Vol. 91, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 14, 1979 Pages 854-861

RECONSTITUTION OF LAC CARRIER FUNCTION IN CHOLATE-EXTRACTED MEMBRANES FROM ESCHERICHIA COLI*

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Received October 17, 1979

SUMMARY: Extraction of Escherichia coli ML 308-225 membrane vesicles with cholate yields a particulate fraction containing 10 to 15% of the phospholipid and about 70% of the protein of intact vesicles. Addition of phospholipid to the particulate fraction in the presence of cholate, followed by sonication and removal of detergent by gel filtration yields a vesicular preparation that exhibits lac carrier function as judged by transient increases in 6'-(N-dansyl)aminohexyl-1-thio-β-D-galactopyranoside fluorescence in the presence of either a lactose diffusion gradient or an artificially-generated membrane potential (interior negative). Activity is not observed in the absence of phospholipid, in the presence of N-ethylmaleimide or in analogous preparations from ML 30 vesicles that do not contain the β-galactoside transport system.

INTRODUCTION: The chemiosmotic hypothesis (1-3) postulates that the immediate driving force for many bacterial transport systems is an electrochemical gradient of protons ($\Delta\bar{\mu}_{H}+$)[1], and studies with intact cells (4) and isolated cytoplasmic membrane vesicles (5-10) have provided strong support for this concept. Since the energetics of bacterial active transport have been resolved to a great extent, further progress necessitates a more mechanistic approach, and recent studies with right-side-out Escherichia coli membrane vesicles (11-13) have provided some

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*A preliminary report on some of this work has been presented [Padan, E. and Kaback, H. R. (1979) Proc. 79th Ann. Meeting of the Amer. Soc. Microbiol. 145(Abst. No. K-1)].

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▲Partially supported by a grant from the United States-Israeli Binational Science Foundation.

[1]Abbreviations: $\Delta\bar{\mu}_{H}+$, electrochemical gradient of protons; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Dns^{6}-Gal, 6'-(N-dansyl)aminohexyl-1-thio-β-D-galactopyranoside; $\Delta\Psi$, membrane potential; CCCP, carbonylcyanide-m-chlorophenylhydrazone; NEM, N-ethylmaleimide.

0006-291X/79/230854-08$01.00/0

insight on this level. It is apparent, however, that solubilization and purification of carriers in a functional state would provide an important tool in unraveling the mechanism of active transport.

In this regard, functionally active components of certain bacterial transport systems have been solubilized and functionally incorporated into proteoliposomes (14-18). However, in no case have impressive results been obtained with either E. coli or Salmonella typhimurium where genetic manipulation can be used to great advantage.

This report demonstrates that cholate-extracted E. coli ML 308-225 membrane vesicles which are depleted of phospholipids regain lac carrier function when reconstituted with exogenous phospholipid. All demonstrable activity is associated with particulate material, however, indicating that none of the lac carrier protein is solubilized in a form that can be subjected to purification.

MATERIALS AND METHODS: E. coli ML 308-225 ($i^-z^-y^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown on minimal medium A containing 1.0% disodium succinate as sole carbon source, and membrane vesicles were prepared as described (19).

A suspension of vesicles containing 8-9 mg of protein was centrifuged at 48,000 x g for 20 min, the supernatant discarded, and the pellet resuspended to 1.0 ml in 50 mM potassium phosphate (pH 7.0) containing 20 mM lactose (unless noted otherwise). Aliquots of a 20% solution (v/v) of sodium cholate (recrystallized 3 times; pH 7.5) were then added stepwise with stirring over about 30 sec to yield a final concentration of 6%, and the suspension was incubated at 4° for 20 min, followed by centrifugation at 48,000 x g for 20 min. The supernatant was discarded and the pellet resuspended in 1.4 ml of 50 mM potassium phosphate (pH 7.0) containing 20 mM lactose (unless noted otherwise) and 6% sodium cholate.

Soy bean phospholipids (Asolectin), partially purified according to Kagawa and Racker (20), were transferred to an appropriate vessel and dried under argon. Cholate-extracted vesicles were added such that the phospholipid:protein ratio was approximately 20:1, and the suspension was homogenized as much as possible with a glass stirring rod, followed by sonication for 10 to 30 sec in bath-type sonicator (Laboratory Supplies Company, Inc., Hicksville, New York; model T-80-80-1 RS). The visually clear sample was then loaded on to a Sephadex G-50 column (1 x 30 cm) that had been equilibrated with 50 mM potassium phosphate (pH 7.0) containing 20 mM lactose (unless noted otherwise), and the column was developed with the same solution. The turbid fraction eluted in the first 5.0 ml after the void volume was collected and centrifuged at 48,000 x g for 40 min. The supernatant was discarded and the pellet resuspended to a concentration of 60-100 mg of protein/ml in 50 mM potassium phosphate (pH 7.0) containing 20 mM lactose (unless noted otherwise) and 4 mg/ml of bovine serum albumin. As demonstrated by analogous experiments with [carboxyl-^{14}C]cholate, the final preparation contained less than 0.01% of the cholate added initially. Preparations were stored at 4° and used within 12 hrs.

For phospholipid determinations, E. coli ML 308-225 was grown on minimal medium A containing disodium succinate as described above, and in addition, 1.0 mM [U-^{14}C]glycerol (0.1 mCi per mmol). Membrane vesicles were prepared and extracted with cholate as indicated. Aliquots of the suspensions containing 5-10 µg of protein were then chromatographed directly on silica gel G coated thin layer plates (Mann Biochemicals) using chloroform:methanol:water (65:25:4, v/v/v) as the solvent system. Known samples of phosphatidylethanolamine and phosphatidylglycerol were used as standards. Spots detected on exposure to iodine vapor or by autoradiography were scraped into scintillation vials and assayed for radioactivity by liquid scintillation spectrometry.

Differential labeling of the lac carrier protein during derepression of the lac operon in E. coli ML 30 was achieved with mixtures of ^{14}C and ^{3}H amino acids, and membrane proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and assayed for radioactivity according to Overath et al. (21).

Fluorescence of 6'-(N-dansyl)aminohexyl-1-thio-β-D-galactopyranoside (Dns^{6}-Gal) was measured as described (22) with a Perkin-Elmer spectrophotofluorimeter (excitation, 340 nm; emission, 500 nm).

Protein was measured according to Lowry et al. (23) using bovine serum albumin as standard.

Sodium cholate was obtained from Sigma Chemical Company, recrystallized 3 times from 70% ethanol (20), and adjusted to pH 7.5 without allowing the pH to exceed 9.0. Soy bean phospholipid (Asolectin) was purchased from Associated Concentrates, [U-^{14}C]glycerol and [carboxyl-^{14}C]cholate from New England Nuclear, and mixtures of ^{14}C and ^{3}H amino acids and [1-^{14}C]lactose from Amersham/Searle. Dns^{6}-Gal was synthesized as described (22). All other materials were of reagent grade obtained from commercial sources.

RESULTS: When E. coli ML 308-225 vesicles are treated with various concentrations of cholate, the resultant particulate fraction is largely depleted of phospholipids (Table I). Over 85% of the phosphatidylethanolamine, which constitutes about 70% of the total phospholipid (24), is solubilized by extraction with 4%

TABLE I

Solubilization of Phospholipids by Cholate Extraction of ML 308-225 Vesicles

[Cholate]	Phospholipid Remaining	
	phosphatidylethanolamine	phosphatidylglycerol
(%)	(%)	(%)
4	13.5	43.3
6	11.5	31.4
10	13.1	28.0

Membrane vesicles containing ^{14}C phospholipids prepared as described in Methods were suspended in 50 mM potassium phosphate (pH 7.0) to a concentration of 6 mg of protein/ml. Sodium cholate was added to given concentrations, and the samples were incubated at 4° for 20 min. The suspensions were centrifuged at 48,000 x g for 20 min, the supernatants discarded, and the pellets assayed for radioactive phosphatidylethanolamine and phosphatidylglycerol as described in Methods. Results are expressed as a percentage of the appropriate phospholipid present in control vesicles.

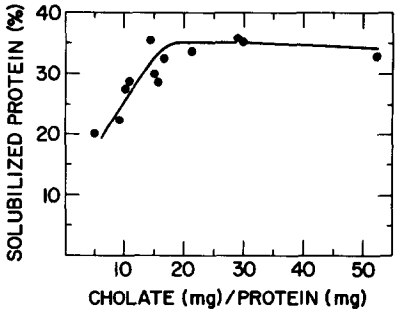

Fig. 1. Solubilization of ML 308-225 vesicle protein by cholate extraction. Membrane vesicles (1.1 to 8.6 mg of protein) were suspended in 1.0 ml of 50 mM potassium phosphate (pH 7.0) containing sodium cholate at concentrations appropriate to yield the cholate:protein ratios given. The samples were incubated at 4° for 20 min, centrifuged at 48,000 x g for 20 min, and the protein released into the supernatant was determined. Results are expressed as a percentage of the total amount of protein added to the reaction mixtures.

cholate, the lowest concentration tested, and extraction with higher detergent concentrations has little additional effect. With phosphatidylglycerol, on the other hand, about 57% is solubilized at 4% cholate, and significantly higher amounts are extracted when the detergent concentration is increased to 10%.

In contrast to phospholipids, only about 35% of the membrane protein is solubilized by cholate extraction (Fig. 1). At a cholate:protein ratio of about 5, approximately 20% of the protein is solubilized. As the ratio is increased to about 15, 35% solubilization is observed, and this value remains constant when the ratio is increased to 50. Variations in ionic strength (from 1 to 50 mM potassium phosphate) and temperature (from 25° to 45°) or addition of urea (up to 5.0 M) do not increase the amount of protein solubilized at any of the cholate:protein ratios tested. It is also noteworthy that the population of membrane proteins solubilized by cholate is relatively specific as evidenced by SDS-PAGE (data not shown).

The *lac* carrier protein can be differentially labeled during derepression of the *lac* operon and electrophoreses as a 30,000 dalton protein on SDS-PAGE (21,25). Although the data will not be presented, a protein exhibiting these properties is demonstrable in vesicles from induced *E. coli* ML 30. Moreover, this protein is retained after the membranes are extracted with cholate.

Examination of cholate-extracted preparations by phase contrast microscopy fails to reveal vesicular structures, but electron microscopy of thin sections

demonstrates that a trilaminar unit membrane structure is retained in non-vesicular fragments (data not shown). Thus, the bacterial membrane, like the mitochondrial inner membrane (26), exhibits the morphological characteristics of a bilayer despite extensive loss of phospholipids.

Treatment of ML 308-225 vesicles with cholate causes complete loss of lac carrier function. Moreover, reconstitution of the cholate-extracted preparations with partially purified soy bean phospholipids fails to yield vesicles that catalyze D-lactate- or reduced phenazine methosulfate-driven lactose accumulation. Since active transport in the presence of these electron donors is dependent upon $\Delta\bar{\mu}_{H}+$ (interior negative and alkaline) (7,8), absence of activity could be due either to inability of the preparations to generate $\Delta\bar{\mu}_{H}+$ or to denaturation of the lac carrier in situ. As demonstrated by the following results, the former possibility is the case.

When ML 308-225 vesicles are extracted with cholate, reconstituted with phospholipids in the presence of 20 mM lactose and diluted 300-fold into a cuvette containing Dns^{6}-Gal, there is a rapid increase in fluorescence that reaches a maximum in about 30 sec and decreases to the baseline within 2-3 min (Fig. 2A). Clearly, this effect is due to the imposition of a lactose diffusion gradient, since it is abolished when the vesicles are diluted 300-fold and incubated for 15 min prior to addition of Dns^{6}-Gal. The phenomenon is not observed with analogous preparations from uninduced E. coli ML 30 nor when reconstituted ML 308-225 vesicles are treated with 1.0 mM N-ethylmaleimide (NEM), indicating that it is also dependent upon functional lac carrier protein. Finally, the reconstituted system exhibits approximately 85% of the activity of intact vesicles at equal protein concentrations and similar phenomena are observed when lactose counterflow activity is assayed by filtration using [1-^{14}C]lactose (11).

The data presented in Fig. 2B demonstrate that $\Delta\bar{\mu}_{H}+$-dependent lac carrier function is also retained in the reconstituted system. In this experiment, reconstituted ML 308-225 vesicles were prepared in potassium phosphate without lactose, diluted 300-fold into choline phosphate containing Dns^{6}-Gal, and valinomycin was

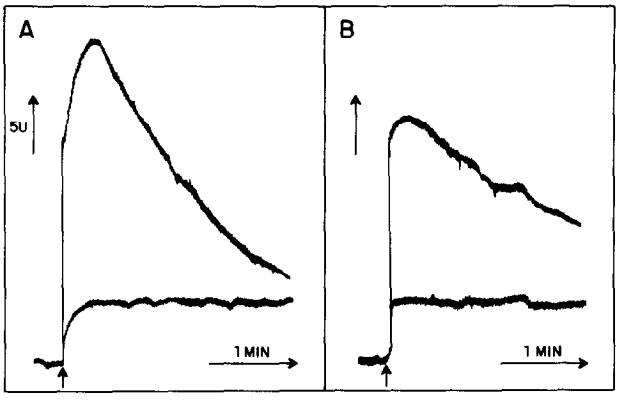

Fig. 2. Effect of lactose efflux (A) and imposed $\Delta\Psi$ (interior negative) (B) on Dns^6-Gal fluorescence in reconstituted ML 308-225 vesicles. A. For the upper curve, reconstituted vesicles (60-100 mg protein/ml) were prepared in 50 mM potassium phosphate (pH 7.0) and 20 mM lactose as described in Methods and diluted 300-fold into a cuvette containing 50 mM potassium phosphate (pH 7.0) and 6.6 μM Dns^6-Gal, as indicated by the arrow. The lower curve was obtained when the vesicles were diluted 300-fold and incubated for 15 min prior to addition of Dns^6-Gal, when ML 308-225 vesicles were treated with 1.0 mM NEM prior to dilution or when reconstituted, lactose-loaded ML 30 vesicles prepared from uninduced cells were subjected to the same operation. B. For the upper curve, reconstituted vesicles (60-100 mg protein/ml) were prepared in 50 mM potassium phosphate (pH 7.0) without lactose as described in Methods, and diluted 300-fold into a cuvette containing 50 mM choline phosphate (pH 7.0) and 6.6 μM Dns^6-Gal. At the time indicated by the arrow, valinomycin was added to a final concentration of 10 μM. The lower curve was obtained under the same conditions in the presence of 10 μM CCCP, when ML 308-225 vesicles were diluted into 50 mM potassium phosphate (pH 7.0), when ML 308-225 vesicles were treated with 1.0 mM NEM prior to dilution or when reconstituted, potassium-loaded ML 30 vesicles prepared from uninduced cells were subjected to the same operation. In all cases, excitation was at 340 nm and emission at 500 nm, and the total volume was 1.5 ml.

added as indicated. Under these conditions, an electrical potential ($\Delta\Psi$, interior negative) is created across the membrane (27), and there is a rapid, transient increase in Dns^6-Gal fluorescence that is not observed when the vesicles are diluted into equimolar potassium or when carbonylcyanide-m-chlorophenylhydrazone (CCCP) is added. In addition, the effect is not observed with similar preparations of uninduced ML 30 vesicles and it is abolished by treatment with 1.0 mM NEM.

DISCUSSION: These results demonstrate that lac carrier function in cholate-extracted E. coli ML 308-225 membrane vesicles which are depleted of 80-90% of their phospholipids and about 35% of their protein can be reconstituted with exogenous phospholipids. As such, the findings indicate that the lac carrier protein is not irreversibly denatured in situ by extensive delipidation of the membrane

nor by the detergent cholate. However, all of the lac carrier activity in the preparations is present in a fraction that sediments at low centrifugal forces, indicating that reconstitution of a soluble, monodisperse protein has not been achieved. In this context, many experiments in this laboratory over the past five years have been directed towards this goal with no success whatsoever. Treatment of vesicles under many different conditions with detergents such as Triton X-100, sarcosyl, and octylglucoside or solvents such as butanol, trifluoroacetate and hexamethylphosphoric triamide, followed by addition of phospholipids to the soluble fraction and removal of detergent or solvent has failed to yield proteoliposomes that exhibit carrier activity. Moreover, we have been unable to confirm the findings of Altendorf et al. (28) who reportedly conferred lactose transport activity on uninduced ML 30 vesicles with an aprotic solvent extract of ML 308-225 vesicles or those of Belaich et al. (29) who claimed to solubilize and partially purify a protein with β-galactoside binding activity by extracting ML 308-225 vesicles with Triton X-100. Thus, although cholate-extracted vesicles may represent a useful system for studying the effect of specific phospholipids on lac carrier activity, it is apparent that another approach must be utilized to achieve solubilization and purification of functional lac carrier protein.

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