The Role of the Carbodiimide-Reactive Component of the Adenosine-5′-triphosphatase Complex in the Proton Permeability of *Escherichia coli* Membrane Vesicles†

Lekha Patel and H. Ronald Kaback*

ABSTRACT: Membrane vesicles isolated from wild-type and dicyclohexylcarbodiimide-resistant strains of *Escherichia coli* exhibit identical respiration-dependent transport activities, and in both cases, this activity is abolished by extraction of the vesicles with 1.0 M guanidine-HCl. Transport activity of extracted wild-type vesicles is completely restored by exposing the vesicles to lipophilic or water-soluble carbodiimides, while transport activity of the mutant vesicles is not restored by exposure to lipophilic carbodiimides. Strikingly, however, complete reactivation of transport in mutant vesicles is observed with water-soluble carbodiimides. Similarly, the Ca^{2+}, Mg^{2+}-stimulated ATPase activity of wild-type vesicles is inhibited by both classes of carbodiimides, while the ATPase activity of mutant vesicles is inhibited by water-soluble carbodiimides, but resistant to inhibition by lipophilic carbodiimides. The carbodiimide-reactive component of the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase complex in wild-type vesicles is readily labeled with *N,N′*-dicyclohexyl[^{14}C]-carbodiimide, while the analogous component in mutant vesicles is not reactive. Alternatively, when vesicles are treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [^{14}C]methiodide, a water-soluble carbodiimide, the carbodiimide-reactive component is labeled to a similar degree in both preparations. The results suggest that the altered carbodiimide-reactive proteolipid in the dicyclohexylcarbodiimide-resistant mutant is specifically defective in its ability to react with lipophilic carbodiimides. In addition, these and other findings indicate that the increase in proton permeability observed on extraction of isolated membrane vesicles with chaotropic agents is due exclusively to an effect on the carbodiimide-reactive component of the Ca^{2+}, Mg^{2+}-stimulated ATPase complex.

Extraction of *Escherichia coli* membrane vesicles with chaotropic agents solubilizes approximately 20% of the membrane protein and causes the vesicles to become specifically permeable to protons, thereby abolishing their ability to generate a membrane potential, interior negative (Patel et al., 1975). As a result, the vesicles are no longer able to catalyze respiration-dependent active transport. Exposure of extracted vesicles to various carbodiimides decreases the proton permeability of the vesicle membrane, causing them to regain their ability to generate a membrane potential. By this means, active transport is completely reactivated. Since vesicles prepared from certain mutants defective in the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase complex also exhibit increased proton permeability and a pleiotropic defect in active transport, both of which are alleviated by exposure to *N,N′*-dicyclohexylcarbodiimide (DCC[1]) (Altendorf et al., 1974; Rosen, 1973; Van Thienen and Postma, 1973), the possibility exists that the effects described above are due to an effect of chaotropic agents on the ATPase complex.

Fillingame (1975) and subsequently Altendorf and Zitzman (1975) described the solubilization and partial purification of the carbodiimide-reactive component of the Ca^{2+}, Mg^{2+}-stimulated ATPase complex of *E. coli*. The protein is extracted from the membrane with chloroform–methanol, and exhibits properties which are similar to those of the analogous protein isolated previously from mitochondria (Cattell et al., 1971). Fillingame (1975) also described the isolation and characterization of *E. coli* mutants which are resistant to the growth-inhibiting properties of DCC, and showed that the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase activity of these mutants is insensitive to inhibition by DCC. Moreover, it was demonstrated (Fillingame, 1975) that the carbodiimide-reactive protein is labeled much less readily in the DCC-resistant mutants, as is also the case for the mutant utilized by Altendorf and Zitzman (1975).

This paper describes experiments carried out with membrane vesicles prepared from the DCC-resistant mutants isolated by Fillingame (1975). The results corroborate and extend the previous findings and demonstrate, furthermore, that the increase in proton permeability observed on extraction of membrane vesicles with chaotropic agents is probably due specifically to an effect on the carbodiimide-reactive component of the Ca^{2+}, Mg^{2+}-stimulated ATPase complex.

Experimental Procedure

Bacterial Strains. The parental strains *E. coli* AN180 and RF-t136 and their respective DCC-resistant derivatives RF-7 (DDC^R) and RF-t263 (DCC^R) were graciously contributed by Dr. Robert H. Fillingame of the University of Wisconsin.

Growth of Cells and Preparation of Membrane Vesicles. Cells were grown on medium 63 (Cohen and Rickenberg, 1956) containing 22 mM sodium succinate, 30 mM potassium acetate, 17 mM potassium L-malate, 0.2 mM arginine, 0.1% yeast extract (Difco), and 2 mg/l. of thiamin. Membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975), using 50 μg/ml of lysozyme for the preparation of spheroplasts.

Guanidine-HCl Extraction and Reactivation with Carbo-

† From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received January 28, 1976.*

[1] Abbreviations used are: DCC, *N,N′*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDCMI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide; DiPC, diisopropylcarbodiimide; CMC, 1-cyclohexyl-3-[2-morpholyl-(4)-ethyl]carbodiimide; P_i, inorganic phosphate.

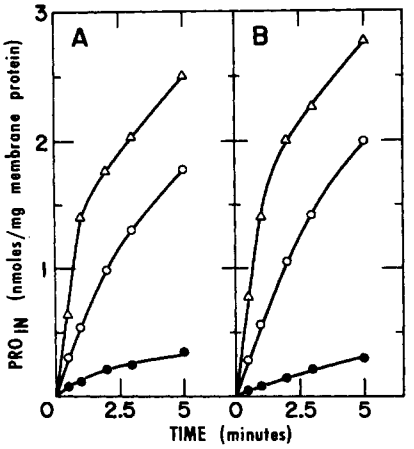

FIGURE 1: Proline transport by membrane vesicles prepared from *E. coli* AN180 (panel A) and RF-7(DCC^R) (panel B). Aliquots (25 μl) of membrane vesicles containing approximately 0.1 mg of membrane protein were diluted to a final volume of 50 μl containing (in final concentrations) 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate. The samples were incubated at 25 °C under oxygen, and lithium ascorbate plus phenazine methosulfate (Δ) or lithium D-lactate (O) were added at final concentrations of 20, 0.1, and 20 mM, respectively, as indicated. (●—●) No exogenous electron donor added. The incubations were continued at 25 °C under oxygen for the times shown, and the reactions were terminated and the samples assayed as described previously (Kaback, 1971; Kaback, 1974).

diimides. Extraction of membrane vesicles with 1.0 M guanidine-HCl and subsequent treatment with indicated carbodiimides was carried out as described by Patel et al. (1975).

Transport Assays. Transport was measured as described previously (Kaback, 1971; Kaback, 1974), using [U-^{14}C]L-proline (232 mCi/mmol) at a final concentration of 8 μM.

Ca^{2+}, Mg^{2+}-Stimulated ATPase Activity and Inhibition by Carbodiimides. Membrane vesicles prepared as described were centrifuged for 20 min at 48 000*g* and resuspended in 30 mM Tris-HCl (pH 8.0) to a final protein concentration of 5.0 mg/ml. Approximately 0.05 mg of membrane protein was incubated with 30 mM Tris-HCl (pH 8.0) containing 2 mM magnesium sulfate and the indicated carbodiimide at the concentration given in a total volume of 0.1 ml for 15 min at 30 °C. Reactions were initiated by addition of 10 μl of [γ-^{32}P]ATP (final concentration 4 mM, containing about 60 000 counts/min), and terminated at 1 min 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 isobutyl alcohol–benzene–acetone (Martin and Doty, 1949), 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.

Labeling of Membrane Vesicles with Radioactive Carbodiimides. Vesicles were labeled with either *N,N'*-dicyclohexyl[^{14}C]carbodiimide ([^{14}C]DCC) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [^{14}C]methiodide (EDC[^{14}C]MI) exactly as described by Fillingame (1975). A suspension of membrane vesicles containing approximately 20 mg of protein/ml in 0.25 M sucrose, 10 mM Tris sulfate (pH 7.6), 5 mM magnesium sulfate, and 0.2 mM dithiothreitol was made 0.1 mM in [^{14}C]DCC (45 mCi/mmol) or 1.0 mM in EDC[^{14}C]MI (47 mCi/mmol) and stirred for 24 h at 0 °C. The suspensions were then diluted 20-fold with suspension buffer, centrifuged, and washed three times.

Preparation of Proteolipid. Membrane vesicles labeled with radioactive carbodiimides were extracted with chloroform–methanol (2:1, v/v) as described by Fillingame (1975). An aliquot of a membrane suspension labeled with the appropriate carbodiimide as described above was added to 25 volumes of chloroform–methanol (2:1, v/v) and stirred at 4 °C for 24 h. The precipitate was removed by filtration and discarded, and the clear chloroform–methanol extract was washed by the procedure of Folch et al. (1957). The lower phase was diluted with one volume of chloroform and the requisite amount of methanol required to keep the solution clear. It was then taken to dryness on a rotoevaporator at 35 °C. The residue was dissolved in chloroform–methanol (2:1, v/v) and four volumes of diethyl ether were added slowly at −10 °C with stirring. After 24 h or more at −20 °C, the proteolipid precipitate was removed by centrifugation at approximately 1 000*g* for 2 h at −20 °C. The precipitate was then dissolved in a small volume of chloroform–methanol (2:1, v/v), and one volume of chloroform was added. The sample was taken to dryness under a stream of argon, 1.0 ml of chloroform was added, and the sample was taken to dryness again. The dried material was dissolved by heating at 45 °C for 16 h in 0.05 M Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate, 10% (v/v) glycerol, and 1% 2-mercaptoethanol, and used for polyacrylamide gel electrophoresis. Aliquots of each fraction were assayed for radioactivity by liquid scintillation spectrometry at a counting efficiency of 80–90%.

Discontinuous Polyacrylamide Slab Gel Electrophoresis. Aliquots of the samples described above are subjected to electrophoresis in the presence of 0.2% sodium dodecyl sulfate as described by Ames (1974), except that the gels contained 13% acrylamide and 0.59% *N,N'*-diallyltartardiamide (Bio-Rad) as a cross-linking agent. The gels were sliced into 1-mm segments with a manual device, and each slice was placed into a scintillation vial and dissolved by addition of 1.0 ml of 2% periodic acid and shaking at room temperature for 1 h. Ten milliliters of Instabray (Yorktown Research) was then added to the vials, and the samples were counted in a liquid scintillation spectrometer.

Protein Determinations. Protein was determined as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Materials. *N,N'*-Dicyclohexyl[^{14}C]carbodiimide (45 mCi/mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide [^{14}C]methiodide (47 mCi/mmol) were synthesized by The Isotope Synthesis Group of Hoffmann-LaRoche, Inc. under the direction of Dr. Arnold Liebman (Patel et al., 1975). [U-^{14}C]L-Proline and [γ-^{32}P]ATP were obtained from New England Nuclear.

All other materials were reagent grade obtained from commercial sources.

Results

Transport Activity of Membrane Vesicles Prepared from Wild-Type and DCC-Resistant Mutants. Membrane vesicles prepared from the DCC-resistant mutant RF-7 catalyze proline transport as effectively as membrane vesicles prepared from the parental strain AN180 (Figure 1). In the absence of exogenous electron donors, there is little proline uptake by either vesicle preparation, while addition of D-lactate or ascorbate and phenazine methosulfate produces marked stimulation of the initial rate and steady-state level of proline accumulation in both preparations. Under each condition tested, both the rate and extent of proline uptake are essentially the same in the two vesicle preparations.

Although not shown, identical results were obtained with vesicles prepared from the isogenic pair RF-t136 and RF-

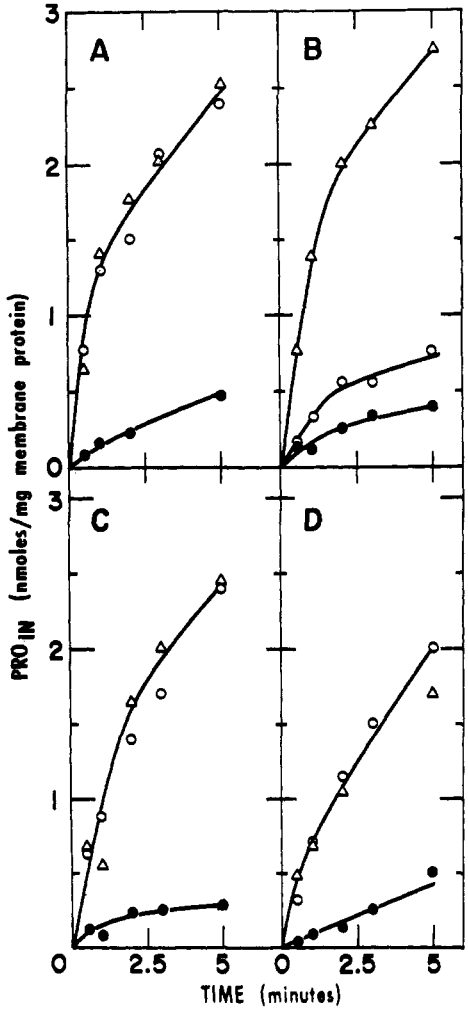

FIGURE 2: Reactivation of proline transport in guanidine-HCl-extracted AN180 (panels A and C) and RF-7(DCC^R) (panels B and D) membrane vesicles by DCC (panels A and B) and EDC (panels C and D). Membrane vesicles were extracted with 1.0 M guanidine-HCl as described under Experimental Procedure (●). Where indicated (○), samples of these preparations were incubated with 0.07 mM DCC (panels A and B) or 1.0 mM EDC (panels C and D) for 30 min at 25 °C prior to assaying proline transport. Proline transport was assayed at 25 °C under oxygen in the presence of lithium ascorbate and phenazine methosulfate as described previously (Kaback, 1971; Kaback, 1974) and in Figure 1. (△) Proline uptake by untreated control vesicles. These concentrations of DCC and EDC had no significant effect on either the rate or extent of proline uptake by the control preparation.

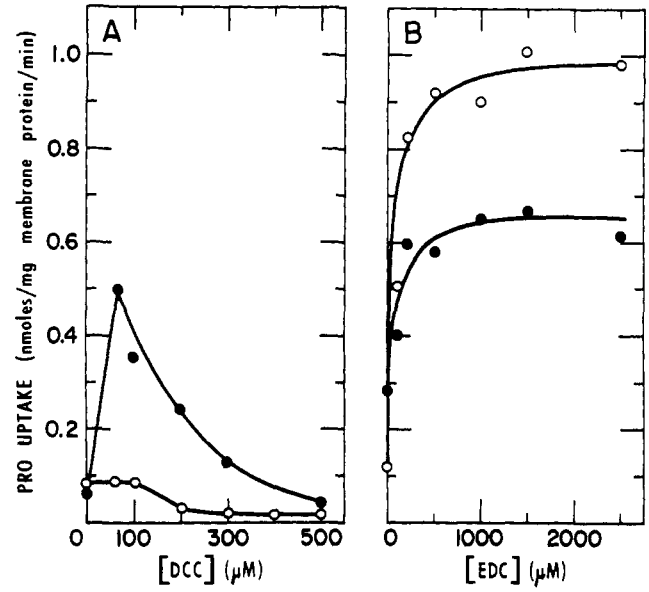

FIGURE 3: Concentration dependence of DCC (panel A) and EDC (panel B) reactivation of proline transport. AN180 (●) and RF-7(DCC^R) (○) membrane vesicles were extracted with 1.0 M guanidine-HCl as described previously (Patel et al., 1975) and under Experimental Procedure. Samples were incubated with given concentrations of DCC (A) or EDC (B) for 30 min at 25 °C. At that time, the rate of proline uptake was measured for 1 min in the presence of lithium ascorbate and phenazine methosulfate, as described previously (Kaback, 1971; Kaback, 1974) and in Figure 1.

t263(DCC^R). It is clear, therefore, that the genetic alteration responsible for DCC resistance does not alter the ability of the vesicles to catalyze active transport.

Effect of Carbodiimides on Active Transport by Guanidine-HCl-Extracted Vesicles. The effect of 1.0 M guanidine-HCl extraction on the transport activity of AN180 and RF-7(DCC^R) membrane vesicles is illustrated in Figure 2. As shown previously with ML 308-225 (Patel et al., 1975) and strain 7 (Boonstra et al., 1975) vesicles, extraction of AN180 vesicles with the chaotrope results in marked inactivation of proline transport in the presence of ascorbate and phenazine methosulfate, and this activity is completely restored when the extracted vesicles are exposed to DCC (panel A) or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (panel C). Extraction of RF-7(DCC^R) vesicles also leads to inactivation of ascorbate–phenazine methosulfate dependent transport, but exposure of the extracted vesicles to DCC (panel B) produces little or no restoration of transport activity. Surprisingly, however, exposure of extracted RF-7(DCC^R) vesicles to EDC, a water-soluble carbodiimide, restores transport to control levels (panel D). It should be emphasized that virtually identical results were obtained with another pair of carbodiimides (data not shown). Diisopropylcarbodiimide (DiPC), a hydrophobic carbodiimide, reactivates transport in guanidine-HCl-extracted AN180 vesicles, but not in RF-7(DCC^R) vesicles, whereas 1-cyclohexyl-3-[2-morpholyl-(4)-ethyl]carbodiimide (CMC), another water-soluble carbodiimide, reactivates transport in both preparations.

The concentration dependence of DCC and EDC for reactivation of proline transport in guanidine-HCl-extracted AN180 and RF-7(DCC^R) membrane vesicles is shown in Figure 3, panels A and B, respectively. With AN180 vesicles, maximal rates of transport are observed with 0.07 mM DCC and 1.0 mM EDC, as reported previously (Patel et al., 1975). With RF-7(DCC^R) vesicles, on the other hand, no restoration of active transport is observed with DCC concentrations as high as 0.5 mM, while EDC yields complete restoration of transport at a concentration of 1.0 mM (i.e., the same concentration as that required for complete reactivation of guanidine-HCl-extracted AN180 vesicles).

With guanidine-HCl-extracted AN180 vesicles, reactivation of transport begins immediately after the addition of 0.07 mM DCC, and by 15–20 min, complete reactivation is observed (Figure 4A). With RF-7(DCC^R) vesicles, no significant reactivation is observed with DCC over the time course of the experiment. Alternatively, as shown in Figure 4B, the time course of EDC reactivation of transport in guanidine-HCl-extracted AN180 and RF-7(DCC^R) vesicles is identical.

Although data will not be presented, results very similar to those presented in Figures 2–4 were obtained with vesicles prepared from the isogenic strains RF-t136 and RF-t263(DCC^R).

Inhibition of Ca^{2+}, Mg^{2+}-Stimulated ATPase Activity by Carbodiimides. As reported by Fillingame (1975), the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase activity of RF-

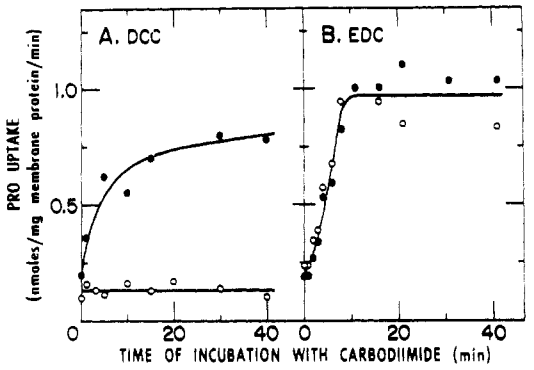

FIGURE 4: Time course of DCC (A) and EDC (B) reactivation of proline transport. AN180 (●) and RF-7(DCC^R) (○) membrane vesicles were extracted with 1.0 M guanidine-HCl as described previously (Patel et al., 1975) and under Experimental Procedure. Samples of these vesicles were incubated with 0.07 mM DCC (A) or 1.0 mM EDC (B) for the times indicated, and proline transport was assayed for 1 min with lithium ascorbate and phenazine methosulfate, as described previously (Kaback, 1971, 1974) and in Figure 1.

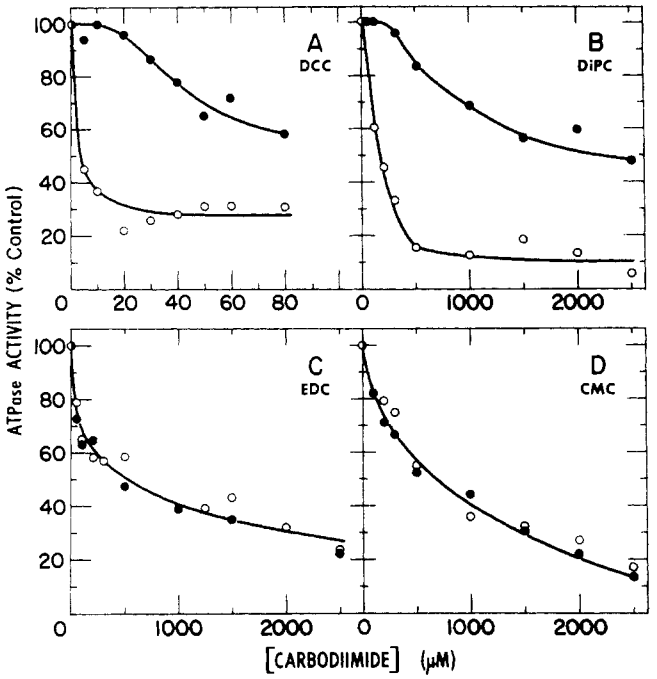

FIGURE 5: Inhibition of Ca^{2+}, Mg^{2+}-stimulated ATPase activity of AN180 (○) and RF-7(DCC^R) (●) membrane vesicles by DCC (A), DiPC (B), EDC (C), and CMC (D). Rates of ATP hydrolysis were measured at 1 min, as described under Experimental Procedure. Activities of control preparations incubated in the absence of carbodiimides were 195 and 259 nmoles P_i released/mg of membrane protein min^{-1} for AN180 and RF-7(DCC^R) vesicles, respectively.

7(DCC^R) and RF-t263(DCC^R) is markedly resistent to inhibition by DCC relative to the ATPase activity of the appropriate parental strains. This finding is corroborated by the data presented in Figure 5A where the difference in sensitivity of Ca^{2+}, Mg^{2+}-stimulated ATPase activity in AN180 and RF-7(DCC^R) vesicles to inhibition by DCC is clearly demonstrated. The ATPase activity of AN180 vesicles is inhibited by concentrations of DCC as low as 0.005–0.01 mM, while little if any inhibition of RF-7(DCC^R) ATPase activity is observed up to 0.02 mM. Although concentrations of DCC higher than 0.02 mM result in progressive inhibition of ATPase activity in RF-7(DCC^R) vesicles, even at 0.08 mM, only about 40% inhibition is observed. A similar difference in sensitivity

TABLE I: Extraction of [^{14}C]DCC-Labeled AN180 and RF-7(DCC^R) Vesicles.[a]

Fraction	AN180[b]	RF-7(DCC^R)[b]
Starting material	2.77 (100%)	2.54 (100%)
$CHCl_3$–MeOH (2:1) extract	2.28 (82%)	1.92 (76%)
Washed $CHCl_3$ layer	2.20 (79%)	1.84 (73%)
Diethyl ether precipitate (proteolipid)	0.20 (7%)	0.03 (1.2%)
	Sp Act. (nmol/mg of proteolipid protein)	
	4.03	0.60

[a] One milliliter samples of AN180 and RF-7(DCC^R) membrane vesicles containing a total of 16 and 18 mg of membrane protein, respectively, were treated with [^{14}C]DCC, as described under Experimental Procedure. The samples were then fractionated according to Fillingame (1975), as described under Experimental Procedure. The values given represent the total radioactivity recovered in each fraction and the percentage in each fraction relative to the amount of [^{14}C]DCC incorporated. A total of 275 and 285 μg of protein was recovered in the AN180 and RF-7(DCC^R) proteolipid fractions, respectively. [b] Counts/min × 10^{-6}.

to inhibition by DiPC is observed in the two vesicle preparations even though the ATPase activities are approximately 50-fold less sensitive to inhibition by this carbodiimide relative to DCC (Figure 5B). In marked contrast, the Ca^{2+}, Mg^{2+}-stimulated ATPase activities of AN180 and RF-7(DCC^R) vesicles exhibit virtually identical sensitivities to inhibition by water-soluble carbodiimides. With various concentrations of either EDC (Figure 5C) or CMC (Figure 5D), no significant differences in inhibition of ATPase activity in the two vesicle preparations are observed.

Extraction and Electrophoresis of Carbodiimide-Reactive Protein from AN180 and RF-7(DCC^R) Vesicles. Fillingame (1975) has demonstrated that the carbodiimide-reactive protein associated with the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase complex in *E. coli* is a proteolipid which is extracted from the membrane with chloroform–methanol (2:1, v/v), and that it has an apparent molecular weight of 9000, as judged by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Moreover, this proteolipid is labeled much less readily with [^{14}C]DCC in the DCC-resistant mutant RF-7. Using the techniques described by Fillingame (1975), we have carried out similar studies with AN180 and RF-7(DCC^R) vesicles labeled with [^{14}C]DCC and EDC[^{14}C]MI. As shown in Table I, approximately 80% of the DCC-reactive material is extended into chloroform–methanol and subsequently into chloroform with vesicles prepared from both AN180 and RF-7(DCC^R), and similar amounts of radioactivity are recovered in both cases. When the washed chloroform phase is treated with diethyl ether, however, the precipitate from the AN180 extract exhibits a much higher specific activity than the analogous fraction from RF-7(DCC^R). Acrylamide gel electrophoresis of the AN180 and RF-7(DCC^R) proteolipid fractions in the presence of sodium dodecyl sulfate (Figure 6) reveals that essentially all of the radioactivity in the AN180 proteolipid is associated with a single rapidly migrating peak, and that this radioactive peak is missing from the RF-7(DCC^R) proteolipid fraction. These results are virtually identical to those presented by Fillingame (1975). On the other hand, when the same procedures are carried out with AN180

TABLE II: Extraction of EDC[^{14}C]MI-Labeled AN180 and RF-7(DCC^{R}) Vesicles.[a]

Fraction	AN180[b]	RF-7(DCC^{R})[b]
Starting material	2.68 (100%)	2.59 (100%)
$CHCl_3$-MeOH extract	0.83 (31%)	0.80 (31%)
Washed $CHCl_3$ layer	0.32 (12%)	0.27 (10%)
Diethyl ether precipitate (proteolipid)	0.11 (4%)	0.08 (3.2%)
	Sp Act. (nmol/mg of proteolipid protein)	
	2.82	2.12

[a] One milliliter samples of AN180 and RF-7(DCC^{R}) membrane vesicles containing a total of 22 and 21 mg of membrane protein, respectively, were treated with EDC[^{14}C]MI as described under Experimental Procedure. The samples were then fractionated according to Fillingame (1975), as described under methods. The values given represent the total radioactivity recovered in each fraction and the percentage in each fraction relative to the amount of EDC[^{14}C]MI incorporated. A total of 410 μg of protein was recovered in each proteolipid fraction. [b] Counts/min × 10^{-6}.

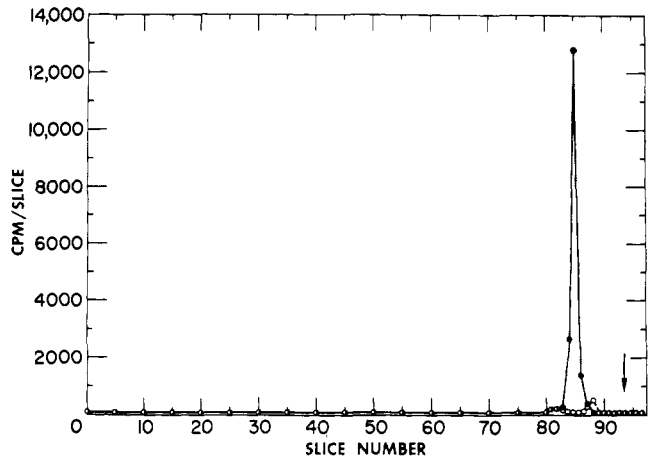

FIGURE 6: Sodium dodecyl sulfate acrylamide gel electrophoresis of proteolipid preparations from AN180 (●) and RF-7(DCC^{R}) (○) membrane vesicles labeled with [^{14}C]DCC. Samples of proteolipid preparations containing 81 μg of protein (AN180) and 85 μg of protein [RF-7(DCC^{R})] at the specific activities given in Table I were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described under Experimental Procedure. The arrow represents the position of the tracking dye bromphenol blue.

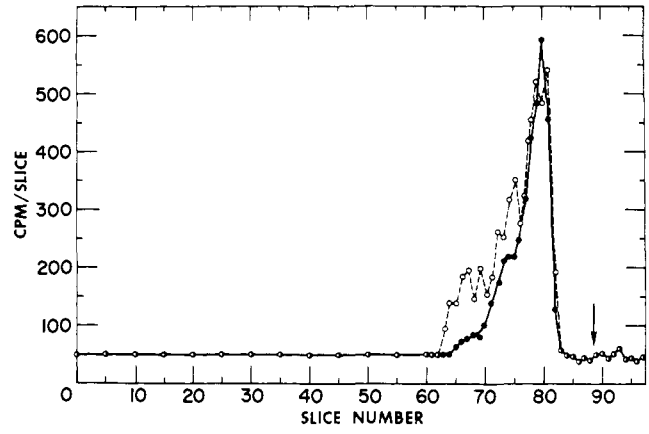

FIGURE 7: Sodium dodecyl sulfate acrylamide gel electrophoresis of proteolipid preparations from AN180 (●) and RF-7(DCC^{R}) (○) membrane vesicles labeled with EDC[^{14}C]MI. Samples of each proteolipid preparation containing 84 μg of protein at the specific activities given in Table II were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described under Experimental Procedure. The arrow represents the position of the tracking dye bromphenol blue. The lack of sharpness of the peaks shown here relative to that shown in Figure 7 is due to experimental variability.

and RF-7(DCC^{R}) vesicles labeled with EDC[^{14}C]MI, the proteolipids extracted from the vesicles have similar specific activities (Table II), and peaks containing similar amounts of radioactivity are observed on sodium dodecyl sulfate acrylamide gel electrophoresis (Figure 7). These results are consistent with the interpretation that the altered carbodiimide-reactive protein in RF-7(DCC^{R}) is specifically defective with respect to its ability to react with hydrophobic carbodiimides, and that it retains a normal ability to react with water-soluble carbodiimides.

Discussion

The results presented in this paper provide a clear demonstration that the carbodiimide-reactive protein associated with the membraneous Ca^{2+}, Mg^{2+}-stimulated ATPase complex of *E. coli* is causally related to the dramatic defect in active transport observed when isolated membrane vesicles are extracted with chaotropic agents. Since the inability of these vesicles to catalyze active transport has been demonstrated to be due to a specific increase in proton permeability, which is reversed by exposure to carbodiimides, it seems reasonable to infer that the carbodiimide-reactive protein plays an important role in proton permeability, as postulated for the analogous proteolipid from mitochondria (Racker, 1972). In addition to the observations presented in this paper, it is important to note that certain other mutations in the Ca^{2+}, Mg^{2+}-stimulated ATPase complex that are unrelated to the carbodiimide-reactive protein cause vesicular transport activity to become impervious to extraction with 1.0 M guanidine·HCl (Boonstra et al., 1975). These findings, considered together, provide a strong indication that the increase in proton permeability observed in vesicles subjected to extraction with chaotropic agents is due solely to an effect on the carbodiimide-reactive component of the ATPase complex. Whether the chaotropes perturb the carbodiimide-reactive protein directly or cause an increase in proton permeability by solubilizing a component of the ATPase complex which inhibits the proton-conducting properties of the carbodiimide-reactive protein cannot be answered at the present time. However, in considering the latter possibility, it is noteworthy that the vesicles used in these experiments have already lost 60–80% of the membrane-bound Ca^{2+}, Mg^{2+}-stimulated ATPase activity (Short et al., 1975).

Surprisingly, although the transport activity of guanidine-HCl-extracted vesicles prepared from the DCC-resistant mutants is not reactivated by lipophilic carbodiimides, completely normal reactivation is observed with either of two water-soluble carbodiimides. In addition, similar effects are observed with respect to inhibition of Ca^{2+}, Mg^{2+}-stimulated ATPase activity. That is, the ATPase activity of vesicles prepared from DCC-resistant mutants is refractory to inhibition by DCC and DiPC, but sensitive to inhibition by the water-soluble carbodiimides EDC and CMC (cf. Figure 5). In view of the observation that the proteolipid isolated from the DCC-resistant vesicles does not react with DCC, but reacts normally with EDCMI, it seems clear that the results are due to the genetic alteration in the carbodiimide-reactive protein itself, and not to a spurious effect of the water-soluble carbo-

diimides on other membrane proteins. The nature of this genetic alteration is by no means clear, however. Possibly, the altered protein is sufficiently changed in its tertiary structure so that carbodiimide-reactive sites become inaccessible from the hydrophobic interior of the membrane in which the protein is embedded, but remain accessible from the aqueous phase. This simplistic interpretation seems to be insufficient to explain the results however, as it is difficult to envisage such a gross change in tertiary structure without a loss of catalytic activity. The DCC-resistant mutants exhibit a slight increase in Ca^{2+}, Mg^{2+}-stimulated ATPase activity (Fillingame, 1975 and Figure 5) and grow normally on aerobic carbon sources (Fillingame, 1975). Furthermore, as indicated previously (Patel et al., 1975), it is not clear that the differences in reactivity of the various carbodiimides are related merely to differences in lipid solubility. Thus, DCC reactivates transport and inhibits ATPase activity at the lowest concentration, while DiPC, another hydrophobic carbodiimide, exhibits concentration optima for reactivation and inhibition that are higher than those observed with water-soluble carbodiimides (cf. Patel et al., 1975, and Figure 5). In any case, the properties exhibited by this DCC-resistant mutant are not apparently unusual. Abrams et al. (1972) reported the isolation of a DCC-resistant mutant of *Streptococcus faecalis* which exhibits DCC- and DiPC-resistant ATPase activity, but no resistance to water-soluble carbodiimides. Although these workers suggested that the water-soluble carbodiimides act by a different mechanism, this does not appear to be the case in the present studies, at least insofar as both types of carbodiimides react with the same proteolipid component of the membrane. Perhaps the two classes of carbodiimides react with different residues in the proteolipid, but an investigation of this possibility must await further purification of the proteolipid and a detailed chemical analysis of the carbodiimide-reactive sites within the molecule.

Acknowledgments

The authors wish to express their appreciation to Dr. Robert H. Fillingame for providing his mutants and for his advice and discussion during the course of the experiments. They also thank Dr. Arnold Liebman and The Isotope Synthesis Group of Hoffmann-La Roche, Inc. for synthesizing the radioactive carbodiimides used in these experiments. Without their generosity, this work would not have been possible.

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