence of oxygen (data not shown).

Active transport by anaerobic membrane vesicles from E. coli strains 7 and NR70. Another ATPase mutant of E. coli, NR70, has been described (36) which, like DL54, exhibits a DCC-reversible defect in active transport under
Ca<sup>2+</sup>, Mg<sup>2+</sup>-stimulated ATPase activity. It has been postulated that the membrane-bound ATPase plays a structural role in maintaining the proton impermeability of the bacterial membrane, and the suggestion has been made that the transport defect observed in mutants DL54 and NR70 is due to an abnormally loose association between the ATPase complex and the membrane (1, 36). It is noteworthy, therefore, that no increase in ATPase activity was observed in DL54 or NR70 vesicles after growth of the mutants under anaerobic conditions in the presence of nitrate. Sonicated anaerobic ML 308-225 and strain 7 vesicles exhibited ATPase activities of approximately 200 nmol/min per mg of protein in the presence of magnesium, whereas anaerobic DL54 vesicles exhibited an activity of less than 3 nmol/min per mg of protein and anaerobic NR70 vesicles exhibited virtually no ATPase activity. Moreover, material that cross-reacted with antiserum prepared against intact catalytic subunits purified from E. coli (27), anti-(α+β), was detected in aerobic and anaerobic vesicles from ML 308-225 and DL54 and in anaerobic vesicles from strain 7 (Fig. 4). Anaerobic vesicles from NR70 that exhibited parental levels of proline transport (Fig. 3) did not exhibit any cross-reacting material. Although not shown, it is also noteworthy that vesicles from strains ML 308-225, DL54, aerobically and in isolated membrane vesicles. In confirmation of Rosen's results (35, 36), we have demonstrated that NR70 vesicles isolated from aerobically grown cells exhibited a severe defect in proline transport in the presence of D-lactate and ascorbate-phenazine methosulfate, and that the defect was partially cured by exposure to DCC (data not shown). Moreover, as demonstrated with DL54, the transport defect exhibited by vesicles isolated from this mutant was suppressed by anaerobic growth in the presence of nitrate (Fig. 3). Proline transport by anaerobic NR70 vesicles in the presence of formate and nitrate did not differ appreciably from that of anaerobic strain 7 vesicles assayed under the same conditions.

![Figure 2](http://jb.asm.org/) Effect of DCC on proline transport in aerobic membrane vesicles of ML 308-225 (A) and DL54 (B) and anaerobic vesicles of ML 308-225 (C) and DL54 (D). Cells were grown aerobically on glucose under forced aeration and anaerobically as described in the text, and transport assays and additions of electron acceptors and donors were performed as described in the text and the legend to Fig. 1. Vesicles were treated with 70 μM DCC as described in the text, and control preparations received 1% ethanol. (A and B) Assayed aerobically with ascorbate and phenazine methosulfate. (C and D) Assayed with formate and nitrate under argon as described in Materials and Methods. Symbols: O, Control; ●, DCC.

![Figure 3](http://jb.asm.org/) Proline uptake in anaerobic membrane vesicles from strain 7 (parent) and NR70 (mutant). Cells were grown, vesicles were isolated, and transport assays and additions were performed as described previously (20) and in the text and legend to Fig. 1. Closed symbols, NR70; open symbols, strain 7; O, ●, formate-nitrate; Δ, ▲, no additions.
and 7 contained material that cross-reacted with antisera prepared against the denatured subunits of ATPase (Kanner et al., in press) —anti-(α), anti-(β), and anti-(γ)—regardless of growth conditions, and that similar preparations of NR70 did not exhibit any material that cross-reacted with these antisera.

**Effect of guanidine hydrochloride extraction.** The effects of 1.0 M guanidine hydrochloride extraction on the transport activity of aerobic and anaerobic ML 308-225 and DL54 vesicles are illustrated in Fig. 5. As shown elsewhere (Patel et al., in press) and in panel A, extraction of aerobic ML 308-225 vesicles with the chaotrope resulted in marked inactivation of proline transport in the presence of ascorbate-phenazine methosulfate, and this activity was completely restored when the extracted vesicles were exposed to DCC. Extraction of aerobic DL54 vesicles also led to inactivation of transport, but the residual activity of these vesicles was five- to sixfold higher than that observed with guanidine hydrochloride-extracted ML 308-225 vesicles. It is also noteworthy that after exposure to DCC, the guanidine hydrochloride-extracted DL54 vesicles catalyzed active transport somewhat better than ML 308-225 vesicles.

Strikingly, the ability of anaerobic DL54 vesicles to catalyze proline transport is essentially unaffected by treatment with 1.0 M guanidine hydrochloride, and subsequent exposure of the vesicles to DCC had virtually no effect (Fig. 5D). On the other hand, proline transport by anaerobic ML 308-225 vesicles was inactivated by guanidine hydrochloride, and activity was restored to control levels after exposure to DCC (Fig. 5C). Although not shown, similar results were obtained with vesicles prepared from NR70 grown anaerobically in the presence of nitrate.

**DISCUSSION**

The studies presented in this report extend previous findings (1, 36) on the transport properties of isolated membrane vesicles from two ATPase mutants of *E. coli*, DL54 and NR70. As opposed to other ATPase mutants described in the literature (30, 33, 38-40), these mutants are unique in that they exhibit defective aerobic transport in isolated membrane vesicles and/or intact cells (1, 36, 42). The data presented here are particularly noteworthy with respect to the proposed relationship between the genetic lesion leading to a deficiency in Ca2+,Mg2+-stimulated ATPase activity and concomitantly to a defect in active transport. As discussed above, the suggestion has been advanced that the ATPase complex serves a structural as well as a catalytic role in the membrane, thereby masking a hydrophobic proton-conducting channel (or carrier). In these mutants, this site is presumably exposed, leading to increased proton permeability, but can be resealed with DCC or ATPase (1, 36). Evidence consistent with this hypothesis has recently been presented by Rosen and Adler (37), who isolated two revertants of an ATPase mutant that regained the ability to catalyze active transport and grow on succinate in proportion to the amount of ATPase activity restored.

It is clear from the data presented here that...
the hypothesis discussed above is insufficient to explain the phenomena observed. Thus, although aerobic DL54 and NR70 vesicles exhibit a defect in their ability to catalyze active transport, parental levels of active transport are observed in vesicles isolated from both mutants when they are grown anaerobically in the presence of nitrate. Moreover, anaerobic vesicles isolated from DL54 and NR70 exhibit almost no ATPase activity, and with NR70 vesicles no material is apparent that cross-reacts with antisera prepared against purified subunits of the ATPase complex. It is apparent, therefore, that anaerobic growth in the presence of nitrate suppresses the defect in active transport without altering the basic genetic lesion. In the same vein, another ATPase mutant of *E. coli* unc405 has been isolated by Cox et al. (8) that lacks the entire ATPase aggregate yet exhibits normal serine and phosphate transport under aerobic conditions. Anaerobic transport in this mutant was observed when fumarate was added to the growth medium and to the assay medium (38). Finally, it should also be emphasized that 60 to 80% of the membrane-bound Ca\(^{2+}\),Mg\(^{2+}\)-stimulated ATPase activity is lost during the preparation of wild-type membrane vesicles (41). It is apparent, therefore, that most of the catalytic subunit of the ATPase complex, at least, is absent from the vesicles although they are impermeable to protons (1; Patel et al., in press) and catalyze active transport as effectively as whole cells in many instances (17).

Growth of DL54 and NR70 under conditions that suppress the defect in active transport also affects the sensitivity of these vesicles to extraction with chaotropic agents. Although aerobic mutant vesicles are somewhat more resistant to the effects of guanidine hydrochloride extraction than corresponding parental preparations, the transport activity of anaerobic vesicles prepared from the ATPase-deficient strains is refractory to extraction. In addition to their implications with respect to the physiology of these mutants, these observations are important with regard to the mode of action of chaotropic agents on the membrane vesicles. Since the transport activity of guanidine hydrochloride-extracted vesicles isolated from a mutant with DCC-resistant ATPase activity is not reactivated by DCC (12; Patel and Kaback, manuscript in preparation) and anaerobic DL54 and NR70 vesicles are resistant to the effects of guanidine hydrochloride, it seems quite possible that the dramatic effects of these chaotropic agents on the vesicles (Patel et al., in press) are related specifically to an alteration in some component of the ATPase complex or to an alteration in a component of the membrane to which the complex is bound.

Mutants of *E. coli* defective in oxidative phosphorylation exhibit a variety of phenotypes with respect to the ATPase complex as well as active transport. The results reported here complicate the issue even further since they indicate that growth under certain conditions can lead to physiological suppression of the transport defect without affecting the primary genetic lesion. It seems apparent, therefore, that attempts to generalize about the precise role of the ATPase complex in the mechanism of active transport based on the

**FIG. 5.** Effect of guanidine hydrochloride extraction and subsequent treatment with DCC on proline uptake in aerobic vesicles of ML 308-225 (A) and DL54 (B) and anaerobic vesicles of ML 308-225 (C) and DL54 (D). Vesicles were treated with 1 M guanidine hydrochloride and subsequently treated with 70 μM DCC as described in the text. Control preparations were treated with ethanol in place of DCC and were not exposed to guanidine hydrochloride. (A and B) Assayed with ascorbate-phenazine methosulfate under oxygen. (C and D) Assayed with formate-nitrate under argon. Symbols: ▼, Control; ●, guanidine hydrochloride followed by DCC; ○, guanidine hydrochloride.
behavior of these mutants must await a more complete understanding of the system.

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LITERATURE CITED

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